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PRODUCTION DE LIPIDES À PARTIR DE BOUES ET DE GLYCÉROL BRUT ET LEUR CONVERSION EN BIODIESEL

Par

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RÉSUMÉ

Le prix croissant des matières premières traditionnelles (huiles végétales et graisses animales) pour la production de biodiesel a poussé les chercheurs et les ingénieurs à chercher des sources alternatives de substrats. Les boues constituent une source abondante de matières organiques produites partout dans le monde. Celles-ci présentent un possible intérêt pour la production de biocarburants car elles contiennent des lipides. Cette étude s'intéresse à la production de biodiesel en utilisant les huiles dérivées de boues. Différents types de boues municipales primaires, secondaires et mixtes, ainsi que des boues secondaires issues de la production de pâtes à papier, ont été collectées et utilisées dans cette étude. Les boues présentaient une teneur en lipides allant de 5% à 11% (p/p). Les effets du type de boue, des concentrations de matières en suspension dans les boues (10 à 30 g/L), du prétraitement des boues (thermique, thermo-acide et thermo-alcalin), ainsi que l'ajout d'une source de carbone (glucose et le glycérol) sur l'accumulation des lipides par les microorganismes ont été étudiés. Une accumulation maximale de lipides (39% p/p de la biomasse) a été obtenue à partir de boues pré-traitées.

Pour approfondir la faisabilité de la production de biodiesel à partir des boues, le bilan énergétique, les émissions de GES et les coûts ont été étudiés. L'étude du bilan énergétique a révélé qu'un gain d'énergie de plus de 29 GJ/tonne de biodiesel produit a été obtenu à partir d'huiles dérivées de boues. L'évaluation des émissions de gaz à effet de serre (GES) a montré que l'utilisation des boues pour la production de biodiesel était une méthode qui permet la réduction des émissions de GES (réduction de plus de 40 tonnes de dioxyde par tonne de biodiesel produit). L'estimation des coûts a été réalisée par le logiciel SuperPro Designer et les résultats ont montré que le coût du biodiesel produit à partir de boues est d'environ 0.5 US$/kg de biodiesel.

Comme la production du biodiesel augmente, la production de glycérol brut, un sous-produit de la production de biodiesel par trans-estérification, augmente également. La grande quantité de

glycérol brut générée nécessite une gestion appropriée. En effet, ce sous-produit représente une grande source de carbone pour de nombreux microorganismes. Le glycérol brut fourni par une usine de production de biodiesel (Québec, Canada) a été utilisé pour l'accumulation de lipides par la levure *Trichosporon oleaginosus*. En raison de la forte teneur en savon dans le glycérol brut, une purification a été réalisée afin de l'enlever en le convertissant en acides gras libres. Une fois purifié, le glycérol a été utilisé pour faire croître les microorganismes. Une accumulation plus élevée des lipides dans la souche microbienne a été obtenue comparativement aux essais réalisés avec le glycérol non purifié (teneur en lipides: 37.2% p/p de la biomasse). La concentration de glycérol ayant donné le rendement le plus élevé de lipides produits (0.19 g de lipides/g glycérol) est de 50 g/L. L'étude du bilan énergétique de la production de biodiesel à partir de glycérol brut a montré un gain énergétique net de 9 GJ/tonne de biodiesel produit. L'estimation des coûts a révélé que le coût de lipides produits à partir de glycérol brut était d'environ 0.44 US$/kg de lipides, ce qui est inférieur à l'huile de soja (matière première de la production de biodiesel utilisée actuellement, 0.88 US$/kg).

L'extraction des lipides est l'étape centrale de production de biodiesel à partir de microorganismes oléagineux. L'extraction par solvant organique nécessite un temps de réaction long. Les ultrasons ont été utilisés pour l'extraction des lipides à partir de *Trichosporon oleaginosus* et du champignon SKF-5 dans les boues. Il a été observé que le temps d'extraction a pu être réduit de 12 h (extraction des lipides classique) à 20 min (extraction des lipides par ultrasons) pour parvenir à une récupération de 95% à 100% des lipides. De plus, l'eau a été testée comme solvant pour effectuer l'extraction par traitement aux ultrasons et des taux de récupération des lipides de 10.2% et 9.3% (p/p de lipides totaux dans la biomasse) ont été obtenus à partir de *Trichosporon oleaginosus* et SKF-5, respectivement. En raison de l'inquiétude croissante sur la manipulation de solvants organiques (notamment le chloroforme), un processus en une étape aussi appelé trans-estérification *in-situ*, en présence des ultrasons a été menée afin d'éviter l'étape d'extraction. Les résultats ont montré que plus de 94% des lipides ont été convertis en biodiesel en 20 à 50 minutes, ce qui est similaire au rendement du procédé en deux étapes dans lequel les lipides sont extraits, puis transformés en FAMEs par trans-estérification.

La détermination de la composition des lipides est essentielle afin de déterminer la faisabilité de la production de biodiesel. Les lipides obtenus à partir de tous les microorganismes cultivés dans la boue, la boue prétraitée et le glycérol brut ont été analysés et sont riches en C_{16} à C_{18} (comme les huiles de graines de plantes qui sont actuellement utilisées pour produire du biodiesel commercial). Un haut degré de saturation a été trouvé dans les lipides. Ceci suggère que le biodiesel produit à partir de ces lipides aurait une grande stabilité à l'oxydation, mais un faible écoulement à froid.

L'étude a montré que les boues et le glycérol brut (sous-produit de production de biodiesel) ont un grand potentiel dans la production de biodiesel. L'utilisation des ultrasons pour l'extraction des lipides et la trans-estérification *in-situ* est une technologie prometteuse car elle réduit largement le temps de traitement nécessaire pour obtenir des performances comparables à celles des technologies actuellement appliquées.

ABSTRACT

Increasing prices of traditional feedstocks (vegetable oils and animal fats) for biodiesel production urge researchers and engineers to seek alternative oil sources. Sludge is naturally, widely, and abundantly produced all over the world and is recently found to contain lipids. In this study, sludge derived oil was used for biodiesel production. Different types of sludge including municipal primary, secondary, mixed, and pulp and paper secondary sludge, were collected in Québec City (Canada), and used as a lipid source or a medium of oleaginous microorganism cultivation. It was found that the original sludge had a lipid content around 5% to 11% (based on weight). Sludge type, sludge suspended solids concentrations (10 to 30 g/L), sludge pre-treatment (thermal, acid-thermal, and alkaline-thermal), and addition of carbon source (glucose and glycerol) were utilized to investigated the effect of lipid accumulation in microorganisms. A maximum lipid accumulation of 39% w/w biomass was achieved with alkaline-thermal pre-treated secondary sludge at a suspended solids concentration of 30 g/L.

To further investigate the feasibility of biodiesel production from sludge, energy balance, greenhouse gas emissions, and cost estimation were studied. The energy balance study found more than 29 GJ of energy gain per tonne of biodiesel produced from sludge derived oil. The greenhouse gas emission evaluation showed that sludge used for biodiesel production resulted in a GHG reduction method, where more than 40 tons of carbon dioxide emissions could be reduced per tonne of biodiesel production. Cost estimations, conducted by SuperPro Designer, showed sludge produced biodiesel to be around 0.5 US$/kg biodiesel when sludge was used as fermentation media or directly as lipid source.

As biodiesel production increases, the production of crude glycerol is simultaneously increasing. This large amount of crude glycerol generation requires a suitable management plan. It has been reported that glycerol is a great carbon source for many microorganisms. In this study, crude glycerol was used as a carbon source for lipid accumulation in yeast *Trichosporon oleaginosus*. Due to the large content of soap in the crude glycerol, purification to remove soap

by converting it to free fatty acid was performed, and the purified glycerol was then utilized for microbial growth. The results showed that purified glycerol (lipid content: 44.3% w/w biomass) provided a higher lipid accumulation in the strain compared to the crude glycerol (lipid content: 37.2% w/w biomass). The optimal glycerol concentration was found to be 50 g/L, with the highest lipid yield of 0.19 g lipid/g glycerol. An energy balance study of biodiesel production from the crude glycerol showed that the net energy gain was around 9 GJ in per tonne of biodiesel produced. Cost estimation revealed that the cost of lipid produced from the crude glycerol was around 0.44 US$/kg, which was lower than the current price (0.88 US$/kg) of soybean oil.

Lipid extraction is the secondary critical step for biodiesel production from oleaginous microorganisms. The existing widely applied conventional organic solvent extraction method requires a long reaction time. In efforts to reduce the reaction time, ultrasonication was employed in lipid extraction from *Trichosporon oleaginosus*, fungus SKF-5, and sludge. Extraction time following ultrasonication was greatly reduced from 12 h (conventional lipid extraction) to 20 min (ultrasonication lipid extraction), achieving 95% to 100% lipid recovery. In addition, green solvent (water) combined with unltrasonication was tested to perform the extraction. Lipid recoveries of 10.2% and 9.3% (w/w total lipid of biomass) were obtained from *Trichosporon oleaginosus* and SKF-5, respectively. Due to the growing concern on handling of organic solvent (especially chloroform), one-stage transesterification, also called in-situ transesterification, in the presence of ultrasonication was conducted to avoid the extraction step. The results showed that more than 94% of lipid was converted to biodiesel within 20 to 50 min, similar yield from the two-stage transesterification where lipid was extracted and then transferred to fatty acid methyle esters through transesterification.

Determination of lipid composition is essential for its feasibility in biodiesel production. Lipids from all the microbes cultivated with sludge, pretreated sludge, and crude glycerol were analyzed and observed to be rich in C16 to C18 vegetable oils, which are currently being used to produce commercial biodiesel. Therefore biodiesel produced from the lipids is suggested to be suitable for biodiesel production.

The study showed that sludge and crude glycerol had great potential in biodiesel production. Ultrasonication application for lipid extraction and *in-situ* transesterification is also a promising technology, largely reducing processing time while maintaining a comparable performance to the currently widely applied technologies.

PUBLICATIONS DANS CETTE THÈSE

- **X.L. Zhang**, S. Yan, R.D. Tyagi, R.Y. Surampalli (2013). Biodiesel production from heterotrophic microalgae through transesterification and nanotechnology application in the production. ***Renewable & Sustainable Energy Reviews***, 26, 216-223.
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Energy balance and greenhouse gas emissions of biodiesel production from oil derived from wastewater and wastewater sludge. ***Renewable Energy***, 55, 392-403.
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Biodiesel production from sludge derived oil. ***Bioresource Technology*** (under review).
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Lipid production from microorganism cultivated with pre-treated wastewater sludge. ***Bioresource Technology*** (under review).
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Crude glycerol application on the production of value added product biodiesel. ***Environmental Science & Technology*** (under review).
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Energy balance of biofuel production from crude glycerol. ***Energy*** (under review).
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Cost estimation of biodiesel production from oil derived from wastewater and wastewater sludge. ***Energy Economics*** (under review).
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Cost estimation of lipid production from crude glycerol. ***Biomass and Bioenergy*** (under review).
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Ultrasonication assisted lipid extraction from oleaginous microorganisms. ***Bioresource Technology*** (under review).
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Ultrasonication aided in-situ transetserification of microbial lipids to produce biodiesel. ***Fuel*** (under review).
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Ultrasonication application in extraction and in-situ transesterification of lipid derived from sludge. ***Fuel***, (under review).

- **X.L Zhang**, Song Yan, R. D. Tyagi, and Rao Y. Surampalli (2013). Chapter 4 Biodiesel Production by Transesterification. In book: ***Biodiesel Production***. American Society of Civil Engineers (submitted).
- **X.L Zhang**, Song Yan, R. D. Tyagi, and Rao Y. Surampalli (2013). Chapter 11 Lipid Extraction Technologies. In book: ***Biodiesel Production***. American Society of Civil Engineers (submitted).

PUBLICATIONS EN DEHORS DE CETTE THÈSE

- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y. Surampalli (2011). Synthesis of nanoparticles by microorganisms and their application ion enhancing microbiological reactions. ***Chemosphere***, 42, 489-494.
- **X.L. Zhang**, S. Yan, R. D. Tyagi, R. Y. Surampalli (2013). Odor control in lagoons. ***Journal of Environmental Management***. 124, 62-71.
- **Xiaolei Zhang**, Satinder Kaur Brar, Song Yan, R. D. Tyagi, R. Y. Surampalli (2013). Fate and transport of fragrance materials in principal environmental sinks. ***Chemosphere***, 93, 857-869.
- **X.L. Zhang**, S. Yan, R.D. Tyagi and R.Y. Surampalli (2010). Chapter 21, Application of Nano-materials and Nano-technology for Biofuels Production. 478-496. In book: ***Bioenergy and Biofuel from Biowastes and Biomass***. Samir K. Khanal, Rao Y. Surampalli, Tian C. Zhang, Buddhi P. Lamsal, R. D. Tyagi, and C. M. Kao (editors), Reston, VA: ASCE / EWRI, 978-0-7844-1089-9.
- **X.L Zhang**, Song Yan, R. D. Tyagi, and Rao Y. Surampalli (2012). Chapter 14, Applications of Membrane Technology for Production of Wastewater Sludge-Based Bioproducts. In book: ***Membrane Technology and Environmental Applications***. American Society of Civil Engineers (in press).
- **X.L Zhang**, Tanaji T. More, Song Yan, R. D. Tyagi, Rao Y. Surampalli, and Tian C. Zhang (2012). Chapter 28 Nanomaterial-based Membranes for Gas Separation and Water Treatment. In book: ***Membrane Technology and Environmental Applications***. American Society of Civil Engineers (in press).
- **X.L Zhang**, Song Yan, R. D. Tyagi, and Rao Y. Surampalli (2013). Chapter 16 Algae-based Carbon Capture and Sequestrations. In book: ***Carbon Capture and Storage: Physical/Chemical and Biological Methods***. American Society of Civil Engineers (in press).
- **X.L Zhang**, Song Yan, R. D. Tyagi, and Rao Y. Surampalli (2013). Chapter 17 Enzymatic Sequestration of Carbon Dioxide. In book: ***Carbon Capture and Storage: Physical/Chemical and Biological Methods***. American Society of Civil Engineers (in press).

- **X.L Zhang**, Song Yan, R. D. Tyagi, and Rao Y. Surampalli (2013). Chapter 21 Management and Reduction of GHGs in Wastewater/Sludge Transport and Treatment. In book: ***Climate Change Modeling, Mitigation, and Adaptation***. American Society of Civil Engineers.
- **X.L Zhang**, Song Yan, R. D. Tyagi, and Rao Y. Surampalli (2013). Chapter 25 greenhouse Gases Emissions from Natural Systems: Mechanisms and Control Strategies. In book: ***Climate Change Modeling, Mitigation, and Adaptation***. American Society of Civil Engineers.

CONGRÈS ET CONFÉRENCES

- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y Surampalli (2011). The potential of wastewater and wastewater sludge in biodiesel production. Conference of 47th Central Canadian Symposium on Water Quality Research, academics presentations, Canada Centre for Inland Waters, 22nd and 23rd Feb. 2011, Burlington, ON, Canada.
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y Surampalli (2011). Biodiesel production from single cell oils derived from wastewater and wastewater sludge. 26th Eastern Regional Conference of Canadian Symposium on Water Quality Research, academics presentations, INRS-ETE, 7th Oct. 2011, Québec, QC, Canada.
- **Xiaolei Zhang**, Song Yan, R. D. Tyagi, R. Y Surampalli (2013). Biodiesel production from wastewater sludge. IWA Conference on Holistic Sludge Management, 6th - 8th, May, 2013, Vasteras Sweden.

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ABRÉVIATIONS

ASTM:	American Society of Testing and Materials
CN:	Cetane Number
Cn:0:	Fatty acid with n carbon atoms and zero double bond
Cn:1:	Fatty acid with n carbon atoms and one double bond
Cn:2.3:	Fatty acid with n carbon atoms and two or three double bonds
FAMEs:	Fatty Acid Methyl Esters
FFA:	Free Fatty Acid
GC-MS:	Gas Chromatograph –Mass Spectrometer
GHG:	Greenhouse Gas
ICP:	Inductively Coupled Plasma
IPCC:	Intergovernmental Panel on Climate Change
PC:	Phosphorus Content
SC:	Sulfur Content
SCO:	Single Cell Oil
SEM:	Scanning Electron Microscopy
SS:	Total Suspended Solid
SV:	Saponification Value
TC:	Total Carbon
TN:	Total Nitrogen
TP:	Total Phosphorus
TS:	Total Solid
WC:	Water Content

INTRODUCTION

La demande de carburants alternatifs a augmenté de façon significative en raison de l'épuisement des combustibles traditionnels, ceci entraînant l'accroissement du prix du pétrole. De plus, la hausse des émissions de gaz à effet de serre (GES) indique qu'il faudra nécessairement les contrôler (Koplow et Dernbach, 2001; Vicente et al., 2009a).

Le biodiesel, esters méthyliques d'acides gras (FAME), a attiré une grande attention en raison des avantages qu'il offre. Il est renouvelable, durable, compatible avec les moteurs diesel actuels. En outre, il offre un excellent pouvoir lubrifiant et une haute densité d'énergie.

Plusieurs technologies ont été développées pour produire du biodiesel comme la pyrolyse, les microémulsions, et la trans-estérification (Doll et al., 2008; Macala et al., 2008; Suarez et al., 2009). La trans-estérification des huiles végétales consistant à faire réagir les FAMEs avec de l'alcool à chaîne courte (méthanol ou éthanol) pour former le biodiesel (Boz et al., 2009; Dizge et al., 2009; Singh et Singh, 2010). Toutefois, le biodiesel produit à partir d'huiles végétales présente de nombreux inconvénients. Le manque de matières premières dû au faible rendement des huiles de culture, limite le développement à grande échelle du biodiesel. De plus, le coût de fabrication élevé lié à celui des huiles végétales représente 70% du coût de production total du biodiesel. Il est évident qu'il y a une concurrence inévitable de l'industrie du biodiesel avec la production alimentaire pour les cultures oléagineuses, et une grande exigence des terres arables disponibles pour atteindre les objectifs actuels en matière de biocarburants. Les principales sources utilisées sont le soja ou le tournesol (Pimentel et Patzek, 2005; Berk, 2008; Sulaiman et al., 2010). Aussi, afin de réduire le coût du litre de biodiesel produit et obtenir un rendement énergétique favorable ainsi qu'une production durable, les matières premières de substitution pour l'obtention de ce biocarburant doivent être considérées.

À ce titre, les boues de traitement des eaux usées pourraient être une matière première prometteuse. Elles présentent plusieurs avantages comme une teneur en lipides comparable à une huile végétale (15 à 25% p/p sec de boues et de 18 à 26% p/p pour une huile végétale). De

plus, ces déchets sont produits mondialement et leur élimination représente un coût appréciable. Leur emploi comme matière première, dont le coût serait nul ou presque, pour la production de biodiésel atténuerait la pression de leur gestion tout en réduisant l'émission des GES. Il a été constaté par ailleurs que de nombreux microorganismes (micro-algues, levures, bactéries, et champignons) ont la capacité d'accumuler des huiles dans certaines conditions de culture particulières. Les huiles microbiennes ont de nombreux avantages par rapport aux huiles végétales: absence de concurrence avec la production alimentaire, libération de terres arables, un taux élevé de croissance, un court cycle de vie, une longue durabilité, non soumis aux conditions climatiques et saisonnières avec un important potentiel d'accumulation de lipides (jusqu'à 85% p/p biomasse) et utilisation de la biomasse résiduelle comme source de carbone (Chisti, 2007; Meng et al., 2009; Karatay et Dönmez, 2010). En outre, la possibilité d'améliorer la teneur en lipides accumulée est réalisable en contrôlant les conditions de culture des microbes (Widjaja et al., 2009). Les huiles microbiennes présentent un potentiel très élevé pour produire du biodiesel à partir des déchets organiques éventuellement utilisés comme source de nutriments pour l'accumulation de lipides.

L'étape importante de la production de biodiesel à partir de substances contenant de l'huile est la séparation des lipides à partir du corps de la cellule. Pour ce faire, l'extraction par solvant organique et l'extraction mécanique sont les deux méthodes largement utilisées mais elles présentent des inconvénients majeurs, tels que de faibles rendements en lipides et leur haute toxicité (Cheng et al., 2011). Par conséquent, il est impératif de développer une méthode d'extraction alternative propre. D'un autre côté, la trans-estérification in-situ est un processus permettant de transférer directement une substance oléagineuse de biodiesel sans l'étape de séparation/extraction des lipides (Ehimen et al., 2012). L'avantage de la trans-estérification est d'éviter l'étape d'extraction ce qui réduit la consommation d'énergie et ainsi réduit le coût de production.

PARTIE I : SYNTHÈSE

1 SYNTHÈSE

1.1 Revue de littérature

1.1.1 Biodiesel

Le biodiesel est un biocombustible capable de s'adapter aux moteurs de pétro-diesel et répond aux exigences d'American Society for Testing and Materials (ASTM) (Tableau 1.1). Les normes de biodiesel ont de légères différences selon les pays ou les régions. Les normes les plus utilisées pour évaluer la qualité du biodiesel sont ASTM D6751 (ASTM, 2008) et le Comité européen de normalisation (EN) 14214 (CEN, 2003).

Table 1.1 Normes de biodiesel

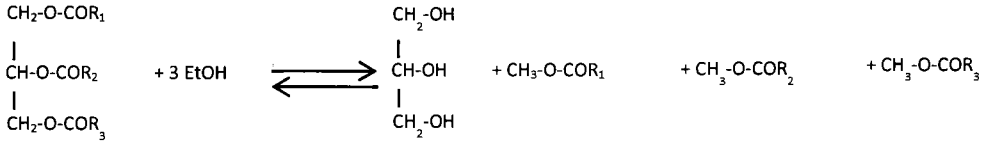
Properties	ASTM D6751[a]	EN 14214[b]	Biodiesel (huiles végétales)
Viscosité (mm^2/s) à 40 ºC	1.9-6.0	3.5-5.0	31.6-51.2[c]
Point d'ignition (ºC)	130.0 min	120.0 min	201-277[c]
Indice de cétane	47 min	51 min	33-66[c]
Point de trouble (ºC)	-2-(-26)	-	-3.9-12.8[c]
Point d'écoulement (ºC)	-	-	-6.1-(-40)[c]
Soufre (ppm)	<15	<10	<10[d]

[a] (ASTM, 2008); [b] (CEN, 2003); [c] (Dunn et al., 1999; Demirbas, 2003b); [d] (http://paultan.org/2011/06/07/b5-biodiesel-palm-biodiesel-sulphur-content-less-than-10ppm/).

La trans-estérification consiste à faire réagir des huiles et des alcools à chaîne courte (méthanol ou éthanol). C'est la méthode la plus populaire pour la production de biodiesel (Equation 1.1). Le méthanol est généralement choisi en raison du fait qu'il est moins cher et qu'il a une polarité minimale tout en fournissant un taux de conversion élevé (Kulkarni et al., 2007). Le biodiesel produit à partir du méthanol consiste en des esters méthyliques d'acides gras (FAMEs). Le biodiesel est un mélange de FAMEs qui contiennent une longueur de chaîne de carbone (de 12 à 20 avec de 0 à 2 doubles liaisons). Les biodiesels commerciaux disponibles sur le marché sont B100, B20, B5, B2 et se réfèrent à 100% de biodiesel, 20% de biodiesel et 80% pétro-diesel, 5% de biodiesel et 95% pétro-diesel et de 2% de biodiesel et 98% pétro-diesel. Normalement

B20, B5, B2 peuvent être directement utilisés dans les équipements de diesel avec ou sans modifications. B100, la forme pure de biodiesel, est également utilisée dans les moteurs diesels, mais peut exiger certaines modifications dans le moteur. Le montant annuel de la production mondiale de biodiesel s'accroît considérablement depuis 1990 (Figure 1.1) (Annie, 2006; Energy, 2010). Selon les prévisions de Perspectives agricoles de l'OCDE et de la FAO, la production de biodiesel devrait augmenter de façon continue au cours des dix prochaines années (http://www.oecd.org/document/9/0) (Figure 1.1).

Équation 1.1



R_1, R_2, et R_3 sont des groupements alkyles. Les produits, CH_3-O-COR_1, CH_3-O-COR_2, et CH_3-O-COR_3 sont des chaines alkyles (methyl, propyl, or ethyl) esters.

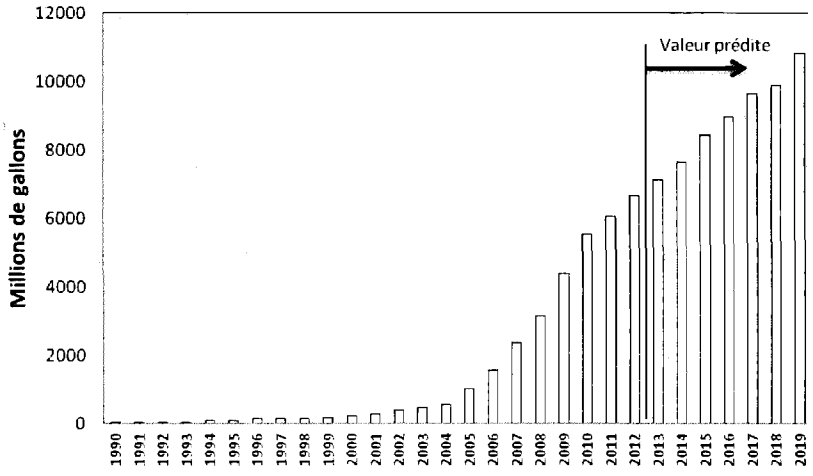


Figure 1.1 **Production annuelle de biodiesel**

1.1.2 Matières premières

1.1.2.1 Source

La matière primaire est la clé de la production de biodiesel car elle en détermine le coût, ce qui représente actuellement plus de 70% du coût total de production (Haas et al., 2006; Kargbo, 2010). Ce biocarburant peut être produit à partir d'huile naturelle ou de matières premières grasse comme les huiles de canola, de ricin, de coprah, de maïs, de coton, de lin, de moutarde, de palme, d'arachide, de colza, de sésame, de soja, de tournesol, de cameline, de jatropha, de lin, de la graisse de poulet, d'huile de poisson, de suif, d'huile de la cellule unique, et d'huiles de restaurants (Meng et al., 2009; Shannon et al., 2009; Sangat et Kevin, 2010).

Présentement, le biodiesel dérive d'huiles de graines de tournesol ou de canola (colza) en raison de la disponibilité sous une forme de haute qualité et de la facilité à les traiter pour la fabrication du biocombustible. À cause du développement de technologies et de la pression croissante des coûts de l'huile comestible, le biodiesel est aussi obtenu à partir de nombreuses matières premières comme des huiles de maïs, de palme, d'algues, etc. L'emploi de matières premières traditionnelles, les usines d'huiles de graines et des graisses animales, pour la production de biodiesel est insoutenable en raison de la forte concurrence pour les besoins alimentaires et qui se traduit par une augmentation du coût d'obtention. En outre, la

production de biodiesel à partir de ce type de matière première est un processus énergivore (Pimentel et Patzek, 2005). L'exploration de matières premières alternatives est donc primordiale. Les boues d'épuration des eaux usées municipales et industrielles ont également été signalées comme une matière première prometteuse pour la production de biodiesel (Kargbo, 2010). Ces déchets contiennent une forte concentration d'huiles ou de graisses (jusqu'à 25% sur la base du poids de boues sèches), et en outre, ils sont générés en grandes quantités dans le monde entier (Jardé et al., 2005; Dufreche et al., 2007; Mondala et al., 2009; Willson et al., 2010). Les microorganismes oléagineux tels que les bactéries, les champignons, les levures et les microalgues, qui sont abondants et durables, ont été trouvés être une source alternative très comparable en raison de leur taux de croissance rapide (quelques heures à quelques jours), de leur grande teneur en lipides (jusqu'à 80% du poids sec). Ils sont aussi propices à la manipulation génétique pour améliorer leurs profils lipidiques. Finalement, cela libérera des terres arables (Sergeeva et al., 2008; Meng et al., 2009; Vicente et al., 2009a; Cheirsilp et al., 2011).

1.1.2.2 Propriétés des matières premières

De manière générale, il est important que la matière première ait une teneur élevée en lipides, une grande productivité et un prix abordable. Cependant, ses propriétés physiques et chimiques sont essentielles dans la production de biodiesel car ils influent sur la qualité et son rendement. Ces propriétés sont, la composition en acides gras, la teneur en acides gras libres, les teneurs en eau, en phosphore et en soufre, ainsi que l'indice de saponification.

Composition en acides gras: la fraction principale des huiles ou des graisses en matières premières comprend des triglycérides (varie de 90% à 98% selon la source de matières grasses) (Srivastava and Prasad, 2000; Canakci and Sanli, 2008). Les triglycérides sont composés de trois acides gras (R-COOH) et un glycérol [$C_3H_5(OH)_3$]. Ces radicaux d'acides gras sont clairement les principaux groupes réactifs dans les triglycérides, ce qui suggère que les acides gras affectent l'huile. En général, les acides gras insaturés comprennent (avec des doubles liaisons), des mono-insaturés (une double liaison, Cn: 1) et des polyinsaturés (plus d'une double liaison, Cn: 2.3), et saturés (pas de double liaison, Cn: 0) des acides gras. Les structures chimiques des acides gras courants sont présentées au Tableau 1.2. La composition en acides gras joue un rôle

important dans les qualités de biodiesel, soit la viscosité, la stabilité à l'oxydation, l'indice de cétane (CN) (indicateur de la qualité de l'allumage), la propriété d'écoulement à froid, le point d'éclair, le pouvoir calorifique (également appelé contenu en chaleur ou densité d'énergie), et la densité du biocarburant. La viscosité indique les caractéristiques des combustibles dans le processus de pulvérisation, la formation du mélange et la combustion. Une viscosité élevée peut provoquer l'injection précoce et augmenter la température dans la chambre de combustion. En règle générale, la viscosité augmente avec l'augmentation de la longueur de la chaîne carbonée et le niveau de saturation des acides gras. Une meilleure stabilité à l'oxydation nécessite un haut niveau de saturation des acides gras (Deng et al., 2010). L'indice de cétane montre la même tendance que la viscosité, ce qui implique que l'augmentation de nombre de cétane est corrélée à l'élévation de la longueur de la chaîne et de la saturation des acides gras (Içingür et Altiparmak, 2003; Knothe, 2005). Les propriétés d'écoulement à froid dépendent du niveau de saturation. Plus le niveau de saturation est important, plus les propriétés d'écoulement à froid sont faibles (Chapagain et Wiesman, 2009; Ramos et al., 2009). Le point d'éclair est bas lorsque la longueur de la chaîne est courte (Karmakar et al., 2010). Toutefois, plus le niveau de saturation est important, plus le pouvoir calorifique augmente (Karmakar et al., 2010). Le niveau de polyinsaturation semble être proportionnel à la densité (Karmakar et al., 2010).

Teneur en acides gras libres: les acides gras libres sont décrits dans le R-COOH. Il est connu que la trans-estérification alcaline est la voie la plus commune de production de biodiesel dans lequel l'huile/graisse réagit avec de l'alcool pour former des esters méthyliques et du glycérol. Basée sur la voie de la production de biodiesel, la présence des acides gras libres dans l'huile/graisse conduit à accroitre l'utilisation d'un catalyseur, la complication dans les phases de séparation et de neutralisation du produit en raison de la formation de savon (Équation 1.2).

Équation 1.2 $\mathbf{RCOOH + KOH/NaOH \rightarrow RCOOK/Na\ (savon) + H_2O}$

Où R représente des chaînes d'acides gras.

Afin d'éviter la formation de savon, la trans-estérification catalytique acide avec un acide prétraité ou la trans-estérification catalytique alcaline doivent être appliquées dans la production de biodiesel lorsque le contenu en acides gras libres est supérieur à 2% (poids/poids) (Canakci et Van Gerpen, 2001; Wang et al., 2005; Naik et al., 2008).

Table 1.2 Structure chimique des acides gras

Acides gras	Formule chimique	Représentant
Acide laurique	$CH_3(CH_2)_{10}COOH$	C12:0
Acide tridécylique	$CH_3(CH_2)_{11}COOH$	C13:0
Acide myristique	$CH_3(CH_2)_{12}COOH$	C14:0
Acide pentadécylique	$CH_3(CH_2)_{13}COOH$	C15:0
Acide palmitique	$CH_3(CH_2)_{14}COOH$	C16:0
Acide margarique	$CH_3(CH_2)_{15}COOH$	C17:0
Acide stéarique	$CH_3(CH_2)_{16}COOH$	C18:0
Acide oléique	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	C18:1
Acide linoléique	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	C18:2
Acide linolénique	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	C18:3
Acide arachidique	$CH_3(CH_2)_{18}COOH$	C20:0

Teneur en eau: L'eau peut causer l'hydrolyse des triglycérides en acides gras libres, ce qui aboutit à la formation de savon (Anderson et al., 2003; Sanford et al., 2009). Par ailleurs, la présence d'eau pourrait aussi provoquer des émulsions. Par conséquent, lorsque sa teneur est supérieure à 0.05% (poids/poids), l'eau doit donc être éliminée (Sanford et al., 2009).

Teneur en phosphore: le phosphore peut endommager les convertisseurs catalytiques présents dans les systèmes de contrôle des émissions des véhicules (del Río, 2007). La teneur en phosphore dans le biodiesel à partir de matières premières doit être contrôlée pour préserver la fonctionnalité des systèmes de traitement des gaz d'échappement au cours de leur durée de vie opérationnelle des véhicules, et donc pour réduire les émissions de polluants au niveau de l'environnement.

Teneur en soufre: La présence de soufre peut altérer significativement le convertisseur catalytique et donc nuire aux systèmes d'échappement des véhicules (http://www.hydro.mb.ca/your_business/mhl/bio_glossary.shtml). D'une manière générale, la teneur en soufre des matières premières de production de biodiesel est proche de zéro, dans le but de réduire la teneur en soufre du pétrodiesel, le biodiesel est utilisé peut être mélangé avec du pétrodiesel (Sanford et al., 2009).

Indice de saponification (SV): Un indice de la taille moyenne des acides gras. Comme mentionné précédemment, les FAMEs avec une longueur de chaîne de 12 à 20 C sont

constitutifs du biodiesel. La valeur de saponification indique la longueur de la chaîne de triglycérides. La longueur de chaîne courte entraîne une augmentation de l'Indice de SV (http://www.thebioenergysite.com/articles/482/feedstock-and-biodiesel-characteristics-report).

Les propriétés de la matière première employée dans la production de biodiesel sont fournies au Tableau 1.3. En comparant les propriétés de la matière première, on voit que l'huile microbienne a des propriétés similaires aux matières premières traditionnelles, huiles végétales et de graisses animales. En outre, comme mentionné précédemment, l'huile microbienne est abondante et durable. Par conséquent, les microorganismes sont considérés comme une alternative favorable pour la production du biocarburant.

Table 1.3 Propriétés de la matière première à la production de biodiesel

Matière première	SL (%)	FFA (%)	WC (%)	PC (ppm)	SC (ppm)	SV (mg KOH g^{-})	Références
Huile de soja	15.34	0.07	0.029	3.7	0.8	195.3	(Canakci et Van Gerpen, 2001; Sanford et al., 2009)
Huile de tournesol	9.34	0.04	0.02	<0.1	0.1	193.14	(Goering et al., 1982; Sanford et al., 2009)
Huile de palme	47.3	0.54	0.049	7.3	1.0	208.62	(Demirbas, 2003a; Sanford et al., 2009)
Huile de canola	4.34	0.34	0.085	17.9	5.7	189.80	(Goering et al., 1982; Sanford et al., 2009)
Huile de maïs	14	12.22	0.153	<0.1	10.5	183.06	(Goering et al., 1982; Demirbas, 2003a; Sanford et al., 2009)
Huile d'arachide	16	<2	<0.5	NA	10	191.50	(Demirbas, 2003a; Barnwal et Sharma, 2005; Ahmad et al., 2009)
Huile de coco	68.7	0.07	0.027	2.0	2.7	267.56	(Demirbas, 2003a; Sanford et al., 2009)
Huile de pourghère	27.1	1.17	0.073	322.9	3.5	200.80	(Elvin-Lewis, 1988; Sanford et al., 2009)
Graisses de volailles	29.69	1.7	0.065	209.3	27.2	188.08	(Exler et al., 1995; Sanford et al., 2009)
Lard	41-50	<18	0.048	<10	100	195	(El-sharkawyL et al., 1993; Lee et al., 1995)
Suif de boeuf	47-63	1.61	0.051	270.8	25.2	198.00	(Lee et al., 1995; Canakci et Sanli, 2008; Sanford et al., 2009)
Graisse brune	37.03	7.38-40	0.485	132.1	30.7	198.36	(Ngo et al., 2007; Sanford et al., 2009)
Déchets d'huile de caisson	55-90	2.72-7.25	0.242	27.0	3.4	198.50	(Rice et al., 1997; Meng et al., 2008; Sanford et al., 2009)
Déchets de friteuse	87.8	5.60	7.3	NA	NA	177.87	(Alcantara et al., 2000; Issariyakul et al., 2007)
Microalgues	12-21	0.45-1.75	0.014-0.021	286.2-339.7	15.4-28.1	160.6-185.82	(Meng et al., 2009; Sanford et al., 2009)
Bactéries	19-22	<1	De	Tr	Tr	NA	(Alvarez et Steinbüchel, 2002)
Levures	12-47	<1	De	Tr	Tr	NA	(Alvarez et Steinbüchel, 2002; Papanikolaou et Aggelis, 2011)
Champignons	9-29	0.5-31.6	De	Tr	Tr	NA	(Alvarez et Steinbüchel, 2002; Papanikolaou et al., 2004; Vicente et al., 2009b)
Huile de boues	75	65	De	NA	NA	NA	(Boocock et al., 1992; Willson et al., 2010)

SL: le niveau de saturation; FFA: acides gras libres; WC: teneur en eau; PC: teneur en phosphore; SC: teneur en soufre; SV: indice de saponification; De: dépend du séchage; Tr: tracer montant; NA: pas disponible.

1.1.2.3 Production microbienne de lipides pour la production de biodiesel

Jusqu'à ce jour, des microbes (autotrophes et hétérotrophes) ont été étudiés pour l'accumulation de lipides dans le but de produire du biodiesel. Les microbes autotrophes principalement des micro-algues sont capables d'absorber le dioxyde de carbone et de l'énergie solaire pour synthétiser des composés organiques tels que les protéines et les lipides pour leur croissance et qui dépend fortement de la lumière. Le faible rendement de la productivité de la biomasse (normalement, 0.15 à 1.5 kg m^{-3} d^{-1}) a également entravé l'application des microbes autotrophes dans l'obtention de biodiesel (Benemann et al., 2006; Alabi et al., 2009). Contrairement aux microbes autotrophes, les microbes hétérotrophes sont plus flexibles pour leurs conditions de culture et pourraient fournir même dix fois plus de biomasse (Chisti, 2007; Johnson et Wen, 2009b). En général, leur teneur en lipides est plus élevée, car ils sont plus faciles à manipuler pour l'accumulation de lipides (Miao et Wu, 2006; Cao et al., 2010). *Rhodococcus opacus*, *Cryptococcus curvata*, *Lipomyces starkeyi*, *Rhodosporidium toruloides*, et *Mortierella isabellina* sont couramment utilisés comme microorganismes oléagineux pour la production des lipides.

Le glucose est normalement utilisé comme source de carbone lors de la culture de micro-organismes pour l'accumulation de lipides, mais il est assez cher (Gouda et al., 2008; Cheng et al., 2009a). Actuellement, les déchets organiques tels que le perméat de lactosérum, la canne à sucre, la paille de riz, les eaux usées, et le glycérol brut (sous-produit de la production de biodiesel), ont été sollicités comme source de carbone ou même comme milieu de culture pour la production de lipides en employant des microorganismes oléagineux (Gouda et al., 2008b; Gao et al., 2010; Liang et al., 2010; Gonzalez-Garcia et al., 2013). L'utilisation des déchets pour l'accumulation de lipides serait une voie prometteuse pour la production de lipides car elle réduit le coût de production et fournit une solution aux problèmes de gestion des déchets.

1.1.3 Extraction des lipides à partir des microorganismes

La production de biodiesel à partir des lipides accumulés par des microorganismes comprend trois étapes, la culture du microbe (accumulation de lipides), l'extraction des lipides (séparation des lipides à partir de biomasse), et la synthèse de biodiesel. L'extraction des lipides est une étape critique dans cette production. Les solvants organiques tels que l'hexane, le méthanol, le chloroforme et le mélange de méthanol sont actuellement mis en œuvre dans l'extraction. Le chloroforme et le méthanol ont été jugés plus efficaces pour l'extraction des lipides à partir de microorganismes (Vicente et al., 2009; Cheirsilp et al., 2011; Cheng et al., 2011; Boyd et al., 2012). Toutefois, les inquiétudes sur l'inflammabilité et la haute toxicité du chloroforme conduisent à rechercher de nouvelles technologies moins dangereuses pour la santé et l'environnement. En outre, l'extraction des lipides à partir des microorganismes avec le chloroforme et le méthanol exige un long temps de réaction (de 4 à 12 h) et s'effectue à haute température (50 à 60°C). Par conséquent, la reduction ou l'élimination de la quantité de solvants toxiques utilisés, ainsi que la diminution du temps d'extraction et de la température est la solution clé du problème.

Les lipides sont un produit intracellulaire des microorganismes présents dans la membrane cellulaire (pour former la bicouche) et le cytoplasme (sous la forme de gouttes de lipides). Afin d'obtenir les produits intracellulaires, la rupture des cellules est nécessaire pour libérer le produit avant d'effectuer une séparation supplémentaire. Par conséquent, la perturbation de la cellule est une étape critique dans séparation des lipides par les cellules. La désintégration des cellules s'effectue avec un broyeur à billes, une homogénéisateur, des micro-ondes ou un bain d'ultrasons avant l'extraction par un solvant, ce qui pourrait réduire la quantité de solvant utilisée ainsi que le temps de procédé (Ranjan et al., 2010; Araujo et al., 2013).

1.1.4 Trans-estérification

Plusieurs technologies ont été développées pour produire du biodiesel, comme l'utilisation de microémulsions, la pyrolyse, et la trans-estérification, qui est le processus le plus commun, car il produit du biodiesel de haute qualité (Doll et al., 2008; Macala et al., 2008; Suarez et al., 2009). On distingue la trans-estérification catalytique de la trans-estérification non-catalytique (présence ou non de catalyseur). En général, le catalyseur favorise un taux convenable de conversion de biodiesel en peu de temps (plusieurs heures), mais le traitement en aval est plus complexe. L'absence de catalyseur nécessite une haute pression et une haute température pour atteindre un taux élevé de conversion, impliquant une grande consommation d'énergie. La sélection de catalyseurs pour la trans-estérification est déterminée par le rapport pétrole brut/propriétés des graisses. La teneur en acides gras libres (AGL) dans la graisse/huile est un facteur majeur car il provoque la formation de savon en présence de catalyseur alcalin, ce qui consomme beaucoup de matériel et réduit le rendement de production du biodiesel. Normalement, la réaction avec le catalyseur alcalin n'est pas préférable lorsque la teneur en acides gras libres est supérieure à 2% d'huile/graisse. Sinon, une préalable étape de trans-estérification pour convertir les AGL en biodiesel en présence d'un catalyseur acide suivie d'une deuxième étape en présence d'un catalyseur alcalin peuvent être appliquées (Sánchez et al., 2011; Chen et al., 2012).

La trans-estérification consiste à convertir les lipides extraits en biodiesel. Récemment, une transformation directe des lipides en biodiesel sans l'étape de séparation/extraction a été étudiée. Le processus est aussi appelé trans-estérification *in-situ*. La technologie est prometteuse puisqu'elle évite l'extraction des lipides, processus nécessitant une grande consommation d'énergie et donc un coût élevé.

La différence de la trans-estérification *in-situ* à la trans-estérification normale est d'utiliser des substances porteuses des lipides à la place de lipides (Figure 1.2). Les lipides sont directement en contact avec le méthanol et le catalyseur, d'où une réaction plus facile que celle *in-situ*. Le long temps de réaction ou la plus grande quantité de méthanol sont constatés durant le processus et les technologies de perturbation des cellules doivent être ajoutées simultanément dans la trans-estérification *in-situ*.

La trans-estérification *in-situ* des lipides à partir des micro-algues, alcaline est faite à 60 °C (8 h) et requiert un temps trois fois plus élevés pour atteindre le taux de conversion similaire à celle en utilisant la trans-estérification normale (moins de 2 heures) dans les mêmes conditions (Ehimen et al., 2010). En outre, il a été signalé que le rapport molaire méthanol par rapport à l'huile (300 à 900:1) est nécessaire pour obtenir le taux de conversion élevé (Samuel et Dairo, 2012). Le temps de réaction élevé ainsi que les grandes quantités d'alcool additionnées, nécessitent l'amélioration de processus de trans-estérification *in-situ*. Le traitement de la rupture des cellules pour améliorer le contact entre lipides et réactif (alcool) permet d'aider le processus.

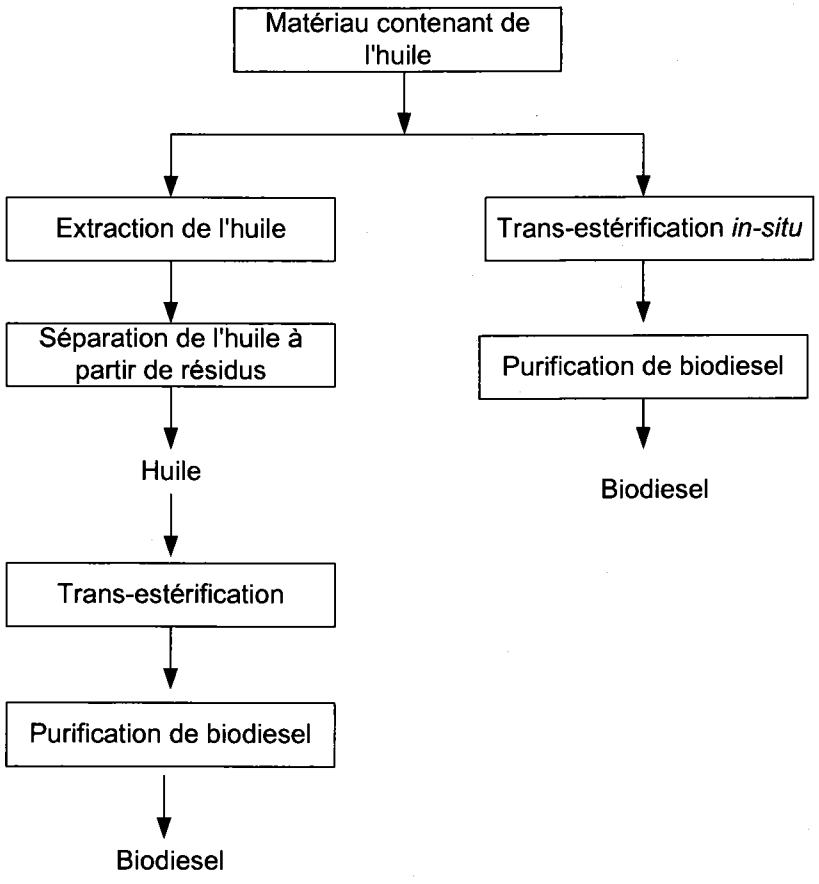


Figure 1.2 **Schéma de trans-estérification normale et trans-estérification *in-situ* pour la production de biodiesel à partir de matériel contenant de l'huile**

1.2 Problématique

À la suite de l'analyse bibliographique, les problèmes suivant a ont été mis en évidence:

1.2.1 Crise énergétique et environnement

Actuellement, les combustibles fossiles jouent un rôle important dans l'industrie, l'agriculture et les transports. Toutefois, 50% du pétrole disponible sur la planète est déjà épuisé (Annie, 2006). Au rythme actuel de la consommation, les réserves seront épuisées d'ici 32 ans (Singh et Singh, 2010). En outre, les émissions de GES causées par la combustion de combustibles conventionnels deviennent une préoccupation majeure pour leur rôle joué dans les changements climatiques. Par conséquent, il existe un besoin croissant pour le développement de sources d'énergies alternatives qui soient durables et respectueuses pour l'environnement.

1.2.2 Valorisation des boues

Les boues, générées en grandes quantités dans le monde entier au cours du traitement des eaux usées municipales et industrielles, ont attiré une grande attention attribuable à leur potentiel de valorisation. Environ un million de tonnes de boues d'épuration sont produites chaque année au Canada. Une petite quantité de boues (30%) sont destinées pour l'agriculture, le reste est enfoui ou incinéré (Jardé et al., 2005), ce qui contribue à des émissions de GES, d'où la nécessité de transformer les boues en produits à valeur ajoutée.

1.2.3 Gestion du glycérol brut (sous-produit de la production de biodiesel)

La trans-estérification de biodiesel génère du glycérol brut qui constitue environ 14% (p/p) du biodiesel produit (Canakci et Sanli, 2008). Comme la demande de biodiesel est croissante, il est à craindre que cela entraîne des problèmes de gestion et de valorisation de glycérol brut. Bien que ce dernier puisse être utilisé pour des applications pharmaceutiques, la production d'éthanol et du savon, les volumes de ce sous-produit générés par les activités de production de biocarburant ont suscité des recherches pour développer d'autres stratégies de récupération des ressources.

1.2.4 Coût élevé des matières premières pour la production de biodiesel

Les huiles végétales et les graisses animales constituent la principale source de production de biodiesel dans l'industrie (Singh et Singh, 2010). Toutefois, leur prix augmente progressivement en raison de la concurrence avec l'industrie alimentaire et la restauration. En outre, la longueur de la durée de vie (deux ans de plus par an) et les grandes terres prises pour la production rendent l'utilisation de ces huiles inadaptée. De ce fait, il est essentiel d'identifier d'autres sources pour la production de biodiesel.

1.2.5 Coût élevé de la source de carbone pour la production d'huile de microorganismes oléagineux

Les recherches antérieures portant sur la production d'huile bactérienne utilisaient un milieu synthétique contenant du glucose comme source de carbone, des minéraux et/ou des sources d'azote organique ainsi que d'autres ingrédients (Meesters et al., 1996; Papanikolaou et al., 2004a). Le prix de ces ingrédients peut représenter de 40 à 60% du coût total de production (Papanikolaou et Aggelis, 2002; Zhao et al., 2010). Pour réduire le coût des huiles microbiennes, la recherche de sources de carbone alternatives permettant d'obtenir un haut rendement et une haute productivité de l'huile est nécessaire. Quelques études ont été effectuées sur la croissance de microorganismes oléagineux sur les déchets après hydrolyse enzymatique (de l'amidon, les déchets de transformation de l'amidon de pomme de terre, les déchets cellulosiques) et l'accumulation de pétrole atteint jusqu'à 50% (Li et al., 2008a). Cependant, l'hydrolyse enzymatique est extrêmement coûteuse car elle implique la production de l'enzyme suivie de l'hydrolyse des déchets. Du point de vue économique, l'accumulation d'huile chez les microorganismes doit donc être obtenue en employant directement les déchets (sans nécessité d'hydrolyse enzymatique) tout en obtenant une forte concentration d'huile pouvant être convertie en biodiesel.

1.2.6 Préoccupation de la méthode actuelle d'extraction des lipides

L'utilisation de produits chimiques toxiques dans le processus d'extraction des lipides conventionnels freine l'application d'huiles à cellule unique (SCO) dans la production de biodiesel.

La trans-estérification d'huile microbienne extraite de microorganismes nécessite une grande quantité de solvant organique généralement le chloroforme et le méthanol, ce qui implique une grande consommation d'énergie. Ceci pourrait entraver la production de biodiesel à partir de microorganismes. Il est donc primordial de développer un procédé plus écologique et rentable pour la production de biodiesel à partir de microorganismes.

1.3 Hypothèses et objectifs de recherche

1.3.1 Hypothèses

Afin d'étudier la faisabilité de la production de biodiesel à partir d'huiles de boues et d'huile obtenues à l'aide de microorganismes oléagineux, mis en culture avec des boues et du glycérol pur (produit intermédiaire de la trans-estérification), les hypothèses suivantes doivent être vérifiées:

- L'utilisation de l'huile de boues et des lipides accumulés par des microorganismes, croissant sur des boues d'eaux usées ou du glycérol brut, pour la production de biodiesel, peut permettre un bilan énergétique favorable et un coût de production plus faible, puisque les boues et le glycérol sont produits en grande quantité et à un faible prix.
- La production de biodiesel en employant des boues comme substrat réduirait grandement les émissions de gaz à effet de serre, puisque cela éviterait l'émission de méthane depuis les décharges de boues.
- De nombreux microorganismes sont connus pour être capables d'accumuler des lipides en utilisant des déchets comme source de carbone. Il est possible de produire des lipides en utilisant des boues (contenant de nombreux nutriments comme le carbone, l'azote et le phosphore) et du glycérol brut.
- Le prétraitement de boues augmenterait la production de lipides par les microorganismes grâce à la libération du carbone disponible. Les surnageants issus du prétraitement peuvent être utilisés pour produire de l'engrais par précipitation de struvite, c'est-à-dire du magnésium ammonium phosphate.
- Le ratio carbone/azote peut avoir un impact important sur l'accumulation des lipides par les microorganismes oléagineux. L'ajout de carbone au sein des boues pour augmenter le ratio C/N pourrait permettre d'atteindre une plus forte accumulation de lipides.
- La méthode conventionnelle d'extraction par le méthanol et le chloroforme requiert une longue période de manipulation (12 h) et l'utilisation d'un produit chimique hautement toxique (chloroforme) pour la séparation des lipides de la biomasse. L'utilisation d'ultrasons réduirait fortement le temps d'extraction puisqu'elle permet de perturber rapidement les

cellules. De plus, une trans-estérification *in-situ* couplée aux ultrasons pourrait rapidement convertir les lipides présents dans la biomasse en biodiesel sans passer par une étape d'extraction des lipides.

1.3.2 Objectifs de recherche

L'objectif principal de ce projet de recherche est d'évaluer la faisabilité de la production de biodiesel à partir des boues et d'huile de microorganismes se développant dans des boues ou du glycérol brut. Par ailleurs, le but de ce travail est d'améliorer le procédé d'extraction des lipides ainsi que la trans-estérification.

Les objectifs spécifiques concernent selon le cas, l'étude, la détermination ou l'évaluation de:

1. La teneur en lipides des différents types de boues d'épuration et évaluation de différentes boues d'épuration et leur effet sur l'accumulation lipidique des microbes;
2. L'effet du prétraitement des boues sur l'accumulation de lipides chez les microbes et la précipitation de struvite;
3. L'équilibre énergétique du biodiesel produit à partir de boues et d'huile de microorganismes suivie par une estimation des émissions de GES;
4. Coûts de biodiesel produit à partir de boues et des huiles de la cellule unique (SCO) à partir de boues (traitée ou non);
5. L'accumulation de lipides chez les microbes cultivés en présence de glycérol brut;
6. Bilan énergétique et de la masse de la production de biodiesel en présence du glycérol brut;
7. Coûts de production des lipides avec du glycérol brut;
8. L'impact des ultrasons sur l'extraction des lipides;
9. Application des ultrasons sur le processus de la trans-estérification *in-situ*.

1.3.3 Originalité du travail

Le bilan énergétique, les émissions de gaz à effet de serre, autant que le coût de la production de biodiesel représentent les paramètres critiques pour la faisabilité du procédé. Aucun travail n'a été réalisé jusqu'à présent au niveau de ces aspects de la production de biodiesel à partir d'huiles provenant de boues et de glycérol brut. Ces études sont donc réalisées pour la première fois.

L'utilisation des boues d'eaux usées brutes et prétraitées comme seule matière première dans le but de produire du biodiesel est nouvelle. La complexité des boues ajoute de nouveaux défis pour la production d'huile en utilisant des microorganismes unicellulaires.

En plus de la production de lipides à partir de microorganismes oléagineux par l'utilisation de boues, une lente libération d'engrais sera générée de manière simultanée, ce qui n'a pas été encore étudié.

Les facteurs d'impact de l'extraction des lipides par ultrasons, incluant la température, la fréquence et la puissance de sonication, le type de solvant et les différentes variétés de microorganismes, n'ont pas été suffisamment étudiés. Ces travaux de recherche révèleront leur effet sur l'extraction.

La trans-estérification *in-situ* pour la production de biodiesel en une étape est une alternative attrayante et économique par rapport à l'actuelle méthode en deux étapes (extraction des lipides et trans-estérification). L'étude a analysé l'ajout d'ultrasons dans la trans-estérification *in-situ* des lipides de levures pour réduire le temps de réaction et la quantité de méthanol à ajouter, ce qui n'a pas été décrit ailleurs.

1.4 Résultats et discussion

Les résultats de cette thèse de l'étude sont divisés en quatre parties: 1) La faisabilité de la production de biodiesel à partir de microorganismes oléagineux en utilisant les boues d'épuration comme matières premières (Étude de l'accumulation des lipides dans les microorganismes et la similarité entre le profil du biodiesel dérivé des boues et celui du biodiesel commercial, estimation du bilan énergétique et du coût de production du biodiesel); 2) Le potentiel de production de biodiesel à partir de microorganismes oléagineux en utilisant le glycérol brut comme matière première (Étude de l'accumulation des lipides dans les microorganismes et la similarité entre le profil du biodiesel dérivé du glycérol brut et celui du biodiesel commercial, estimation du bilan énergétique et du coût de production du biodiesel); 3) L'extraction des lipides par ultrasons avec la variation de la température, du solvant, de la fréquence et la puissance des ultrasons, et les matières premières; 4) La trans-estérification *in-situ* des lipides accumulés dans les microorganismes et les boues en biodiesel, assistée par l'ultrasons.

1.4.1 Faisabilité de la production de biodiesel à partir de microorganismes oléagineux en utilisant les boues d'épuration comme matière première

1.4.1.1 Production de biodiesel à partir d'huile dérivée de boues (objectif 1)

La hausse du prix des matières premières traditionnelles, y compris les huiles végétales et les graisses animales, pour la production de biodiesel incitent la société en général à chercher des sources alternatives de pétrole. Les boues sont naturellement et largement produites, partout dans le monde, et contiennent des lipides. Dans cette étude, les huiles dérivées de boues ont été utilisées pour la production de biodiesel. Différents types de boues, comprenant en autre des rejets municipaux primaires, secondaires, mixtes, et des boues secondaires de pâte à papier collectées dans la ville de Québec (Canada) ont été utilisées pour étudier la teneur en lipides dans les boues d'origine et l'impact sur l'accumulation de lipides par *Pichia amethionina* sp., *Galactomyces* sp. et *Trichosporon oleaginosus* (Chi et al., 2011) parce que leur grande adaptation aux boues, quand elles sont utilisées comme milieux de culture. Il a été constaté que les teneurs en lipides étaient respectivement de 6.8%, 5.3% 6.4% et 10.9% p/p dans les

boues municipales primaires, les boues secondaires, les boues mixtes, et les boues secondaires de pâtes à papier. Les lipides contenus dans les boues primaires proviennent principalement des rejets de l'homme et des déchets ménagers. Par contre, les lipides des boues secondaires (municipales ou de pâte à papier) contiennent principalement de la biomasse. Par conséquent, les lipides dans les boues secondaires proviennent principalement des cellules. La boue mixte est un mélange de (1:1 v/v) de boue primaire et de boue secondaire. Les lipides de la boue mixte sont donc un mélange des lipides contenus dans ces deux types de boues.

L'effet des différents types de boues sur l'accumulation de lipides a montré que la teneur maximale en lipides (30.1%, 31.6% et 36.0% p/p dans *Pichia amethionina* sp., *Galactomyces* sp. et *Trichosporon oleaginosus*, respectivement) a été obtenue dans les boues secondaires municipales. Ceci est dû au fait que les boues secondaires sont plus biodégradables que les autres boues testées. Le contenu en lipides dans un milieu synthétique est plus élevé (58.6%, 53.3% et 61.7% p/p dans *Pichia amethionina* sp., *Galactomyces* sp. et *Trichosporon oleaginosus*, respectivement) que celui dans les boues. Ce fait résulte de la présence de substances non biodégradables telles que les fibres dans les boues, et qui persistent durant la fermentation. La teneur en lipides a été calculée en se basant sur la quantité de lipides dans les matières en suspension sèches totales, qui contiennent principalement les fibres et la biomasse. Par conséquent, la teneur en lipides dans la boue est faible.

De plus, l'effet de la concentration initiale en matières en suspension des boues secondaires municipales (10 à 30 g/L) sur l'accumulation de lipides a été étudié. Des teneurs maximales en lipides de 30.2 et 32.4% p/p sec, par *Pichia amethionina* sp. et *Galactomyces* sp., respectivement ont été obtenues à une concentration en matières solides en suspension de 25 g/L. Par contre, la teneur maximale en lipides accumulée par *Trichosporon oleaginosus* a été de 37.7% p/p sec en utilisant une concentration en matières solides en suspension de 30 g/L. Le rapport carbone-azote (C/N) a été ajusté avec l'ajout de glucose ou de glycérol. Il a été observé que le rapport C/N avait plus d'effet sur l'accumulation des lipides dans les trois souches, quand la concentration en matières solides en suspension était faible (10 g/L).

Le profil des acides gras a révélé que les principaux composés des huiles dérivées à partir des boues (boue municipale primaire, boue municipale secondaire, boue municipale mixte, et les

boues secondaires de pâte à papier) sont le C16:0 et C18:0. Ces acides gras sont fortement présents dans l'huile de soja. Cependant, les fractions de saturation dans les boues d'origine et les boues fermentées par *Pichia amethionina* sp. *Galactomyces* sp. et *Trichosporon oleaginosus* étaient plus élevées que celles dans l'huile de soja. Ceci suggère que le biodiesel dérivé de boue a une stabilité à l'oxydation et une densité plus élevées que celles du biodiesel dérivé de l'huile de soja (le volume de la chambre pouvant être réduit dans les véhicules). En outre, le biodiesel dérivé d'huile de soja a une viscosité plus faible que celle du biodiesel provenant des boues.

1.4.1.2 Production de lipides à partir de microorganismes cultivés dans les boues prétraitées (objectif 2)

Des études ont montré que les microorganismes peuvent utiliser les boues d'épuration pour produire des lipides. Ainsi, des traitements thermiques et chimiques (acide et basique) ont été effectués pour améliorer la disponibilité des nutriments dans les boues. Les boues municipales secondaires à différentes concentrations de solides en suspension (10 à 30 g/L) ont été utilisées comme matière première pour la production de lipides par *Trichosporon oleaginosus*. Les résultats ont montré que le prétraitement chimique et thermique conduit à une forte augmentation de la concentration de carbone organique dissous (de 1.5 à 6.0 g/L) et de l'azote dissous (0.45 à 1.8 g/L) dans les boues. Une augmentation en teneur de lipides (environ 39% p/p) a été révélée par *Trichosporon oleaginosus* pendant 48 h pour les boues thermiques prétraitées, la teneur maximale en lipides étant atteinte en 42 h dans les boues thermochimiques prétraitées.

En outre, un engrais (struvite) formée dans le surnageant de boues prétraitées a été marquée par l'addition de Mg^{2+}. Après l'élimination de la struvite, le liquide résiduel a été rajouté à la boue solide pour l'étude de l'accumulation de lipides. La même teneur en lipides est obtenue en utilisant des boues prétraitées sans formation de struvite.

Le biodiesel produit par les boues prétraitées avec ou sans formation de struvite contient principalement C16 et C18, qui sont également riches dans le biodiesel commercial produit à partir de graines de soja, de canola et d'huiles de tournesol. Il montre ainsi, que les boues d'épuration peuvent être utilisées comme matière première pour la production de biodiesel.

1.4.1.3 Bilan énergétique et émissions de GES de la production de biodiesel à partir d'huiles produites avec des eaux usées et des boues d'épuration (objectif 3)

Les huiles issues de microorganismes et les boues d'épuration sont connues comme des matières premières pour la production de biodiesel, lesquelles étaient jusqu'à ce jour énergivores et coûteuses. Le bilan énergétique et les émissions de gaz à effet de serre (GES) sont des facteurs essentiels pour évaluer la faisabilité d'un procédé de production de biodiesel. Cette étude a évalué le bilan énergétique et les émissions de GES de la production de biodiesel à partir d'huile microbienne et des boues des eaux usées. Les résultats de la production de biodiesel à partir de boues d'origine ont montré que l'étape de trans-estérification *in-situ* et l'étape d'extraction des lipides suivie par trans-estérification ont fourni respectivement un gain énergétique net de 26.2 GJ et 29.4 GJ par tonne de biodiesel produit. Pour les boues d'origine et les boues prétraitées utilisées par les microorganismes comme milieux nutritifs pour accumuler des lipides (plus convertie en biodiesel), les gains nets d'énergie étaient 37.0 GJ et 15.6 GJ par tonne de biodiesel produit. Le faible gain d'énergie pour l'utilisation des boues prétraitées est du à l'utilisation de produits chimiques (NaOH) et de la vapeur (condition d'état thermique). Dans le même cas, le bilan énergétique de la production de biodiesel avec des micro-algues a été également étudié. Pour les microorganismes phototrophes (micro-algues), étang ouvert et système de bioréacteur, ont montré respectivement un gain énergétique net de 19.1 GJ et 15.6 GJ par tonne en biodiesel produit. Pour les microorganismes hétérotrophes, le bilan énergétique dépend du type de source de carbone. Le gain énergétique net par tonne en biodiesel produit est -1.5 GJ pour l'amidon, 11.8 GJ pour la cellulose, et 27.2 GJ pour l'amidon des eaux usées industrielles (SIW). L'étude indique que les boues utilisées comme éléments nutritifs par les microorganismes pour la production de lipides est la méthode la plus favorable en termes de bilan énergétique en comparant avec d'autres méthodes de production de biodiesel.

Ainsi, les études des GES ont montré que la production de biodiesel à partir de microorganismes ou par les huiles accumulées, est un procédé de capture du dioxyde de carbone net dans le cas ou l'amidon est utilisé comme matière première pour la production

d'huile microbienne, avec un taux de capture qui est de l'ordre de 40 tonnes de dioxyde de carbone par tonne de biodiesel produit.

1.4.1.4 Estimation des coûts de production du biodiesel à partir des huiles derivées de boues d'épuration (objectif 4)

Le coût de production du biodiesel à partir des boues d'épuration utilisées comme matière première avec une capacité de 260 tonnes des boues sèches par jour a été estimée en utilisant le logiciel SuperPro Designer.

Selon les résultats obtenus, les boues d'origine ont une teneur en lipides d'environ 10% p/p de boues sèches, alors que le coût du biodiesel produit à partir de lipides extraits de boues d'origine est d'environ 0.4 US$/kg de biodiesel. De plus, les boues peuvent être employées pour accumuler des lipides par des microorganismes oléagineux avec une teneur en lipides d'environ 40% (p/p biomasse). En conséquence, le coût de production de biodiesel est de 0.5 US$/kg. Il a été noté que l'utilisation directe des boues comme source de lipides est plus rentable pour l'accumulation de lipides, même si la teneur en lipides est faible (seulement 11%), car les boues utilisées comme sources nutritives pour l'accumulation de lipides nécessitent la culture et la récolte de microorganismes coûteux.

Par ailleurs, les études de sensibilité ont montré que le contenu lipidique des boues de biomasse a un effet positif sur les coûts d'obtention. Lorsque la teneur en lipides est augmentée, le coût de production est diminué. Les prix sont respectivement de 0.5, 0.4 et 0.3 US$ kg pour des teneurs en lipides de 40%, 50% et 60% (p/p boues-biomasse). Le traitement de biomasse résiduelle a montré une légère variation sur le coût. Les coûts sont réduits autour de 3 à 6 cents par kilogramme de biodiesel produit lorsque les boues résiduelles sont utilisées comme engrais par rapport à la mise en décharge résiduelle.

1.4.2 Faisabilité de la production de biodiesel à partir de microorganismes oléagineux avec le glycérol brut

1.4.2.1 Valorisation du glycérol brut dans la production du biodiesel (objectif 5)

L'augmentation spectaculaire de la demande de biodiesel conduit à sa production en grande quantité. Le glycérol brut est un sous-produit de la production de biodiesel par trans-estérification, qui est généré simultanément (environ 0.10 à 0.14 kg par kilogramme de biodiesel produit). En fait, de nombreux microorganismes peuvent utiliser le glycérol comme source de carbone pour la croissance. Le glycérol brut, fourni par une usine de production de biodiesel (Québec, Canada), a été utilisé pour l'accumulation de lipides dans *Trichosporon oleaginosus*. Dans un premier temps, la composition de glycérol brut a été évaluée et une grande teneur en savon (21.1% p/p) a été trouvée. Une purification a été effectuée pour enlever le savon en le transformant en acide gras libre à faible pH, et le glycérol purifié a été utilisé pour faire croître les microorganismes. Les résultats ont montré que le glycérol purifié (teneur en lipides: 44.3% p/p de la biomasse) a permis une accumulation des lipides dans la souche microbienne plus élevée que celle obtenue par le glycérol brut (teneur en lipides: 37.2% p/p de la biomasse). Il serait dû à la présence de savon dans le glycérol brut. Le savon ainsi que la surface des cellules sont polaires, et donc le savon pourrait se fixer facilement sur la surface des cellules, ce qui affectera négativement sur la croissance cellulaire, la couche de savon pouvant provoquer l'inhibition du transfert des nutriments.

L'effet de la concentration du glycérol (25 à 100 g/L) sur l'accumulation des lipides a été réalisée avec du glycérol purifié, en raison de sa performance dans l'accumulation de lipides plus élevée que celle du glycérol brut. La concentration optimale du glycérol a été de 50 g/L. Cette concentration a donné le plus haut rendement de lipides (0.19 g de lipides/g glycérol). Il a été observé que l'augmentation de la concentration de glycérol réduit le rendement des lipides, qui pourrait être dû à l'inhibition par l'excès du substrat.

Le profil des acides gras a démontré que les acides gras majoritaires sont C16:0 et C18:1 comme l'huile de graines de Jatropha, qui est utilisé dans la production du biodiesel commercial. Ceci

suggère que les lipides accumulés par *Trichosporon oleaginosus* cultivée sur du glycérol sont adaptés à l'utilisation comme matières premières pour la production de biodiesel.

1.4.2.2 Bilan énergétique net de la production de biocarburants à partir de glycérol brut (objectif 6)

Le glycérol brut a été largement étudié dans la production de biocarburants. Le but de cette étude est d'évaluer le bilan énergétique de la production de biodiesel, d'hydrogène, de biogaz et d'éthanol à partir de glycérol brut. Le calcul est basé sur l'utilisation de 3.5 millions de litres de glycérol brut (80% p/p) par an, ce qui correspond à produire 925 650 kg de biodisel, 1 513 346 m^3 d'hydrogène, 556 948 m^3 de biogaz, et 1 030 353 kg d'éthanol. Il a été constaté que l'utilisation de glycérol brut pour produire des lipides et les transformer par la suite en biodiesel fournit un gain net d'énergie de 8 430.56 GJ pour 0.93 million kg de biodiesel produit. La production d'hydrogène en utilisant le glycérol brut a donné un gain énergétique net de 3 GJ par litre d'hydrogène produit. Le glycérol brut utilisé pour la production d'hydrogène, la production de biogaz et la production d'éthanol a un bilan énergétique négatif (l'apport est supérieur à la production). Les résultats indiquent que l'utilisation de glycérol brut pour la production de biodiesel est faisable en termes de gain d'énergie.

1.4.2.3 Estimation des coûts de production de lipides à partir du glycérol brut (objectif 7)

L'accumulation de lipides dans les microorganismes oléagineux en utilisant le glycérol brut comme source de carbone a été observée en laboratoire. La charge de biodiesel actuellement utilisée est principalement l'huile de soja qui est coûteuse et utilisée dans la production alimentaire. Afin d'évaluer la faisabilité des coûts d'utilisation du glycérol brut comme matière première dans la production du biodiesel, le logiciel SuperPro Designer, a été employé dans l'étude. Un procédé de production des lipides comprenant la fermentation et la séparation des lipides a été conçu avant le calcul. L'étude a été réalisée sur la base 1 000 000 kg de lipides produits par an.

L'estimation a révélé que le coût du biodiesel produit à partir de glycérol était d'environ 0.44 US$/kg de lipides lorsque le temps de fermentation était de 48 h, le rendement de la biomasse était de 0.63 g/g de glycérol, la teneur en lipides est de 60% (p/p biomasse), et la

récupération des lipides est de 95% (p/p). Une étude de sensibilité a montré que les taux de lipides et la taille de l'usine ont un grand impact sur le coût de production unitaire des lipides. Le coût unitaire des lipides était respectivement de 0.91, 0.44 et 0.16 US$ pour 0.4, 1 et 5 millions de kg de lipides produits. Chaque augmentation du contenu en lipides de 10% a entraîné une réduction du cout de 0.1 à 0.2 US$ par kg de lipides produite selon la taille de l'usine de production.

1.4.3 Extraction des lipides par ultrasons à partir de microorganismes oléagineux et des boues (objectif 8)

La production de biodiesel à partir de microorganismes comprend trois étapes: la culture du microorganisme (accumulation des lipides), l'extraction des lipides (séparation des lipides à partir de la biomasse), et la synthèse du biodiesel. L'extraction des lipides, qui est une étape centrale dans la production, est critique. Le traitement par chloroforme et méthanol est une méthode couramment employée pour la séparation des lipides à partir de microorganismes. Ce procédé est efficace mais lent (environ 12 h) et nécessite une température modérée (50 à 60 °C). Dans cette étude, des ultrasons ont été appliqués pour améliorer l'extraction. Différents solvants, y compris l'eau, l'hexane, le méthanol et un mélange chloroforme méthanol (1:1 v/v), ont été testés pour identifier l'efficacité de l'extraction des lipides à partir de *Trichosporon oleaginosus*, *Trichoderma* sp. (SKF-5), et les boues sous ultrasons (520 kHz 40 W et 50 Hz 2800 W). L'efficacité d'extraction par ultrasons a été comparée à la méthode d'extraction conventionnelle par le mélange chloroforme méthanol (2:1 v/v). Les images réalisées en microscopie électronique à balayage (MEB) ont montré que les cellules éclatent sous l'action des ultrasons. Des récupérations maximales de lipides de 10.2-11.75% et de 9.3% avec de l'eau, 34.6-43.2% et 33.2% à l'hexane, 62.0-75.7% et 65.1% avec du méthanol, 95.3-100% et 100% p/p de biomasse avec le mélange chloroforme/méthanol ont été obtenus à partir de *Trichosporon oleaginosus* et SKF-5, respectivement, pour une intensité d'ultrasons de 50 Hz 2800 W. La récupération des lipides par des ultrasons à haute fréquence, était légèrement inférieure à celle de basse fréquence. Ceci est dû au fait qu'une fréquence plus basse peut produire des forces de cisaillement plus agressives que celle produites à haute fréquence (Chanamai et al., 2000).

L'extraction par chloroforme/méthanol et ultrasons a permis la récupération du contenu total en lipides en peu de temps (20 minutes) et à basse température (25 °C) tandis que la récupération du contenu total en lipides par extraction conventionnelle avec chloroforme/méthanol nécessite un temps de 12 h à 60 °C. En outre, la composition des acides gras obtenus à partir de l'extraction par ultrasons était similaire à celle obtenue en employant l'extraction conventionnelle avec le mélange chloroforme/méthanol. Ceci prouve que les ultrasons ne change pas les propriétés du produit final. En outre, ce fait suggère que l'extraction par chloroforme/méthanol et les ultrasons serait une méthode prometteuse pour l'extraction des lipides des microorganismes.

1.4.4 Ultrasons trans-estérification *in-situ* pour la production de biodiesel (objectif 9)

De nombreuses études ont permis de transformer l'huile de microorganismes en biodiesel (Karatay et Donmez, 2010; Liang et al., 2010). Ce procédé comprend normalement 4 étapes: la culture des microorganismes, la récolte des microorganismes, l'extraction des lipides, et la trans-estérification. L'extraction des lipides à partir de microorganismes nécessite une grande quantité de solvants organiques (chloroforme et méthanol). En raison de l'inquiétude croissante concernant la manipulation de solvants organiques (notamment le chloroforme), un processus en une seule étape appelé trans-estérification *in-situ*, est devenue prioritaire tout en évitant l'étape d'extraction. La trans-estérification in-situ avec ou sans ultrasons a été réalisée en variant le ratio molaire méthanol/lipide, la quantité de catalyseur (NaOH ou H_2SO_4) ajoutée, et le temps de réaction. Les résultats ont montré que l'utilisation d'ultrasons pourrait aboutir à un rendement de production de FAMEs élevé de 92.1% (p/p lipides) avec un ratio molaire méthanol/lipide de 60:1, une quantité de NaOH ajoutée de 1% (p/p de lipides) et durant 20 min. Tandis que pour obtenir un rendement similaire par trans-estérification in situ sans ultrasons, il faut utiliser un ratio molaire méthanol/lipide de 360:1, une teneur de NaOH 1% (p/p de lipides) et un temps de réaction de 12 h. En outre, il a été constaté que le rendement de FAMEs par ultrasons-trans-estérification in-situ (94% p/p de lipides) était supérieur à celui obtenu par la procédure en deux étapes (93.8% p/p de lipides) dans laquelle le lipide a été extrait et par la suite transformé en FAMEs par trans-estérification. Les compositions de FAMEs

obtenues par ultrasons et trans-estérification in-situ étaient similaires à celles obtenues par trans-estérification en deux étapes.

1.5 Conclusions et recommandations

1.5.1 Conclusions

- L'étude de l'emploi des boues comme milieu nutritif pour la production de lipides a montré que la teneur maximale en lipides a été obtenue dans les boues secondaires municipales parmi tous les types de boues testés (municipale primaire, secondaire, mixte, et les boues secondaires de pâte à papier collectées de la ville de Québec, Canada).
- L'effet de la concentration initiale en matières en suspension des boues (10 à 30 g/L) sur l'accumulation de lipides a montré que des teneurs maximales en lipides de 30.2 et 32.4% p/p du poids sec, ont été obtenues par *Pichia amethionina sp.* et *Galactomyces sp.*, respectivement, à une concentration de matières solides en suspension de 25 g/L. Par contre une teneur maximale en lipides de 37.7% p/p sec a été accumulée par *Trichosporon oleaginosus* à une concentration en matières solides en suspension 30 g/L.
- Le prétraitement thermo-alcalin conduit à une forte augmentation de la concentration de carbone organique dissous (de 1.5 à 6.0 g/L) et de l'azote dissous (0.5 à 1.8 g/L) dans la boue. Une augmentation en teneur de lipides (environ 39% p/p) a été révélée par *Trichosporon oleaginosus* en 48 h pour les boues prétraitées thermiquement, alors que la teneur maximale en lipides est atteinte en seulement 42 h dans les boues thermo-chimiquement prétraitées.
- La composition d'acides gras de lipides produit à partir de boues brutes et des microorganismes cultivés avec des boues et des boues prétraitées était similaire à celle du biodiesel actuellement commercialisé.
- Les résultats des bilans énergétiques et des émissions de gaz à effet de serre de production de biodiesel à partir de différentes sources (boues brutes, microorganismes oléagineux phototrophes et microorganisme oléagineux hétérotrophes cultivés avec des eaux usées et boues d'épuration) ont montré que le gain énergétique net le plus élevé (36.96 GJ par tonne

de biodiesel produit) et une réduction des émissions de gaz à effet de serre (90 tonnes de CO_2 par tonne produite biodiesel) ont été obtenus à partir de cultures de microorganismes hétérotrophes croissant sur des boues.

- L'estimation des coûts de production de biodiesel à partir des lipides provenant des boues a révélé que le contenu lipidique en microorganismes cultivés avec de la boue a un impact sur le prix. Les coûts sont respectivement de 0.5, 0.4 et 0.3 US$/kg pour des teneurs en lipides de 40%, 50% et 60% (p/p boues-biomasse).
- La production de biodiesel à partir de glycérol brut a montré que le glycérol purifié (teneur en lipides: 44.3% p/p de la biomasse) permet une accumulation des lipides dans la souche microbienne plus élevée que celle obtenue pour le glycérol brut (teneur en lipides: 37.2% p/p de la biomasse). La concentration optimale du glycérol purifié a été de 50 g/L. Cette concentration a donné le plus haut rendement de lipides (0.19 g de lipides/g glycérol).
- Les compositions de biodiesel produit à partir de microorganismes cultivés avec le glycérol brut et le glycérol purifié étaient similaires à celle du biodiesel actuellement commercialisé.
- L'étude du bilan énergétique de la production de biocarburants a montré que le glycérol brut utilisé pour la production de biodiesel (1.32) a le taux le plus élevé en énergie, appelée aussi production d'énergie/entrée nette d'énergie, par rapport à l'utilisation de l'hydrogène (0.22), le biogaz (0.27) et d'éthanol (0.52).
- Les taux de lipides et la taille de l'usine ont un grand impact sur le coût de production unitaire des lipides. Le coût unitaire était de 0.91, 0.44 et 0.16 US$ pour 0.4, 1, et 5 millions de kg de lipides produits respectivement. Chaque augmentation du contenu en lipides de 10% entraînant une réduction du coût de 0.1 à 0.2 US$ par kg de lipides produits selon la taille de l'industrie de production.
- L'extraction par le mélange chloroforme/méthanol et les ultrasons a permis de récupérer totalement le contenu en lipides en peu de temps (15 min) et à basse température (25°C) tandis que la récupération du contenu total en lipides par extraction conventionnelle avec le mélange chloroforme/méthanol nécessite un temps de 12 h à 60°C. En outre, la composition des acides gras obtenus à partir de l'extraction avec le mélange chloroforme

méthanol par ultrasons était similaire à celle obtenue à partir de l'extraction conventionnelle avec le mélange chloroforme /méthanol.

- L'utilisation d'ultrasons pourrait aboutir à un rendement de production de FAMEs plus élevé de 92.1% (p/p de lipides) avec un ratio molaire méthanol/lipide de 60:1 et une quantité de NaOH ajoutée de 1% (p/p de lipides) durant 20 min. Tandis que pour obtenir un rendement similaire par trans-estérification *in-situ* sans ultrasons, il faut utiliser un ratio molaire méthanol/lipide de 360:1, une teneur de NaOH 1% (p/p de lipides) et un temps de réaction de 12 h.

1.5.2 Recommandations

À partir des études sur l'accumulation de lipides par les microorganismes oléagineux avec les boues d'eaux usées et de glycérol brut, l'extraction des lipides avec les ultrasons, et ultrasons et trans-estérification in-situ, les recommandations suivantes peuvent être envisagées:

- Les microorganismes ayant un contenu lipide élevé (plus de 60% p/p biomasse) doivent être isolés avec de la boue et du glycérol brut.
- Le prétraitement des boues par les ultrasons, les micro-ondes, l'oxydation et les champs électriques pulsés doit être effectué pour étudier l'impact sur l'accumulation de lipides de microorganismes oléagineux.
- Le glycérol brut (source de carbone) avec addition de boues comme supplément nutritif doit être utilisé pour l'étude de l'accumulation de lipides par les microorganismes oléagineux.
- La réalisation d'essais de production de lipides, de biodiesel et d'extraction en présence d'ultrasons à plus grande échelle est recommandée.

1.6 Bibliographie

Ahmad, M., Rashid, S., Khan, M. A., Zafar, M., Sultana, S., Gulzar, S., 2009. Optimization of base catalyzed transesterification of peanut oil biodiesel. African Journal of Biotechnology 8, 441-446.

Alabi, A. O., Tampier, M., Bibeau, E., 2009. Microalgae technologies and processes for biofuelsbioenergy production in British Columbia. Report Seed Science Ltd, Victoria, Canada.

Alcantara, R., Amores, J., Canoira, L., Fidalgo, E., Franco, M. J., Navarro, A., 2000. Catalytic production of biodiesel from soy-bean oil, used frying oil and tallow. Biomass and Bioenergy 18, 515-527.

Alvarez, S., 2002. Triacylglycerols in prokaryotic microorganisms. Applied Microbiology and Biotechnology 60, 367-376.

Anderson, D., Masterson, D., McDonald, B., Sullivan, L., 2003. Industrial Biodiesel Plant Design and Engineering: Practical Experience. the Chemistry and Technology Conference, Session Seven: Renewable Energy Management International Palm Oil Conference (PIPOC), 24-28 August 2003, Putrajaya Marriot Hotel, Putrajaya, Malaysia.

Annie, D., 2006. Biofuels production, trade and sustainable development: emerging issues. Environmental Economics Programme/Sustainable Markets Group September 2006, London, England.

Araujo, G. S., Matos, L. J. B. L., Fernandes, J. O., Cartaxo, S. J. M., Gonçalves, L. R. B., Fernandes, F. A. N., Farias, W. R. L., 2013. Extraction of lipids from microalgae by ultrasound application: Prospection of the optimal extraction method. Ultrasonics Sonochemistry 20, 95-98.

ASTM, 2008. ASTM Standard specification for biodiesel fuel (B100) blend stock for distillate fuels. Annual Book of ASTM Standards ASTM International, West Conshohocken, Method D6751-08. New York, USA.

Barnwal, B. K., Sharma, M. P., 2005. Prospects of biodiesel production from vegetable oils in India. Renewable and Sustainable Energy Reviews 9, 363-378.

Benemann, J. R., Van Olst, J. C., Massingill, M. J., Weissman, J. C., Brune, D. E., 2006. The Controlled Eutrophication Process: Using Microalgae for CO_2 Utilization and Agricultural Fertilizer Recycling. http://www.oilgae.com/blog/2006/10/microalgae-for-co2-utilization.html.

Berk, Z., 2008. Food process engineering and technology. Food Science and Technology 1, 180.

Boocock, D. G. B., Konar, S. K., Leung, A., Ly, L. D., 1992. Fuels and chemicals from sewage sludge: 1. The solvent extraction and composition of a lipid from raw sewage sludge. Fuel 71, 1283-1289

Boyd, A. R., Champagne, P., McGinn, P. J., MacDougall, K. M., Melanson, J. E., Jessop, P. G., 2012. Switchable hydrophilicity solvents for lipid extraction from microalgae for biofuel production. Bioresource Technology 118, 628-632.

Boz, N., Degirmenbasi, N., Kalyon, D. M., 2009. Conversion of biomass to fuel: Transesterification of vegetable oil to biodiesel using KF loaded nano-gamma-Al_2O_3 as catalyst. Applied Catalysis B-Environmental 89, 590-596.

Canakci, M., Sanli, H., 2008. Biodiesel production from various feedstocks and their effects on the fuel properties. Journal of Industrial Microbiology and Biotechnology 35, 431-441.

Canakci, M., Van Gerpen, 2001. Biodiesel production from oils and fats with high free fatty acids. Transaction of the ASAE 44, 1429-1436.

Cao, Y., Yao, J., Li, J., Chen, X., Jinyong, W., 2010. Breeding of high lipid producing strain of *Geotrichum robustum* by ion beam implantation. Electronic Journal of Biotechnology 13, 2-3.

CEN, 2003. Committee for Standardization Automotive fuels-fatty acid methyl esters (FAME) for diesel engines-requirements and test methods. European Committee for Standardization, Brussels, Method EN 14214.

Chanamai, R., Herrmann, N., McClements, D. J., 2000. Probing floc structure by ultrasonic spectroscopy, viscometry, and creaming measurements. Langmuir 16, 5884-5891.

Chapagain, B. P., Wiesman, Z., 2009. MALDI-TOF/MS fingerprinting of triacylglycerols (TAGs) in olive oils produced in the Israeli Negev desert. Journal of Agricultural and Food Chemistry 57, 1135-1142.

Cheirsilp, B., Suwannarat, W., Niyomdecha, R., 2011. Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for lipid production from industrial wastes and its use as biodiesel feedstock. New Biotechnology 28, 362-368.

Chen, L., Liu, T., Zhang, W., Chen, X., Wang, J., 2012. Biodiesel production from algae oil high in free fatty acids by two-step catalytic conversion. Bioresource Technology 111, 208-214.

Cheng, C.-H., Du, T.-B., Pi, H.-C., Jang, S.-M., Lin, Y.-H., Lee, H.-T., 2011. Comparative study of lipid extraction from microalgae by organic solvent and supercritical CO_2. Bioresource Technology 102, 10151-10153.

Cheng, Y., Lu, Y., Gao, C., Wu, Q., 2009. Alga-Based Biodiesel Production and Optimization Using Sugar Cane as the Feedstock. Energy and Fuels 23, 4166-4173.

Chi, Z., Zheng, Y., Ma, J., and Chen, S. (2011). Oleaginous yeast Cryptococcus curvatus culture with dark fermentation hydrogen production effluent as feedstock for microbial lipid production. International Journal of Hydrogen Energy 36, 9542-9550.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnology Advances 25, 294-306.

del Río, M. I. T., 2007. An analysis of the influence of phosphorus poisoning on the exhaust emission aftertreatment systems of light-duty diesel vehicles. M.S. thesis of the Faculty of Science Nelson Mandela Metropolitan University, Port Elizabeth, Germany, 1-167.

Demirbas, A., 2003a. Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey. Energy Conversion and Management 44, 2093-2109.

Demirbas, A., 2003b. Chemical and fuel properties of seventeen vegetable oils. Energy Sources 25, 721-728.

Deng, X., Fang, Z., Liu, Y.-h., 2010. Ultrasonic transesterification of Jatropha curcas L. oil to biodiesel by a two-step process. Energy Conversion and Management 51, 2802-2807.

Dizge, N., Keskinler, B., Tanriseven, A., 2009. Biodiesel production from canola oil by using lipase immobilized onto hydrophobic microporous styrene-divinylbenzene copolymer. Biochemical Engineering Journal 44, 220-225.

Doll, K. M., Sharma, B. K., Suarez, P. A. Z., Erhan, S. Z., 2008. Comparing biofuels obtained from pyrolysis, of soybean oil or soapstock, with traditional soybean biodiesel: density, kinematic viscosity, and surface Tensions. Energy and Fuels 22, 2061-2066.

Dufreche, S., Hernandez, R., French, T., Sparks, D., Zappi, M., Alley, E., 2007. Extraction of lipids from municipal wastewater plant microorganisms for production of biodiesel. Journal of the American Oil Chemists Society 84, 181-187.

Dunn, R., Shockley, M., Bagby, M., 1999. Improving the low temperature properties of alternative diesel fuels: vegetable oil-derived methyl esters. Journal of the American Oil Chemists' Society 73, 1719-1728.

Ehimen, E. A., Sun, Z., Carrington, G. C., 2012. Use of ultrasound and co-solvents to improve the *in-situ* transesterification of microalgae biomass. Procedia Environmental Sciences 15, 47-55.

Ehimen, E. A., Sun, Z. F., Carrington, C. G., 2010. Variables affecting the in situ transesterification of microalgae lipids. Fuel 89, 677-684.

Elvin-Lewis, M., 1988. Non-traditional oilseeds and oils of India. Economic Botany 42, 540-540.

Energy, U. S. D. o., 2010. 2009 Renewable energy data book. Energy Effiency and Renewable Energy 2010, August, New York, USA.

Exler, J., Lemar, L., Smith, J., 1995. Fat and fatty acid content of selected foods containing trans-fatty acids. U.S. Department of Agriculture, pp.1-38.

Gao, C., Zhai, Y., Ding, Y., Wu, Q., 2010. Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Applied Energy 87, 756-761.

Goering, C., Schwab, A., Daugherty, M., Pryde, E., Heakin, A., 1982. Fuel properties of eleven vegetable oils. Trans ASAE 25, 1472-1477.

Gonzalez-Garcia, Y., Hernandez, R., Zhang, G., Escalante, F. M. E., Holmes, W., French, W. T., 2013. Lipids accumulation in *Rhodotorula glutinis* and *Cryptococcus curvatus* growing on distillery wastewater as culture medium. Environmental Progress & Sustainable Energy 32, 69-74.

Gouda, M., Omar, S., Aouad, L., 2008. Single cell oil production by *Gordonia* sp. DG using agro-industrial wastes. World Journal of Microbiology and Biotechnology 24, 1703-1711.

Haas, M. J., McAloon, A. J., Yee, W. C., Foglia, T. A., 2006. A process model to estimate biodiesel production costs. Bioresource Technology 97, 671-678.

http://paultan.org/2011/06/07/b5-biodiesel-palm-biodiesel-sulphur-content-less-than-10ppm/.

http://www.hydro.mb.ca/your_business/mhl/bio_glossary.shtml.

http://www.oecd.org/document/9/0, en_36774715_36775671_45438665_1_1_1_1.00.html.

http://www.thebioenergysite.com/articles/482/feedstock-and-biodiesel-characteristics-report.

Içingür, Y., Altiparmak, D., 2003. Effect of fuel cetane number and injection pressure on a DI Diesel engine performance and emissions. Energy Conversion and Management 44, 389-397.

Issariyakul, T., Kulkarni, M., Dalai, A., Bakhshi, N., 2007. Production of biodiesel from waste fryer grease using mixed methanol/ethanol system. Fuel Processing Technology 88, 429-436.

Jardé, E., Mansuy, L., Faure, P., 2005. Organic markers in the lipidic fraction of sewage sludges. Water Research 39, 1215-1232.

Johnson, M. B., Wen, Z., 2009. Production of biodiesel fuel from the microalga *Schizochytrium limacinum* by direct transesterification of algal biomass. Energy Fuels 23, 5179-5183.

Karatay, S. E., Dönmez, G., 2010. Improving the lipid accumulation properties of the yeast cells for biodiesel production using molasses. Bioresource Technology 101, 7988-7990.

Kargbo, D. M., 2010. Biodiesel production from municipal sewage sludges. Energy and Fuels 24, 2791-2794.

Karmakar, A., Karmakar, S., Mukherjee, S., 2010. Properties of various plants and animals feedstocks for biodiesel production. Bioresource Technology 101, 7201-7210.

Knothe, G., 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Processing Technology 86, 1059-1070.

Koplow, D., Dernbach, J., 2001. Federal fossil subsidies and greenhouse gas emissions: A case study of increasing transparency for fiscal policy. Annual Review of Energy and the Environment 26, 361-389.

Kulkarni, M. G., Dalai, A. K., Bakhshi, N. N., 2007. Transesterification of canola oil in mixed methanol/ethanol system and use of esters as lubricity additive. Bioresource Technology 98, 2027-2033.

Lee, I., Johnson, L., Hammond, E., 1995. Use of branched-chain esters to reduce the crystallization temperature of biodiesel. Journal of the American Oil Chemists' Society 72, 1155-1160.

Liang, Y., Cui, Y., Trushenski, J., Blackburn, J. W., 2010. Converting crude glycerol derived from yellow grease to lipids through yeast fermentation. Bioresource Technology 101, 7581-7586.

Macala, G., Robertson, A., Johnson, C., Day, Z., Lewis, R., White, M., Iretskii, A., Ford, P., 2008. Transesterification catalysts from iron doped hydrotalcite-like precursors: Solid bases for biodiesel production. Catalysis Letters 122, 205-209.

Meng, X., Chen, G., Wang, Y., 2008. Biodiesel production from waste cooking oil via alkali catalyst and its engine test. Fuel Processing Technology 89, 851-857.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., Xian, M., 2009. Biodiesel production from oleaginous microorganisms. Renewable Energy 34, 1-5.

Miao, X., Wu, Q., 2006. Biodiesel production from heterotrophic microalgal oil. Bioresource Technology 97, 841-846.

Mondala, A., Liang, K. W., Toghiani, H., Hernandez, R., French, T., 2009. Biodiesel production by in situ transesterification of municipal primary and secondary sludges. Bioresource Technology 100, 1203-1210.

Naik, M., Meher, L. C., Naik, S. N., Das, L. M., 2008. Production of biodiesel from high free fatty acid Karanja (*Pongamia pinnata*) oil. Biomass and Bioenergy 32, 354-357.

Ngo, H. L., Zafiropoulos, N. A., Foglia, T. A., Samulski, E. T., Lin, W., 2007. Efficient two-step synthesis of biodiesel from greases. Energy and Fuels 22, 626-634.

Papanikolaou, S., Aggelis, G., 2011. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. European Journal of Lipid Science and Technology 113, 1031-1051.

Papanikolaou, S., Sarantou, S., Komaitis, M., Aggelis, G., 2004. Repression of reserve lipid turnover in *Cunninghamella echinulata* and *Mortierella isabellina* cultivated in multiple-limited media. Journal of Applied Microbiology 97, 867-875.

Pimentel, D., Patzek, T. W., 2005. Ethanol production using corn, switchgrass, and wood; Biodiesel production using soybean and sunflower. Natural Resources Research 14, 65-76.

Ramos, M. J., Fernández, C. M., Casas, A., Rodríguez, L., Pérez, Á., 2009. Influence of fatty acid composition of raw materials on biodiesel properties. Bioresource Technology 100, 261-268.

Ranjan, A., Patil, C., Moholkar, V. S., 2010. Mechanistic assessment of microalgal lipid extraction. Industrial and Engineering Chemistry Research 49, 2979-2985.

Rice, B., Frohlich, A., and Leonard, R., 1997. Bio-diesel production based on waste cooking oil: promotion of the establishment of an industry in Ireland. Teagasc, Final Report Sept, 1997, Carlow, Ireland.

Sánchez, E., Ojeda, K., El-Halwagi, M., Kafarov, V., 2011. Biodiesel from microalgae oil production in two sequential esterification/transesterification reactors: Pinch analysis of heat integration. Chemical Engineering Journal 176–177, 211-216.

Samuel, O. D., Dairo, O. U., 2012. A critical review of *in-situ* transesterification process for biodiesel production. The Pacific Journal of Science and Technology 13, 72-79.

Sanford, S. D., White, J. M., Shah, P. S., Wee, C., Valverde, M. A., Meier, G. R., 2009. Feedstock and biodiesel characteristics report. Renewable Energy Group Report. Denver, USA. 1-136.

Sangat, J., and Kevin, N., 2010. Innovative Canadian process technology for biodiesel production. AMEC Americas Limited, Oakville, ON, Canada, 1-10.

Sergeeva, Y., Galanina, L., Andrianova, D., Feofilova, E., 2008. Lipids of filamentous fungi as a material for producing biodiesel fuel. Applied Biochemistry and Microbiology 44, 523-527.

Shannon, D. S., James, M. W., Parag, S. S., Claudia, W., Marlen, A. V., and Glen, R. M., 2009, Feedstock and biodiesel characteristics report. Renewable Energy Group, 1-136. Ames, USA.

Singh, S. P., Singh, D., 2010. Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: A review. Renewable & Sustainable Energy Reviews 14, 200-216.

Srivastava, A., Prasad, R., 2000. Triglycerides-based diesel fuels. Renewable and Sustainable Energy Reviews 4, 111-133.

Suarez, P. A. Z., Moser, B. R., Sharma, B. K., Erhan, S. Z., 2009. Comparing the lubricity of biofuels obtained from pyrolysis and alcoholysis of soybean oil and their blends with petroleum diesel. Fuel 88, 1143-1147.

Sulaiman, S., Abdul Raman, A. A., Mohammed Kheireddine Aroua, M. K. A., 2010. Coconut waste as a source for biodiesel production. In *Chemical, Biological and Environmental Engineering (ICBEE), 2010 2nd International Conference on*, pp.254-256.

Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., Sádaba, I., Ruiz-Vázquez, R. M., Torres-Martínez, S., Garre, V., 2009a. Biodiesel production from biomass of an oleaginous fungus. Biochemical Engineering Journal 48, 22-27.

Wang, P., Tat, M., Van Gerpen, J., 2005. The production of fatty acid isopropyl esters and their use as a diesel engine fuel. Journal of the American Oil Chemists' Society 82, 845-849.

Widjaja, A., Chien, C. C., Ju, Y. H., 2009. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. Journal of the Taiwan Institute of Chemical Engineers 40, 13-20.

Willson, R. M., Wiesman, Z., Brenner, A., 2010. Analyzing alternative bio-waste feedstocks for potential biodiesel production using time domain (TD)-NMR. Waste Management 30, 1881-1888.

PARTIE II : PRODUCTION DE BIODIESEL À PARTIR DES LIPIDES ACCUMULÉS PAR DES MICROORGANISMES

BIODIESEL PRODUCTION FROM HETEROTROPHIC MICROALGAE THROUGH TRANSESTERIFICATION AND NANOTECHNOLOGY APPLICATION IN THE PRODUCTION

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2 BIODIESEL PRODUCTION FROM HETEROTROPHIC MICROALGAE THROUGH TRANSESTERIFICATION AND NANOTECHNOLOGY APPLICATION IN THE PRODUCTION

2.1 Résumé

Les huiles végétales et les graisses animales sont les matières premières les plus souvent utilisées dans la production de biodiesel. Toutefois, elles sont également utilisées dans la production alimentaire, ce qui se traduit par l'augmentation du prix des matières premières à cause de la compétition avec les huiles végétales. Par conséquent, des matières premières alternatives sont nécessaires pour la production de biodiesel. Les micro-algues hétérotrophes sont trouvés capables d'accumuler des teneurs éleveés en lipides (jusqu'à 57% en p/p). Ils peuvent utiliser des carbones complexes tels que le sorgho à sucré et le *Jerusalem artichoke* comme nutriments pour produire de l'huile de quantité équivalente à celle résultante de l'utilisation de glucose, ce qui fournit une stratégie de production de biodiesel moins cher. Actuellement, il a été constaté que les nanomatériaux pourraient stimuler le métabolisme des microorganismes, ce qui suggère que les nanomatériaux présents dans la culture pourraient améliorer la production de lipides des micro-algues. En outre, l'utilisation des nanomatériaux pourrait améliorer l'efficacité de l'extraction des lipides et même l'accomplir sans nuire aux micro-algues. Les nanomatériaux tels que CaO et MgO ont été utilisés comme porteurs de biocatalyseur ou comme catalyseur hétérogène dans la trans-estérification d'huile de biodiesel. Dans ce document, les facteurs qui pourraient avoir une incidence sur l'accumulation de lipides chez les micro-algues hétérotrophes, l'extraction et la trans-estérification de l'huile de biodiesel sont abordés.

Mots clés : Microalgues hétérotrophes; huile microbienne; trans-estérification; biodiesel; nanotechnologie

2.2 Abstract

Vegetable oils and animal fats are the most often used feedstock in biodiesel production; however, they are also used in food production, which results in increasing the feedstock price due to the competition. Therefore, alternative feedstock is required in biodiesel production. Heterotrophic microalgae are found capable of accumulating high lipid (up to 57% w/w). They can use complex carbons such as sweet sorghum and Jerusalem artichoke as nutrient to produce equivalent quantity oil as that of using glucose, which provides a cheap biodiesel production strategy. It was found that nanomaterials could stimulate microorganism metabolism, which suggested that nanomaterial addition in the cultivation could enhance lipid production of microalgae. Furthermore, the use of nanomaterials could improve the efficiency of the lipid extraction and even accomplish it without harming the microalgae. Nanomaterials such as CaO and MgO nanoparticles have been used as biocatalyst carriers or as heterogeneous catalyst in oil transesterification to biodiesel. In this paper, the factors that could impact on lipid accumulation of heterotrophic microalgae are critically reviewed; the advances on application of nanotechnology in microalgae lipid accumulation, extraction, and transesterification are addressed.

Keywords: Heterotrophic microalgae; microbial oil, transesterification; biodiesel; nanotechnology

2.3 Introduction

At present, transesterification using plant oils, animal fats, or lipids from oleaginous microalgae are the major method of biodiesel production [1-3]. Among all the feedstock, oleaginous microalgae have gained a growing interest because of that conventional feedstock, plant oils which at present are the main source of biodiesel production, is becoming more and more unsustainable due to the strong competition with food production and kitchen utilization. The faster growth rate and greater lipid content of microalgae compared to oilseed crops urge researchers to develop the microalgae utilization in biodiesel production instead of plant oils [2,4]. In addition, the possibility of increasing lipid content of microalgae by controlling their cultivation condition, while which is not possible for plant, offers another significant advantage [5]. Rodolfi et al. [6] selected four among thirty strains of microalgae to investigate the impact of cultivation condition such as irradiation and nutrient on lipid accumulation of the microalgae and found that lipid content significantly varied with the change of cultivation conditions.

Numerous studies have been reported on autotrophic microalgae used for production of biodiesel [7,8]. Autotrophic microalgae are capable of using carbon dioxide and solar energy to synthesize organics such as protein and lipid for their growth. Most of the production of autotrophic microalgae for biodiesel production occurs in indoor photobioreactors. The heavily light-dependent growth characterization of autotrophic microalgae resulting in energy consuming for illumination, as well as the low efficiency in the biomass productivity, has hindered autotrophic microalgae application in biodiesel production. In comparison, heterotrophic microalgae are more flexible for the cultivation condition (can grow under light free condition), and was found capable of accumulate higher lipid in the cells [9-11]. Miao and Wu [9] reported that the lipid content of heterotrophic *Chlorella protothecoides* was 3 times higher than that of the autotrophic one. Up to now, *Chlorella protothecoides* is the most studied heterotrophic algae as lipid source for biodiesel production [12-14].

Nanotechnology is the technique to devise, synthesize, manufacture and apply the matters with atomic or molecular precision at dimensions of 100 nanometers (nm) or smaller [15]. Nanomaterials have the surface area several hundred times more than their equal weight of

macroscale materials. Not only is the surface area extensively increased, the tenacity, elasticity, strength and electricity are also enhanced.

There are many research fields and several potential applications that involve nanotechnology due to its unique behaviors and properties. Nanotechnology application in biodiesel production from microalgae mainly includes nanomaterial utilization on lipid accumulation, extraction and on transesterification process as catalyst support or catalyst as shown in Figure 2.1 [16-19]. In tradition, organic solvents having great affinity to lipid such as chloroform, hexane, isopropanol, and methanol are utilized in lipid extraction from microalgae, however, the use of toxic material (solvents), the difficulty of the complete recover of the material, and the demand of the energy intense solvent-lipid separation step requires the development on extraction technology. The mechanism of solvent extraction is that solvent can weaken/break cell wall, and thus enhance lipid diffusion to the outer environmental/dissolve the lipid. Nanomaterails are favorable carrier in immobilization due to the high surface area, and solid nanomaterials can be easily recovered from liquid phase by filtration or centrifugation. Therefore, immobilizing organic solvent-like chemicals onto solid nanomaterials would solve the problems in organic solvent extraction. The immobilized chemicals as function group achieve the lipid extraction and recovered as nanomaterials recovered. A research revealed that modified nanosphere silica accomplished the extraction from alive microalgae which would be sent back for lipid accumulation again, and hence the process avoids recultivation [16]. It would be the immobilization of chemicals which weaken the cell wall (but not to kill the microalgae) and thus lead to lipid diffusion from inside to outside of the cells.

The most employed catalyst in transesterification is acid or base, however, the corrosion (aggressive acid utilization) and soap formation (free fatty acid reacts with base) need alternative catalysts. Lipase, a biocatalyst, is environmentally friendly and efficient, but rather expensive, while the cost can be reduced when lipase immobilization is applied because of the possibility of lipase reuse. Nanomaterials have large surface area for immobilization and can be easily separated from products, hence, immobilizing lipase onto nanomaterials would benefit reducing the cost of using lipase [20]. Apartment from as carrier, nanomaterials selves such as CaO, Al_2O_3, and MgO nanoparticles are heterogeneous catalyst and can achieve high conversion

rate (> 99%) with less addition amount (<1% of oil addition) [21,22]. It is contributed by it high surface area which increased contacting chances with the reagent lipid. Moreover, comparing with the bulk materials, activity, lifetime, and resistance to poisons of their nanomaterials are improved [23,24] Therefore, it suggested that nanomaterials catalysts could have high performance in transesterrification.

Biodiesel production from heterotrophic microalgae includes microalgae lipid production (also called feedstock production) and the lipid transesterification to biodiesel. The lipid production including lipid accumulation and extraction is essential step as feedstock takes up to 70% of the overall cost [25,26]. Researchers have reviewed the methods including cultivation temperature, pH, the presence of radiation, and nutrient limitation, for enhancing lipid accumulation in microalgae [27]. Carbon and nitrogen source, carbon to nitrogen ratio, mineral presence, and nanomaterial effect on the lipid accumulation have not been well addressed. The review on organic solvent extraction or pre-treatment (sonication, homogenization, microwave, bead milling) followed by solvent extraction for lipid extraction from microalgae have been reported [28,29]. Nanomaterial application on the extraction hasn't been discussed. Transesterification of lipid to biodiesel with various catalysts the homogeneous and the heterogeneous have been well analyzed [30,31]. Nanomaterial as a promising catalyst in transesterification should be paid significant attention. In this paper, the factors that could impact on lipid accumulation of heterotrophic microalgae are critically reviewed, and the advances of nanomaterial's application in lipid production are addressed. Additionally, the potential application of nanomaterials in biodiesel synthesis (transesterification) is proposed as well.

2.4 Lipid production from heterotrophic microalgae and nanomaterial application in the production

Lipid production from heterotrophic microalgae mainly includes the cultivation and lipid extraction process. It is known that lipid production takes a major part of the overall cost of biodiesel production. Therefore, researchers and engineers have been working on increasing lipid content in microalgae by manipulating the cultivation conditions and improving lipid extraction efficiency by controlling the extraction steps [10,32,33].

2.4.1 Factors affecting lipid accumulation

Lipid content is the key factor of biodiesel production from heterotrophic microalgae. Strategies such as selection of carbon source and nitrogen source for enhancing lipid accumulation in microalgae have been reported [10-12,33]. Glucose is the most often used carbon source in heterotrophic microalgae cultivation, however, its high cost requires replacement which is cheaper and at least equally efficient [33]. Cheng et al. [12] investigated the effect of sucrose and sugar cane juice as carbon source on lipid production of heterotrophic microalgae, *Chlorella protothecoides*. It was found that lipid content was only slightly affected by the carbon source (Table 2.1). It indicates that sugar cane could be a suitable substitute of carbon source for producing heterotrophic microalgae oil. In addition, more complicated carbon sources such as Jerusalem artichoke and corn powder have been investigated for lipid accumulation of microalgae [14,33,34]. Cheng et al. [33] found that the lipid content of the microalgae *Chlorella protothecoides* cultivated with Jerusalem artichoke (44%), known as tuberous plant rich in carbohydrates, had almost no difference with that using glucose (45.2%) as carbon source. Xu et al. [14] obtained higher cell concentration and higher lipid content fed with corn powder (3.92 g/L and 55.3%), than those with glucose (3.74 g/L and 54.7%), respectively, at 144 h cultivation. Sweet sorghum is a well-known plant producing sugar-rich stems, of which the sugar is mainly sucrose (55% w/w) and cellulose (22.6% w/w) [35]. It was found that lipid accumulation content and yield in *Chlorella protothecoides* cultivated using sweet sorghum juice or glucose as carbon source was no remarkable difference (52.7% w/w, 0.54 g/L/d for sweet sorghum juice and 53.3% w/w, 0.39 g/L/d for glucose, respectively) [34]. Complex carbon sources have shown good results in lipid accumulation of microalgae, which implies that it is feasible to use these cheap carbon sources for microalgae oil production. Shen et al. [11] studied the influence of nitrogen source (urea, yeast extract, and nitrate) on lipid productivity of heterotrophically cultivated *Chlorella protothecoides*. It was observed that the lipid yield in microalgae varied from a hundred to several hundred milligrams per liter per day according to the difference of the nitrogen source, and the highest yield (654 mg/L/day) was gave with nitrate. It suggests that nitrogen type significantly affects the lipid accumulation (lipid content varies from 25% to 46% w/w with different types of nitrogen source), which could be due to the

impact of composition of nitrogen source on metabolic pathway of microalgae. Carbon to nitrogen (C/N) ratio has also been studied to optimize lipid accumulation of microalgae [12,13,33]. It was shown that higher C/N ratio led to higher lipid accumulation (Table 2.1). Nitrogen is an important nutrient in cell growth and division of microalgae. The size and number of cells in microalgae would increase under appropriate ratio of carbon and nitrogen supply. However, when carbon is sufficient but nitrogen is deprived, the cell division would be forced to cease and cell size growth would take place. The deprivation of nitrogen would inhibit the protein formation in the cell and thus result in lipid accumulation in the cells. Rodolfi et al. [6] reported that microalgae cultivated in nitrogen deficient condition (50% w/w lipid content) had given 18% w/w more lipid content than the one cultivated in nitrogen sufficient condition (32% w/w lipid content) with other conditions the same. Widjaja et al. [5] stated the similar result. Though heterotrophic microalgae have shown great capacity of lipid accumulation, the related study is rather limited. Except the impact of carbon source and nitrogen source on lipid accumulation, no other factors have been investigated till date. It was reported that environmental stress such as silica deprivation, pH, temperature, significantly affected lipid accumulation in autotrophic microalgae and fungi [5,6,36-38]; therefore, the factor can also impact on lipid accumulation of heterotrophic microalgae. Impropriate pH could inhibit microorganism activities and hence affects lipid production. Temperature effect on lipid accumulation is probably due to the self-protection that microorganisms accumulate lipid, which is major energy supplier for the living beings, for maintaining the normal life activities under the low temperature. Mineral concentration in culture medium could also affect lipid accumulation of heterotrophic microalgae. Some researchers have indicated that iron is an important substance in metabolism of living beings [39,40]. Menzyanova et al. [41] studied the iron effect on growth rate, protein content, and lipid content of autotrophic microalgae, *Dunaliella viridis Teod.*, and reported that iron concentration in cultural medium showed impact on lipid content of the microalgae. It can be predicated that lipid content in heterotrophic microalgae could possibly be manipulated by adjusting iron concentration of the medium. Additionally, other minerals such as calcium and magnesium had also impact on lipid

accumulation [42]. Moreover, periodic carbon depletion could also lead to vary of lipid content of the heterotrophic microalgae as it may adjust the metabolic pathway of lipid.

2.4.2 The effect of nanomaterial addition on lipid accumulation

Nanomaterials are found capable of enhancing microbe activities [43,44], and hence, it could be speculated that the addition of nanomaterials to microalgae cultivation medium could impact on lipid accumulation. It has been revealed that stress in cultivation such as low temperature (less than 20 °C), nutrient (nitrogen) depletion, high metal concentration (Fe), etc. triggered lipid accumulation [45,46]. The addition of nanoparticle such as silica or iron oxide nanoparticle in the medium causes strong sheer between cell and the nanoparticle which would be considered as competitor of nutrients by the cell. It threatens the cell to rapidly uptake nutrients and result in lipid accumulation.

Enhancing growth rate of heterotrophic microalgae would be an alternative for enhancing lipid productivity. Gao et al. [34] have proved that high growth rate could result in high lipid yield. It was reported that nanomaterials such as metal oxide nanoparticles (AgI/TiO_2) and single-walled carbon nanotubes were toxic to microbes [47,48]. However, Jin et al. [49] did not observe toxicity of nanomaterials on living cells. Williams et al. [50] investigated nanoparticle (silica, silica/iron oxide, gold) effects on growth and activity of *Escherichia coli* and reported that the addition of nanoparticles had no negative impact on growth and activity of *E. coli*. These studies indicated that appropriate selection of nanomaterials could possibly assist heterotrophic microalgae growth.

Lipid content directly affects the biodiesel production cost; therefore, the factors affecting lipid accumulation of heterotrophic microalgae should be further studied. Nanomaterial application in microalgae cultivation has great potential to increase lipid content and should be studied in future.

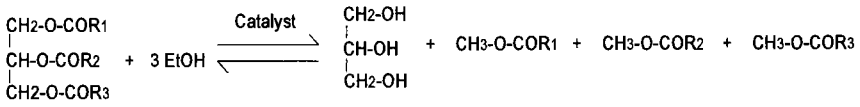
2.4.3 Nanomaterial application on lipid extraction

Lipid extraction is one of the major fractions of the cost of biodiesel production from microalgae. At present, the most common used method is solvent extraction in which organic solvents such as hexane, chloroform, methanol, or the combinations of the solvents, were used

to reiteratively wash the wet or dried biomass of microalgae to obtain lipid [12,14,51]. It was displayed that the extraction yield of lipids varied a lot while using different solvents [52]. The result showed that the lipid yield using chloroform and methanol was 20% w/w but it was only 15% w/w using hexane in the extraction. It suggests that the lipid extraction method is rather important in lipid production from microalgae, and should be paid attention for high yield. In addition to the selection of the solvents, the extraction condition could be also considered to improve lipid extraction yield. The utilization of irradiation and ultrasonication in extraction could assist the lipid yield [53]. However, these methods require additional energy consumption which may increase the extraction cost compared to solo solvent extraction. Recently, use of nanomaterials to enhance the extraction from microalgae has been introduced. Lu [18] reported that a type of nanomaterial had been synthesized and would be used in the extraction process from microalgae. It was predicated that this would be a favorable approach for lipid extraction because using nanomaterial could prevent the use of toxic materials (organic solvents) and the demand of complex solvent-lipid separation step in conversional extraction process. Furthermore, it has been reported that use of sphere nanomaterial to extract the lipid from living microalgae with no impact on microalgae lives which would be continuously used for lipid accumulation [16]. It indicates that nanomaterial instead of solvent, which kills the microalgae, lipid extraction would reduce the cost which is required for microalgae re-cultivation in solvent extraction case.

2.5 Nanomaterial application on transesterification

Transesterification is the most applied technology in biodiesel production. It is a process using oils derived from animal, plant, or oleaginous microorganisms to react with alcohol (mainly methanol) for synthesizing fatty acid methyl esters - FAMEs (biodiesel). The reaction occurs either under extreme condition of high temperature and pressure or under mild condition in the presence of catalyst. Currently, biodiesel is mainly synthesized through catalytic method. There are four types of catalyst, acid, base, enzyme, and heterogeneous catalysts, which have been studied in the synthesis. Biodiesel synthesis through transesterification is shown in Equation 2.1.



Equation 2.1

Where R_1, R_2, and R_3 are fatty acid chains; CH_3-O-COR_1, CH_3-O-COR_2, and CH_3-O-COR_3 are alkyl (methyl) esters.

Acid such as H_2SO_4 and HCl is usually used as catalyst in the reaction in lab scale studies [14,52], while bases such as NaOH and KOH are usually employed in industrial biodiesel production [54]. However, studies have pointed out that acid catalyzed process needs extra care in reactor due to the aggressive characteristic of employed acid, and additionally, it usually requires large amount excess methanol (molar ratio of methanol to oil is around 60:1) [52]. While base catalyzed biodiesel production consumes base due to the soap formation [55]. Compared to acid and base catalyzed processes, transesterification catalyzed by biocatalyst lipase is more environmentally friendly and efficient [56,57]; however, the use of costly raw material for lipase production has inhibited enzymatic biodiesel production. There are three ways to reduce enzymatic biodiesel production cost. One is to reduce lipase production cost through development of a cheap and efficient method for lipase production; the second is to enhance lipase efficiency; and the last is the reuse of lipase. Among all, lipase reuse is the most feasible way. Immobilizing lipase on carriers, porous materials, is an effective method for lipase reuse. Various materials, such as fiber cloth, acrylic resin, silica gel, hydrotalcite, and macroporous or microporous materials, have been used as lipase carrier [1,58-60]. It has been indicated that the reused lipase could perform in terms of stability and activity as the initial one [58,61,62], which suggests that immobilization is a promising approach for lipase reuse.

Among all the carriers, nanomaterials have gained a great interest in the immobilization of lipase (Table 2.2). Nanomaterials are characterized with extensively large surface to volume ratio, which reveals that nanomaterials are capable of providing enormous surface area for lipase immobilization. In addition, extremely small pore sizes in nanomaterials enhance reactant diffusion rate to the active site of lipase because of that the diffusion rate is determined by the square of diffusion path accessing the active site (Equation 2.2).

$$R_{df} \propto \frac{1}{D^2}$$

Equation 2.2

Where R_{df} is diffusion rate of reactant to active sites of enzymes, D is diameter of diffusion path of reactants accessing to active sites of enzymes.

Thus the smaller D leads to greater R_{df} [63,64]. A high diffusion rate of reactants would accelerate transesterification process. Furthermore, researchers have reported that nanomaterials used for lipase immobilization would retain or even enhance enzyme activity, selectivity, and stability [65-69].

The activity of the lipase immobilized onto carbon nanotubes (CNTs) in transesterification of ethyl butyrate and found that 97% activity of the lipase was retained as well as a high enantioselectivity (360) was shown [65]. It could probably be due to that the hydrophobic CNTs lead the active sites of lipase, which are located on the opposite direction with hydrophobic pocket of lipase, to an accessible orientation [70-72]. Moreover, it is predicated that terminal group of CNTs could be responsible for the retention of enzyme activity and stability [73]. Lipase encapsulated by polymer nanogel retained 80% activity after 2 h reaction, while free lipase retained less than 10% activity after 30 min reaction in transesterification of dextran and vinyl decanoate [67]. According to the result from molecule stimulation, the high activity retention could be due to the lipase structure perseveration under nanogel environment protection, while the high stability of the lipase was most probably attribute to firm lipase immobilization onto the network structure polymer gel. Kwon et al. [74] reported that lipase immobilized onto nanosized silica kept 93% activity yet free lipase only remained 40% activity after 7 months storage. In addition, it was reported that enhanced activity was achieved after immobilizing lipase onto surface modified zirconia nanoparticles and the activity retained as high as initially after reusing 8 times [66].

Immobilizing lipase onto nanomaterials showed rather encouraging results. Lipase reuse is accomplished by settling and centrifuging. The fact that nanomaterials have small particle sizes causes time consuming in settling and energy consuming in centrifuging. In order to overcome

the problem, it was introduced the application of nanomagnetic materials in enzyme immobilization and found that the recovery of lipase could be easily and rapidly (within 1 min) completed by the addition of external magnetic fields [64,75]. It implies that immobilizing lipase onto nanomagnetic materials could be a strategy of enhancing the reusability of lipase.

In addition, it has been suggested that the use of whole microbial cells containing lipase in transesterification was comparable with free lipase [76]. Utilization of whole cells instead of free lipase is more economical because of the prevention in lipase extraction, separation, and purification. Moreover, studies on whole cell immobilization utilization in transesterification have been reported [77-79]. It could be predicated that immobilized microbial whole cells containing lipase onto nanomaterials could be a cost-efficient method of biodiesel production.

Apart from biocatalyst (lipase), heterogeneous catalyst is found to be another efficient catalyst. Numerous heterogeneous catalysts, such as calcined Li-CaO, Mg-Al hydrotalcites, calcium oxides, magnesia-rich magnesium aluminate spinel, Mg/Zr, which are the most solid acid or base, have been investigated in biodiesel production [21,80,81]. Among all heterogeneous catalysts, nanocatalysts have become a competitive candidate because of the high catalytic efficiency and ease in separation from products (Table 2.3). Biodiesel production through nanocatalytic transesterification from various oils such as plant oils and waste oils have been reported [21,22,80,82,83]. It was revealed that to obtain similar reaction (transesterification) conversion, the amount of nanocatalyst required is only 30% of that of common catalysts, and additionally, the reaction is less affected by the moisture of the oil and not influenced by free fatty acid content [21,83-85]. Recently, nanocatalyst application in the transesterification of microalgae oil has also been reported. A novel bifunctional (acid-base) mesoporous silica nanomaterial catalyst was introduced and planned to use the catalyst in biodiesel synthesis from microalgae [19]. It was reported that lipid extracted from microalgae was converted into biodiesel using nanoparticle silica catalyst in pilot plant [16].

Nanotechnology application in biodiesel production could significantly impact on the current edible oil, microalgae lipid and biodiesel market. The growing price of edible oil leads to biodiesel production unaffordable. As discussed above, nanomaterial could improve lipid accumulation (increasing lipid content in biomass) in microalgae and hence increase lipid

production from equal amount of microalgae biomass, which would reduce the production cost. In addition, implementing nanomaterial in lipid extraction without harm on microalgae prevents the cost which is demanded for recultivating microalgae after extraction in current system. The utilization of nanomaterial as carrier for whole cell lipase or as catalyst in transesterification would provide high quality biodiesel and by-product glycerol due to the ease recovery of the solid nanomaterial compared to the generally used acid or base catalyst. Moreover, the downstream purification of biodiesel and glycerol is simplified which reduces the cost. On the other hand, the elimination of the usage of acid or base catalyst, which is not possible to be recovered and has to be neutralized with the addition of chemicals, could further reduce the biodiesel production cost. Overall, nanomaterial utilization could bring a revolution in biodiesel market.

2.6 Research needs and future prospect

Heterotrophic microalgae have 10 to 20 folds higher growth rate than oleaginous crops and showed high lipid accumulation ability (up to 50% w/w of dry microalgae weight). The work on enhancing lipid accumulation through manipulating cultivation condition such as pH, temperature, carbon to nitrogen ratio, etc. should be performed as carbon and nitrogen sources were the only two factors have been reported till date.

The utilization of nanomaterial could enhance lipid production and transesterification, Specifically, in lipid production, the study on the fortification of nanomaterial in cultivation medium to stimulate lipid production/accumulation of heterotrophic microalgae should be conducted, and the utilization of nanomaterial instead of organic solvent which has safety and health issue, to achieve lipid extraction without killing the microalgae should be developed.

Immobilizing lipase onto nanomaterials has found to enhance lipid stability and reuse potential; however, the studies are mainly focusing on the utilization of nanoparticles and their separation from the products is difficult. Therefore, different types of nanomaterials such as the materials with nanosized pore or channels should be investigated for lipase immobilization; in order to accomplish easy separation of immobilized lipase from products, magnetic nanomaterials should be applied. Extracting lipase from microorganism is a costly process;

therefore, whole cell lipase has been reported utilizing in transesterification. In order to complete its recovery from products, immobilizing the whole cell lipase onto nanomaterials should be investigated. The studies of nanomaterial application in biodiesel production are in lab-scale, for the practical utilization, pilot-scale study is required. As nanotechnology application in biodiesel production develops, biodiesel production using heterotrophic microalgae will be more sustainable than current biodiesel production method.

2.7 Summary

Utilization of heterotrophic microalgae as feedstock is a promising way of biodiesel production. However, it has been hampered due to the costly lipid extraction process. Nanomaterials could efficiently achieve the extraction from microalgae cells, and appropriate selection on nanomaterials could even prevent harming microalgae. In addition, nanomaterial addition in the cultivation medium could enhance lipid accumulation of microalgae because it may affect the lipid metabolism.

Enhancing biodiesel production from heterotrophic microalgae through nanotechnology is still in the infant stage. Further research on the addition of nanomaterial such as nanosized silica and iron oxide to cultivation medium of heterotrophic microalgae should be investigated on the effect of lipid accumulation. The effort should be made on manipulation of the synthesis of nanomaterial containing function groups weakening/breaking cell walls and dissolving lipid. Whole cell lipase immobilization on nanomaterials should be studied and optimized.

2.8 Aknowledgments

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2.9 References

[1] N. Dizge, B. Keskinler, A. Tanriseven, Biodiesel production from canola oil by using lipase immobilized onto hydrophobic microporous styrene-divinylbenzene copolymer, Biochem. Eng. J. 44 (2009) 220-225.

[2] X. Meng, J. Yang, X. Xu, L. Zhang, Q. Nie, M. Xian, Biodiesel production from oleaginous microorganisms, Renewable Energy 34 (2009) 1-5.

[3] S.P. Singh, D. Singh, Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: A review, Renewable Sustainable Energy Rev. 14 (2010) 200-216.

[4] Y. Chisti, Biodiesel from microalgae, Biotechnol Adv 25 (2007) 294–306.

[5] A. Widjaja, C.-C. Chien, Y.-H. Ju, Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*, J. Taiwan Instit. Chem. Eng. 40 (2009) 13-20.

[6] L. Rodolfi, G. Chini Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, M.R. Tredici, Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor, Biotechnol. Bioeng. 102 (2009) 100-112.

[7] N.M. Verma, S. Mehrotra, A. Shukla, B.N. Mishra, Prospective of biodiesel production utilizing microalgae as the cell factories: A comprehensive discussion, Afr. J. Biotechnol. 9 (2010) 1402-1411.

[8] A.B.M.S. Hossain, A. Salleh, A.N. Boyce, P. Chowdhury, M. Naqiuddin, Biodiesel fuel production from algae as renewable energy, Am. J. Biochem. Biotechnol. 4 (2008) 250-254.

[9] X. Miao, Q. Wu, High yield bio-oil production from fast pyrolysis by metabolic controlling of Chlorella protothecoides, J. Biotechnol. 110 (2004) 85-93.

[10] X. Miao, Q. Wu, Biodiesel production from heterotrophic microalgal oil, Bioresour. Technol. 97 (2006) 841-846.

[11] Y. Shen, W. Yuan, Z. Pei, E. Mao, Heterotrophic culture of *Chlorella protothecoides* in various nitrogen sources for lipid production, Appl. Biochem. Biotechnol. 160 (2010) 1674-1684.

[12] Y. Cheng, Y. Lu, C. Gao, Q. Wu, Alga-based biodiesel production and optimization using sugar cane as the feedstock, Energy Fuels 23 (2009) 4166-4173.

[13] X. Li, H. Xu, Q. Wu, Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors, Biotechnol. Bioeng. 98 (2007) 764-771.

[14] H. Xu, X. Miao, Q. Wu, High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters, J.Biotechnol. 126 (2006) 499-507.

[15] F. Richard, G. Nigel, M.Q. James, S. Anthony, Nanoscience and nanotechnologies: opportunities and uncertainties, Report: The Royal Society and The Royal Academy of Engineering July 2004.

[16] V. Lin, Nanofarming technology extracts biofuel oil without harming algae, News released from Office of Public Affairs, April 14, 2009.

[17] V. Lin, Nanoparticle technology for biorefinery of non-food source feedstocks, http://www1.eere.energy.gov/industry/nanomanufacturing/pdfs/nanoparticle_tech_biorefinery.pdf (2009).

[18] F. Lu, Developing innovative photobioreactor and extraction technology for production of biodiesel feedstock using microalgae, http://www.reeis.usda.gov/web/crisprojectpages/217877.html (2009).

[19] E. Andrijanto, Novel bifunctional mesoporous silica nanomaterial catalyst for biodiesel production, Poster of University of Huddersfield Research Festival, 23rd March - 2nd April 2009 (2009).

[20] W. Xie, N. Ma, Immobilized lipase on Fe_3O_4 nanoparticles as biocatalyst for biodiesel production, Energy Fuels 23 (2009) 1347-1353.

[21] C.R. Venkat Reddy, R. Oshel, J.G. Verkade, Room-temperature conversion of soybean oil and poultry fat to biodiesel catalyzed by nanocrystalline calcium oxides, Energy Fuels 20 (2006) 1310-1314.

[22] L. Wen, Y. Wang, D. Lu, S. Hu, H. Han, Preparation of KF/CaO nanocatalyst and its application in biodiesel production from Chinese tallow seed oil, Fuel 89 (2010) 2267-2271.

[23] T. Mahmood, S.T. Hussain, Nanobiotechnology for the production of biofuels from spent tea, Afri. J. Biotechnol. 9 (2010) 858-868.

[24] T. Mahmood, S.T. Hussain, S.A. Malik, New nanomaterial and the process for the production of biofuel from metal hyper accumulator water hyacinth, Afri. J. Biotechnol. 9 (2010) 2381-2391.

[25] D. Pimentel, T.W. Patzek, Ethanol production using corn, switchgrass, and wood; biodiesel production using soybean and sunflower, Nat. Resour. Res. 14 (2005) 65-76.

[26] S. Sulaiman, A.A. Abdul Raman, M.K.A. Mohammed Kheireddine Aroua, Coconut waste as a source for biodiesel production, Chemical, Biological and Environmental Engineering (ICBEE), 2010 2nd International Conference on, 2010, pp. 254-256.

[27] K.K. Sharma, H. Schuhmann, P.M. Schenk, High lipid induction in microalgae for biodiesel production, Energies 5 (2012) 1532-1553.

[28] R. Halim, M.K. Danquah, P.A. Webley, Extraction of oil from microalgae for biodiesel production: A review, Biotechnology Advances 30 (2012) 709-732.

[29] C. Wang, L. Chen, B. Rakesh, Y. Qin, R. Lv, Technologies for extracting lipids from oleaginous microorganisms for biodiesel production, Front. Energy 6 (2012) 266-274.

[30] H. Fukuda, A. Kondo, H. Noda, Biodiesel fuel production by transesterification of oils, Journal of Bioscience and Bioengineering 92 (2001) 405-416.

[31] D.Y.C. Leung, X. Wu, M.K.H. Leung, A review on biodiesel production using catalyzed transesterification, Applied Energy 87 (2010) 1083-1095.

[32] X.-M. Shi, F. Chen, High-yield production of lutein by the green microalga *Chlorella protothecoidesin* heterotrophic fed-batch culture, Biotechnol. Progress 18 (2002) 723-727.

[33] Y. Cheng, W. Zhou, C. Gao, K. Lan, Y. Gao, Q. Wu, Biodiesel production from Jerusalem artichoke (Helianthus Tuberosus L.) tuber by heterotrophic microalgae *Chlorella protothecoides*, J. Chem. Technol. Biot. 84 (2009) 777-781.

[34] C. Gao, Y. Zhai, Y. Ding, Q. Wu, Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*, Appl. Energy 87 (2010) 756-761.

[35] E. Billa, D.P. Koullas, B. Monties, E.G. Koukios, Structure and composition of sweet sorghum stalk components, Indust. Crops Prod. 6 (1997) 297-302.

[36] U. Riebesell, A.T. Revill, D.G. Holdsworth, J.K. Volkman, The effects of varying CO_2 concentration on lipid composition and carbon isotope fractionation in *Emiliania huxleyi*, Geochim. Cosmochim. Acta 64 (2000) 4179-4192.

[37] J. Sheehan, T. Dunahay, J. Benemann, P. Roessler, A look back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae, Report, prepared by National Renewable Energy Laboratory (1998).

[38] A. Converti, A.A. Casazza, E.Y. Ortiz, P. Perego, M. Del Borghi, Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production, Chem. Eng. Process. 48 (2009) 1146-1151.

[39] S. La Fontaine, J.M. Quinn, S.S. Nakamoto, M.D. Page, V. Gohre, J.L. Moseley, J. Kropat, S. Merchant, Copper-dependent rron assimilation pathway in the model photosynthetic *Eukaryote chlamydomonas reinhardtii*, Eukaryot. Cell 1 (2002) 736-757.

[40] Y. Paz, E. Shimoni, M. Weiss, U. Pick, Effects of iron deficiency on iron binding and internalization into acidic vacuoles in *Dunaliella salina*, Plant Physiol. 144 (2007) 1407-1415.

[41] N.G. Menzyanova, A.V. Goltvyansky, Y.A. Kuznetsova, E.I. Sysenko, Season variability of iron effects on periodic culture of microalgae *Dunaliella viridis Teod.*, Front. Biology Chin. 4 (2009) 328-336.

[42] X. Deng, X. Fei, Y. Li, The effects of nutritional restriction on neutral lipid accumulation in *Chlamydomonas* and *Chlorella*, Afr. J. Microbiol. Res. 5 (2011) 260-270.

[43] G. Shan, J. Xing, H. Zhang, H. Liu, Biodesulfurization of dibenzothiophene by microbial cells coated with magnetite nanoparticles, Appl. Environ. Microbiol. 71 (2005) 4497-4502.

[44] W. De Windt, P. Aelterman, W. Verstraete, Bioreductive deposition of palladium (0) nanoparticles on *Shewanella oneidensis* with catalytic activity towards reductive dechlorination of polychlorinated biphenyls, Environ. Microbiol. 7 (2005) 314-325.

[45] L. Xin, H. Hong-ying, Z. Yu-ping, Growth and lipid accumulation properties of a freshwater microalga Scenedesmus sp. under different cultivation temperature, Bioresource Technology 102 (2011) 3098-3102.

[46] Z.Y. Liu, G.C. Wang, B.C. Zhou, Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*, Bioresour. Technol. 99 (2008) 4717-4722.

[47] S. Kang, M. Pinault, L.D. Pfefferle, M. Elimelech, Single-Walled Carbon Nanotubes Exhibit Strong Antimicrobial Activity, Langmuir 23 (2007) 8670-8673.

[48] C. Hu, J. Guo, J. Qu, X. Hu, Photocatalytic Degradation of Pathogenic Bacteria with AgI/TiO2 under Visible Light Irradiation, Langmuir 23 (2007) 4982-4987.

[49] Y. Jin, M. Wu, X. Zhao, Toxicity of nanomaterials to living cells, Nanotech. 1 (2005) 274-277.

[50] D. Williams, S. Ehrman, T. Pulliam Holoman, Evaluation of the microbial growth response to inorganic nanoparticles, J. Nanobiotechnol. 4 (2006) 3.

[51] M. Zhu, P.P. Zhou, L.J. Yu, Extraction of lipids from *Mortierella alpina* and enrichment of arachidonic acid from the fungal lipids, Bioresour. Technol. 84 (2002) 93-95.

[52] G. Vicente, L.F. Bautista, R. Rodríguez, F.J. Gutiérrez, I. Sádaba, R.M. Ruiz-Vázquez, S. Torres-Martínez, V. Garre, Biodiesel production from biomass of an oleaginous fungus, Chem. Eng. J. 48 (2009) 22-27.

[53] F. Pernet, R. Tremblay, Effect of ultrasonication and grinding on the determination of lipid class content of microalgae harvested on filters, Lipids 38 (2003) 1191-1195.

[54] G. Jon Van, Biodiesel processing and production, Fuel Process. Technol. 86 (2005) 1097-1107.

[55] F. Ullah, A. Nosheen, I. Hussain, A. Bano, Base catalyzed transesterification of wild apricot kernel oil for biodiesel production, Afr. J. Biotechnol. 8 (2009) 3289-3293.

[56] W. Du, Y. Xu, D. Liu, J. Zeng, Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors, J. Mol. Catal. B: Enzym. 30 (2004) 125-129.

[57] A.V. Lara Pizarro, E.Y. Park, Lipase-catalyzed production of biodiesel fuel from vegetable oils contained in waste activated bleaching earth, Process Biochem. 38 (2003) 1077-1082.

[58] H. Noureddini, X. Gao, R.S. Philkana, Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil, Bioresour. Technol. 96 (2005) 769-777.

[59] Y. Gao, T.-W. Tan, K.-L. Nie, F. Wang, Immobilization of Lipase on Macroporous Resin and Its Application in Synthesis of Biodiesel in Low Aqueous Media, Chin. J. Biotechnol. 22 (2006) 114-118.

[60] Y.-X. Bai, Y.-F. Li, Y. Yang, L.-X. Yi, Covalent immobilization of triacylglycerol lipase onto functionalized novel mesoporous silica supports, J. Biotechnol. 125 (2006) 574-582.

[61] A. Salis, M. Pinna, M. Monduzzi, V. Solinas, Comparison among immobilised lipases on macroporous polypropylene toward biodiesel synthesis, J. Mol. Catal. B: Enzym. 54 (2008) 19-26.

[62] K.R. Jegannathan, S. Abang, D. Poncelet, E.S. Chan, P. Ravindra, Production of biodiesel using immobilized lipase-a critical review, Crit. Rev. Biotechnol. 28 (2008) 253-264.

[63] M. Qhobosheane, S. Santra, P. Zhang, W. Tan, Biochemically functionalized silica nanoparticles, Analyst 126 (2001) 1274-1278.

[64] S.C. Tsang, C.H. Yu, X. Gao, K. Tam, Silica-encapsulated nanomagnetic particle as a new recoverable biocatalyst carrier, J. Phys. Chem. B 110 (2006) 16914-16922.

[65] S. Shah, K. Solanki, M. Gupta, Enhancement of lipase activity in non-aqueous media upon immobilization on multi-walled carbon nanotubes, Chem. Cent. J. 1 (2007) 30.

[66] Y.Z. Chen, C.T. Yang, C.B. Ching, R. Xu, Immobilization of lipases on hydrophobilized zirconia nanoparticles: highly enantioselective and reusable biocatalysts, Langmuir 24 (2008) 8877-8884.

[67] J. Ge, D. Lu, J. Wang, Z. Liu, Lipase nanogel catalyzed transesterification in anhydrous dimethyl sulfoxide, Biomacromolecules 10 (2009) 1612-1618.

[68] S.-I. Matsuura, R. Ishii, T. Itoh, S. Hamakawa, T. Tsunoda, T. Hanaoka, F. Mizukami, Immobilization of enzyme-encapsulated nanoporous material in a microreactor and reaction analysis, Chem. Eng. J. 167 (2011) 744-749.

[69] A. Macario, M. Moliner, A. Corma, G. Giordano, Increasing stability and productivity of lipase enzyme by encapsulation in a porous organic-inorganic system, Microporous Mesoporous Mater. 118 (2009) 334-340.

[70] R. Fernandez-Lafuente, P. Armisén, P. Sabuquillo, G. Fernández-Lorente, J. M. Guisán, Immobilization of lipases by selective adsorption on hydrophobic supports, Chem. Phys. Lipids 93 (1998) 185-197.

[71] S.S. Karajanagi, A.A. Vertegel, R.S. Kane, J.S. Dordick, Structure and Function of Enzymes Adsorbed onto Single-Walled Carbon Nanotubes, Langmuir 20 (2004) 11594-11599.

[72] B. Dragoi, E. Dumitriu, Candida antarctica lipase B immobilization onto hybrid organic-inorganic mesoporous materials, Acta Chim. Slov. 55 (2008) 277-285.

[73] I.V. Pavlidis, T. Tsoufis, A. Enotiadis, D. Gournis, H. Stamatis, Functionalized multi-wall carbon nanotubes for lipase immobilization, Adv. Eng. Mater. 12 (2010) B179-B183.

[74] S.S. Kwon, S.H. Jeon, J.K. Shon, D.H. Kim, I. Chang, J.M. Kim, Preparation and stabilization of chitosan-lipase composite within mesoporous silica material, Solid State Phenom. 124-126 (2007) 1717-1720.

[75] R. Konwarh, N. Karak, S.K. Rai, A.K. Mukherjee, Polymer-assisted iron oxide magnetic nanoparticle immobilized keratinase, Nanotechnol. 20 (2009) 225107.

[76] W. Li, W. Du, D. Liu, Rhizopus oryzae whole-cell-catalyzed biodiesel production from oleic acid in tert-butanol medium, Energy Fuels 22 (2007) 155-158.

[77] K. Ban, M. Kaieda, T. Matsumoto, A. Kondo, H. Fukuda, Whole cell biocatalyst for biodiesel fuel production utilizing Rhizopus oryzae cells immobilized within biomass support particles, Biochem. Eng. J. 8 (2001) 39-43.

[78] J. Zeng, W. Du, X. Liu, D. Liu, L. Dai, Study on the effect of cultivation parameters and pretreatment on *Rhizopus oryzae* cell-catalyzed transesterification of vegetable oils for biodiesel production, J. Mol. Catal. B: Enzym. 43 (2006) 15-18.

[79] Q. He, Y. Xu, Y. Teng, D. Wang, Biodiesel production catalyzed by whole-cell lipase from *Rhizopus chinensis*, Chin. J. Catal. 29 (2008) 41-46.

[80] Y. Wang, S.-y. Hu, Y.-p. Guan, L.-b. Wen, H.-y. Han, Preparation of mesoporous nanosized KF/CaO–MgO catalyst and its application for biodiesel production by transesterification, Catal. Lett. 131 (2009) 574-578.

[81] R. Sree, N.S. Babu, P. Saiprasad, N. Lingaiah, Transesterification of edible and non-edible oils over basic solid Mg/Zr catalysts, Fuel Processing Technology 90 (2009) 152-157.

[82] M. Verziu, B. Cojocaru, J. Hu, R. Richards, C. Ciuculescu, P. Filip, V.I. Parvulescu, Sunflower and rapeseed oil transesterification to biodiesel over different nanocrystalline MgO catalysts, Green Chem. 10 (2008) 373-381.

[83] N. Boz, N. Degirmenbasi, D.M. Kalyon, Conversion of biomass to fuel: Transesterification of vegetable oil to biodiesel using KF loaded nano-γ-Al_2O_3 as catalyst, Appl. Catal. B-Environ. 89 (2009) 590-596.

[84] V.B. Veljković, O.S. Stamenković, Z.B. Todorović, M.L. Lazić, D.U. Skala, Kinetics of sunflower oil methanolysis catalyzed by calcium oxide, Fuel 88 (2009) 1554-1562.

[85] J. Li, X. Wang, W. Zhu, F. Cao, $Zn_{1.2}H_{0.6}PW_{12}O_{40}$ nanotubes with double acid sites as heterogeneous catalysts for the production of biodiesel from waste cooking oil, ChemSusChem 2 (2009) 177-183.

[86] J. Breznak, J. Switzer, H. Seitz, 150:, *Sporumusa termitida* sp. nov., an H_2 / CO_2 utilizing acetogen isolated from termites, Archives of Microbiology 150 (1988) 282–288.

[87] J. Liu, J. Huang, K.W. Fan, Y. Jiang, Y. Zhong, Z. Sun, F. Chen, Production potential of *Chlorella zofingienesis* as a feedstock for biodiesel, Bioresour.Technol. 101 (2010) 8658-8663.

[88] J. Zhu, P.M. Ndegwa, A. Luo, 2001. . , Effect of solid-liquid separation on BOD and VFA in swine manure, Environ. Technology. 22 (2001) 1237-1243.

[89] W. Xiong, X. Li, J. Xiang, Q. Wu, High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production, Appl. Microbiol. Biotechnol. 78 (2008) 29-36.

[90] Y.-H. Chen, T. Walker, Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol, Biotechnol. Lett. 33 (2011) 1973-1983.

[91] M.B. Johnson, Z. Wen, Production of Biodiesel Fuel from the Microalga Schizochytrium limacinum by Direct Transesterification of Algal Biomass, Energy Fuels 23 (2009) 5179-5183.

[92] Z. Chi, D. Pyle, Z. Wen, C. Frear, S. Chen, A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation, Process Biochem. 42 (2007) 1537-1545.

[93] D.J. Pyle, R.A. Garcia, Z. Wen, Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition, J. Agric. Food. Chem. 56 (2008) 3933-3939.

[94] K. Solanki, M.N. Gupta, Simultaneous purification and immobilization of *Candida rugosa* lipase on superparamagnetic Fe_3O_4 nanoparticles for catalyzing transesterification reactions, New J. Chem. (2011).

[95] A. Dyal, K. Loos, M. Noto, S.W. Chang, C. Spagnoli, K.V.P.M. Shafi, A. Ulman, M. Cowman, R.A. Gross, Activity of *Candida rugosa* lipase immobilized on γ-Fe2O_3 magnetic nanoparticles, J. Am. Chem. Soc. 125 (2003) 1684-1685.

[96] C.G.C.M. Netto, L.H. Andrade, H.E. Toma, Enantioselective transesterification catalysis by *Candida antarctica* lipase immobilized on superparamagnetic nanoparticles, Tetrahedron: Asymmetry 20 (2009) 2299-2304.

[97] N. Miletić, V. Abetz, K. Ebert, K. Loos, Immobilization of Candida antarctica lipase B on Polystyrene Nanoparticles, Macromol. Rapid Commun. 31 (2010) 71-74.

[98] S. Luz Martínez, R. Romero, J.C. López, A. Romero, V.c. Sánchez Mendieta, R. Natividad, Preparation and characterization of CaO nanoparticles/NaX zeolite catalysts for the transesterification of sunflower oil, Ind. Eng. Chem. Res. 50 (2010) 2665-2670.

[99] J. Montero, K. Wilson, A. Lee, Cs promoted triglyceride transesterification over MgO nanocatalysts, Top. Catal. 53 (2010) 737-745.

[100] S. Hu, Y. Guan, Y. Wang, H. Han, Nano-magnetic catalyst KF/CaO–Fe_3O_4 for biodiesel production, Appl. Energy 88 (2011) 2685-2690.

[101] H. Han, Y. Guan, Synthesis of biodiesel from rapeseed oil using K_2O/γ-Al_2O_3 as nano-solid-base catalyst, Wuhan Univ. J. Nat. Sci. 14 (2009) 75-79.

[102] Q.Y. Zhang, D.M. Qi, H. Zhang, D.W. Ba, Z.J. Chen, Z.Z. Wu, Ma. P.H, Synthesis of biodiesel catalyzed by K_2CO_3/CaO nanocatalyst from soybean oil, Chem. Eng. Chin. 4 (2011) 15-17.

[103] M. Kaur, A. Ali, Lithium ion impregnated calcium oxide as nano catalyst for the biodiesel production from karanja and jatropha oils, Renewable Energy 36 (2011) 2866-2871.

[104] L. Wang, J. Yang, Transesterification of soybean oil with nano-MgO or not in supercritical and subcritical methanol, Fuel 86 (2007) 328-333.

[105] A. Yacob, M. Khairul, A. Amat, N. Samadi, Calcination temperature of nano MgO effect on base transesterification of palm oil, Eng. Technol. 56 (2009) 408-412.

Table 2.1 **Lipid accumulation in microorganisms**

Microbe	Lipid content (% w/w)	Carbon source (g/L)	Nitrogen source	C/N ratio	pH	Cultivation conditions			Reference
						Temp. (ºC)	Shaking rate (rpm)	Period (h)	
Chlorella zofingiensis	52	Glucose (50)	Nitrate	143	6.5	25	150	144	[87]
	22	Lactose (50)	Nitrate	150	6.5	25	150	144	
	25	Galactose	Nitrate	143	6.5	25	150	144	
	51	Fructose	Nitrate	143	6.5	25	150	144	
	51	Sucrose	Nitrate	150	6.5	25	150	144	
	50	Mannose	Nitrate	143	6.5	25	150	144	
Chlorella protothecoids	55.2	Glucose (10)	Glycine	214	6.5	28	180	240	[14]
	54.7	Glucose (5)	Glycine	107	6.0	28	180	240	
	55.3	Corn powder (5)	Glycine	-	6.0	28	180	240	
Chlorella protothecoids	57.9	Glucose (10)	Glycine	214	6.5	25	200	240	[9]
Chlorella protothecoids	46.7	Glucose (20)	Yeast extract	31	6.3	28	200	108	[12]
	44.4	Sugar cane (20)	Yeast extract	12.5	6.3	28	200	108	
	53	Sugar cane (16.8)	Yeast extract	21	6.3	28	200	108	
	49	Sugar cane (16.8)	Yeast extract	15	6.3	28	200	108	
	42	Sugar cane (16.8)	Yeast extract	9	6.3	28	200	108	
Chlorella protothecoids	*50.5*	Glucose (40)	Nitrate	22.86	6.8	28	250	216	[11]
	27.3	Glucose (40)	Urea	19.8	6.8	28	250	216	
	33.4	Glucose (40)	Yeast extract	44.8	6.8	28	250	216	
Chlorella protothecoids	*52.5*	Sweet sorghum juice (10)	Yeast extract	16.7	-	28	220	120	[34]
	53.3	Glucose (10)	Yeast extract	11.8	-	28	220	120	
Chlorella protothecoids	*44*	Jerusalem artichoke tuber (30)	Yeast extract	Yeast extract (4g/L)	6	28	200	96	[33]
	45.2	Glucose (30)	Yeast extract	35.3	6	28	200	96	
Chlorella protothecoids	*44.3*	Glucose (10)	Yeast extract	21	6.5	28	200	200	[13]
Chlorella protothecoids	*57.8*	Glucose (15-60)	Yeast extract	17.6-70.4	6.5	28	200	184	[89]

Chlorella protothecoids	*23.5*	Glycerol (30)	Yeast extract	34.4	6.8	28	200	144	[90]
Schizochytrium limacinum	*51*	Glycerol (90)+ corn steep solid (10)	0	-	8	-	-	-	[91]
Schizochytrium limacinum	*18*	Glycerol (106)	0	-	7.5	20	170	-	[92]
Schizochytrium limacinum	*50.57*	Glycerol (75)	Nitrate + ammonia nitrogen	2000	7.5-8	20	170	-	[93]

Table 2.2 Nanomaterial application in lipase immobilization

Lipase source	Nanomaterials	Activity remaining (%)	Times of IRTA[a] of immobilized to free lipase	Times of TCR[b] of immobilized to free lipase	Reuse ability	Reference
Candida rugosa	Carbon nanotubes	97	2.2-14	4.44	-	[65]
Candida rugosa	Nanogel	85	-	7.67	-	[67]
Candida rugosa	Fe_3O_4 nanoparticles	80	110	20.5	4	[94]
Candida rugosa	ZrO_2	214	-	3.3	8	[66]
Candida rugosa	γ-Fe_3O_4 nanoparticles	<100	-	-	-	[95]
Candida antarctica	Fe_3O_4 nanoparticles	200	-	-	4	[96]
Candida antarctica	Polystyrene nanoparticles	204	-	-	-	[97]
Pseudomonas cepacia	ZrO_2		-	3.6	-	[66]
Thermomyces lanuginosus	Nanosized silica	93	-	-	-	[74]
Thermomyces lanuginosa	Fe_3O_4 nanoparticles	70	-	1.05	4	[20]

[a] *Initial rates of transesterification activity.*

[b] *Transesterification conversion rate.*

Table 2.3 Nanocatalyst in transesterification

Heterogeneous catalyst (nanosized)	Oil	Catalyst addition C/O[c] ratio (% w/w)	Reaction time (h)	Yield (%)	Reference
CaO	Poultry fat	0.6	12	99	[21]
CaO	Soybean oil	16	6	93.5	[98]
$Cs_2Mg(CO_3)_2$	Butter	-	3	100	[99]
KF/Al_2O_3	Soybean oil	3	8	99.8	[83]
$KF/CaO–Fe_3O_4$	Stillingia oil	4	3	95	[100]
KF/CaO–MgO	Rapeseed oil	3	3	95	[80]
KF/CaO	Tallow seed oil	-	-	96.8	[22]
$K_2O/\gamma\text{-}Al_2O_3$	Rapeseed oil	3	3	94	[101]
K_2CO_3/ CaO	Soybean oil	3	1	99	[102]
Li-CaO	Karanja and jatropha oils	5	1	100	[103]
MgO	Soybean oil	2	17	99	[104]
MgO	Sunflower oil and rapeseed oil	1.5	6	90	[82]
MgO	Palm oil	0.5	4	51.3	[105]
$Zn_{1.2}H_{0.6}PW_{12}O_{40}$	Waste cooking oil	2.5	12	97.2	[85]

[c] *ratio presents catalyst to oil ratio.*

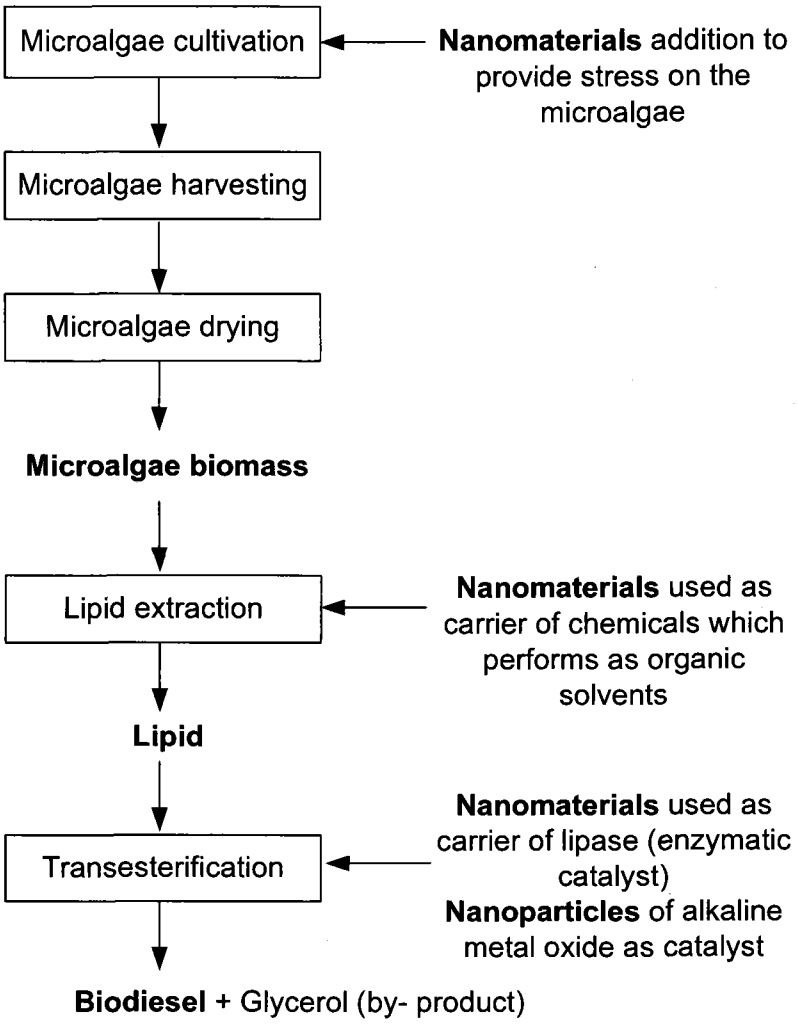

Figure 2.1 **Nanotechnology application in biodiesel production from heterotrophic microalgae**

LIPID EXTRACTION TECHNOLOGIES

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CHAPTER 11 OF THE BOOK: BIODIESEL PRODUCTION: TECHNOLOGIES, CHALLENGES, AND FUTURE PROSPECTS (ACCEPTED)

3 LIPID EXTRACTION TECHNOLOGIES

3.1 Résumé

Ce chapitre passe en revue les technologies d'extraction des lipides, y compris les technologies physiques et chimiques. L'extraction physique permet de rompre les cellules en fournissant la force pour libérer les lipides, tandis que l'extraction chimique permet d'utiliser un solvant pour extraire les lipides à partir des cellules. Chacune de ces méthodes présente des avantages et des désavantages. La méthode physique est propre mais présente une faible efficacité d'extraction des lipides (environ 70% des lipides totaux) avec une consommation d'énergie élevée. L'extraction chimique présente un fort potentiel de contamination des lipides en raison de la présence de solvants résiduels. L'utilisation des solvants tels que le CO_2 supercritique permet de donner des lipides de haute qualité, mais il dépend normalement du procédé de pré-traitement ou de la présence des co-solvants, afin d'atteindre une efficacité d'extraction plus élevée. Les nouvelles technologies comme l'utilisation des nanomatériaux pour l'extraction montrent un rendement élevé.

Mots clés: Lipides; extraction physique; extraction chimique

3.2 Abstract

The chapter reviews the lipid extraction technologies including physical and chemical ones. Physical extraction breaks the cells by providing force to release the lipid, while chemical extraction is to utilize solvent to pull out lipid from cells. Each of the method has its advantage and disadvantage. Physical method is clean but has low lipid extraction efficiency (around 70% of total lipid) and high energy consumption. Chemical extraction has high possibility on contamination of the lipid due to the presence of the residual solvents. Clean solvent such as supercritical $CO_{2\,g}$ives high quality lipid but it normally depends on pre-treatment or co-solvent addition in order to achieve high extraction efficiency. New technologies such as switchable solvent and nanomaterial extraction have also been reported, but detailed information is not available as it could be still in the infant research stage.

Keywords: Lipid; physical extraction; chemical extraction

3.3 Introduction

Current biodiesel is converted from oils or fats contained in plants seeds, microorganisms, or animals. Therefore, extraction of the oils and fats from oil bearing materials is an essential step of biodiesel production. The extraction methods should be rapid, efficient, and can preserve the originality of the oil/fat.

Several methods have been established to achieve the extraction. Mechanical pressing was the leading technology before 1900's. It is still applied in present age as it requires low cost and provides high quality products (oil and residual cakes). However, the extraction efficiency (50 to 80%), especially for the substances with low oil/fat content (<20%), is undesirable. To enhance the efficiency, mechanical pressing followed by solvent extraction has been established and widely used in oil extraction from oilseeds. The process could achieve a 98% oil recovery (Amalia Kartika et al. 2010). The application of solvents has significantly enhanced the efficiency of oil extraction. Therefore, mechanical pressing has been slowly replaced by solvent extraction. Organic solvents can dissolve oil and be readily evaporated. Methanol-chloroform, hexane, and hexane-isopropanol are normally utilized solvents (Cheng et al. 2011; Boyd et al. 2012). Currently, solvent extraction is the most often applied method in industry. However, the utilization of organic solvents has raised health and environmental concerns due to their flammability and toxicity.

Technologies with less threat to the human being and environment are demanded. Therefore, to lower or eliminate the toxic solvent utilization amount becomes the key solution of the problem. The assistances of ultrasonification and microwave in oil extraction avoid the large amount of solvent utilization and enhance the oil yield (Ranjan et al. 2010; Araujo et al. 2013). Fatty acid methyl ester which is non-toxic, renewable, and biodegradable, has been studied in oil extraction from sunflower seeds and showed comparable performance as the conventional solvents (hexane, chloroform, methanol, and isopropanol) (Amalia Kartika et al. 2010). This chapter reviewed the technologies of oil extraction and discussed their advantages and disadvantages.

3.5 Cell disruption

Biological products synthesized by cells are intracellular and extracellular. Extracellular products are easy to be separated from the cells by filtration or centrifugation. Intracellular products are either in the cytoplasm or as inclusion bodies such as lipid. Lipid is mainly present in cell membrane (to form the bilayer) and cytoplasm (in the form of lipid droplets). In order to obtain the desired intracellular products, cell disruption has to be conducted to release these products before further separation to be carried out. Therefore, cell disruption is a critical step of lipid separation from cells.

Blade homogenizer, bead milling, liquid homogenization, sonication, and freeze/thaw are the most utilized physical approaches (Prabakaran and Ravindran 2011; Dhanani et al. 2013). Blade homogenizer uses rotating blades to grind cells and achieves the disruption. Generally, higher energy input provides higher disruption efficiency. Beat milling is normally used along with agitation and the cell disruption efficiency is determined by bead size and agitation speed (Klimek-Ochab et al. 2011). Liquid homogenizer is widely applied in the disruption of microorganism cells. It lyses the cells by forcing the cell suspension passing through a narrow space and then shearing the cells (Zheng et al. 2011). Sonication utilizes sound waves to form microscope vapor bubbles and then obtains the disruption. The method is efficient and suitable for the disruption of small size materials such as bacterial, spores, cells, and finely diced tissues (Choonia and Lele 2011). Freeze/thaw completes the disruption by freezing the cells to cause the swell and contract during thaw, and ultimately breaks the cells (Shin et al. 1994; Schwede et al. 2011). The physical methods show the disadvantages on preventing the product quality as the methods tend to increase the local temperature and lead to oxidation and denaturisation.

Chemical methods of cell disruption are the processes with the addition of chemicals such as solvents, detergents, and enzymes. Detergents such as triton-X series and tween series are capable of solubilize phospholipid and thus cause the cell membrane disruption. However, a pre-treatment to weaken the cell wall is required before detergent can act (Northcote and Horne 1952). Organic solvent cell disruption works in the similar way as detergent to solubilize the cell membrane. Normally the solvent can disrupt the cell wall; therefore, pre-treatment is

not demanded (Klimek-Ochab et al. 2011). Enzyme such as lysozyme has the ability to disrupt the cell wall, but cannot break the cell membrane; hence, it is generally used combined with detergent addition (Jin et al. 2012). Some of the cell disruption methods are presenting in Table 3.1.

3.6 Physical technical technologies of lipid extraction

3.6.1 Expeller pressing

Expeller pressing is a mechanical method for separating lipid from raw materials such as nuts. Different types of expeller such as hand bridge press, hydraulic press, ram press, and screw press, have been used. The structures of the expellers vary from one type to the other. The principle of expeller lipid extraction is that the target materials fed between two heave metal plates is grinded, crushed, and pressed as the plates rotate towards each other driven by manual or power, which results in the lipid separation from the oleaginous materials. The pressure generated by the driving force (manual, motor or engine) is the main factor on the extraction efficiency as it is the main cause of cell disruption.

Expeller pressing lipid extraction is clean and cost-efficient. However, there are two major disadvantages namely low efficiency and oil flavor change. The general lipid recovery from expeller pressing is normally less than 70% w/w (more than 90% w/w for solvent extraction) (Bamgboye and Adejumo 2007). In order to recover more lipids from the raw materials, solvent extraction has to be performed after the pressing. The other concern of pressing method is the high temperature generation during the pressing. The temperature increase depends on the hardness of the raw materials. Harder the material is, higher the temperature reaches in the process. The lipid extraction with temperature controlled expeller is called cold pressing in which the temperature will not rise above 50 °C. Cold pressing is generally used to obtain lipid from delicate materials such as olive. The expeller pressing efficiency on different raw materials is shown in Table 3.2.

Expeller is suitable for lipid extraction from any type of oleaginous material. It is specially used for lipid extraction from soybean, sunflower seed, and nuts in farms and small scale industries

of rural area. Up to date, no report on its utilization on lipid extraction from microorganisms has been released. As no special requirement on raw material is demanded for lipid extraction; therefore, expeller pressing could be used in lipid separation from oleaginous microorganisms.

3.6.2 Thermal lipid extraction

Hot water floatation is the simplest and oldest method of lipid extraction. Raw materials are immerged in boiling water and kept simmering for certain period (normally several hours). As temperature going down, the raw material becomes a paste. Lipid floats to the surface and then can be skimmed off. Generally, in this process, the lipid is required to be reheated to 100 °C to drive off the trace amount of water. The extraction efficiency of the method is depending on the lipid content of the materials and the lipid property (liquid or solid form at room temperature). The extraction efficiency is high when the oil bearing substance has high lipid content and lipid is in solid form at room temperature. Thermal extraction is normally applied in lipid separation from animal fat and fish, and salt could be added to enhance the separation (Bimbo 2012). Thermal extraction has also applied in lipid extraction from groundnut. With vegetable oils, the method is undesirable due to the formation of oil-water emulsions, which makes the floating of oil from the water is difficult. Microorganism lipid content could reach 80% w/w and the lipid is generally in solid form at room temperature. Hence, the method could be utilized for the lipid separation from microorganisms.

3.6.3 Ultrasonication lipid extraction

Ultrasonication provides cavitation phenomena. Microscopic bubbles at various nucleation sites in fluid were formed during ultrasonication which has two phase, namely, rarefaction and compression phase. The bubbles grow during the rarefaction and are compressed during compression phase which cause the collapse of the bubbles. A violent shock wave was formed by the collapse of the bubbles, and then tremendous heat, pressure, and shear were generated.

Ultrasonication has been widely applied in industry and is grabbing more and more attentions as it has accomplished protein extraction, chemical synthesis, disinfection, and cell disruption with reducing or eliminating chemical addition, which is considered as green chemistry. The application of ultrasonication in cell disruption for the intracellular products recovery is not

new. The method has been widely used in protein (especially enzymes such as β-Galactosidase) and lactase releasing from cells (Becerra et al. 2001; Benov and Al-Ibraheem 2002; Choonia and Lele 2011). Generally, cells harvested from fermentation will have to be washed before being subjected to ultrasonification in order not to contaminate the products. Filtration and centrifugation are performed after ultrasonication to separate the products from the impurities.

Study has performed in utilization of ultrasonication on lipid extraction from *Nannochloropsis oculata (Adam et al. 2012)*. Response surface methodology was used to obtain the optimal condition. Parameters including extraction time (10 to 30 min), biomass concentration (10 to 50 g/L), and ultrasound power (450 to 1000 W) were varied. The optimal condition was found at the power of 1000 w for 30 min with biomass concentration 50 g/L. After the extraction salt was added to enhance the separation of lipid from the solution. The highest lipid yield was 0.21% w/w which is a lot lower than solvent (chloroform and methanol) extraction yield (5.47% w/w). More efforts are required to increase lipid recovery with ultrasonication.

3.7 Chemical technologies of lipid extraction

3.7.1 Organic solvent extraction

So far, many methods can be found on lipid extraction from various materials such as animal and plant tissues, and microorganism cells. The first popular lipid extraction is described in 1879 by Franz von Soxhlet. They invented a special apparatus called Soxhlet Apparatus to extract lipid from solid materials (Soxhlet 1879). The extraction is accomplished by boiling solvent to generate the vapor which constantly flows over the solid to extract the lipid. At the end, solvents containing lipid are collected, and then lipid will be obtained after evaporating of the solvents. The method is simple while there is risk of lipid oxidation due to the high temperature. In fact, the method is often used in the extraction of pesticides and polychlorinated biphenyls (PCB) rather than in lipid extraction (Zhao et al. 2005; Zhou et al. 2008). So far, the most cited two lipid methods are reported by Folch et al. (1957) and Bligh and Dyer (1959). The common points of the two methods are the utilization of chloroform and

methanol aiming to estimate the total lipid. The both methods have been well established. The method of Folch et al. (1957) is known due to its simplicity (one step extraction); while the one of Bligh and Dyer (1959) is considered as a rapid method (no requirement on pre-drying). There is an adverse effect on environment of the both methods due to the utilization of chloroform.

Therefore, the mixture of hexane and isopropanol, which are less toxic and cheaper than chloroform and methanol, was studied on lipid extraction (Hara and Radin 1978). However, it was observed that the method couldn't extract gangliosides. In fact, ganglioside is just a minor fraction of the total lipid; hence the method is still widely used and recommended by US-EPA for field studies. Another method which has similar procedure as method of Bligh and Dyer (1959) has also been reported, but mixture of isopropanol and cyclohexane was employed instead of chloroform and methanol (Smedes 1999). However, the method was found not suitable for specific tissues such as liver, as the possibility of emulsion formation. Another halogenate free solvent extraction employing 2-propanol (Sree et al.), diethyl ether (DEE), and n-hexane completes the extraction with the first IPR and DEE extraction, the second n-hexane/DEE and IPR, and the last n-hexane/DEE (Jensen et al. 2003). The advantages of the method are that there is no requirement on heating and it is easy to handle. The method is normally utilized for large samples (>10 g) and the suitability to small samples is not studied.

Accelerate solvent extraction is similar as Soxhlet extraction but the method is not only applies high temperature but also high pressure to keep the solvent in liquid phase (Richter et al. 1996). The method is time saving but expensive, and generally utilized for extracting environmental contaminants such as PCBs, dioxins, and pesticides. Current organic solvent extraction is either the original method described above or the modification. Modification is mainly embodied on the combination of the above mentioned methods with assistance of cell disruption treatment such as bead milling, ultrasonication, microwave, and so on.

3.7.1.1 Solvent type effect on lipid extraction

Solvent extraction efficiency is up to 96% (Ferraz et al. 2004; Dufreche et al. 2007). The most utilized solvents include alcohols (mainly referring to methanol), chloroform, hexane, petroleum ether, and diethyl ether. The selection of the solvents is critical as it impacts on the

extraction efficiency, lipid property preservation, and solvent recovery. To extract lipid from tissues, it is necessary to create enough force to break cell membrane and lipoprotein to release the lipids. However, it is also required that the solvents would not react chemically with the lipids.

Cell membrane has a double lipid layer. Each lipid layer is composed of polar head and non-polar tail. The tail is oriented inwards and the head faces outwards (toward the aqueous cytosol of the cell or the outside environment). These tails or heads are grouping together to form the bilayer. The structure of the cell membrane determines that non-polar solvent cannot perform the extraction as it cannot approach and pull out the lipid from cell membrane, and hence cannot rupture the membrane. However, if cell disruption is performed prior to non-polar solvent extraction, the extraction will be possible to complete. Polar solvent (as water) could approach to the membrane but if the polarity of the solvent is lower, then cannot pull out the lipid as the tails are tightly bonding (hydrophobic interaction) together. Therefore, mixture of polar and non-polar solvent is required. The polar solvent interacts and pulls apart the cell membrane, and the non-polar solvent excesses to the non-polar tail and dissolve the lipid. So far, many solvent extractions have been used (Table 3.3). It clearly shows that the mixture of polar and non-polar solvents provide high extraction efficiency (around 95%) at mild condition (around 25 °C). For the extraction with single polar or non-polar solvent, the extraction efficiency (less than 75%) is normally low.

The lipid droplets, also called lipid bodies, in oil bearing tissues are mainly triglycerides (TAG) which is a non-polar substance. They are soluble in hexane, cyclohexane, diethyl ether, and chloroform. Therefore, when the focus is on TAG extraction, non-polar solvent should be employed. However, it is necessary to break the cell first to allow non-polar solvent to excess TAG. Therefore, either polar solvent should be added along with non-polar solvent or other cell disruption methods such as milling and ultrasonification should be employed.

3.7.1.2 Oil bearing substance effect on lipid extraction

It can be also seen that type of oleaginous substances also has effect on the extraction (Table 3.3). The structure differences of the cells of plants, animals, and microorganisms are the main

cause of the difference on extraction efficiency. Unlike plant and microorganism, animal cells have no cell wall. It makes the lipid extraction easier (such as short extraction time and high efficiency) from animal tissues than from plant and microorganisms (Ferraz et al. 2004; Vicente et al. 2009). Cell wall of plants is formed by cellulose-hemicellulose network with embedded pectin matrix. For fungus, the cell wall consists largely of β(1-3) and β(1-6)-D-glucans, chitin, and protein. The linkage between β(1-3), β(1-6)-D-glucans and chitin forms the cell wall, and protein is normally embedded. The solubility of the cell wall in the solvent or solvent mixture determines the extraction efficiency. Lipid extraction with n-hexane from fungus is easier compared from soybean as the extraction time (1 h for fungus and 2.5 h for soybean) was shorter and the required temperature was lower (25 °C for fungus and 70 °C for soybean) (Nikolić et al. 2009; Vicente et al. 2009). In addition, the extraction efficiency is higher for fungus (70.7%) than for soybean (68.7%). This would be due to that the cell wall of soybean is harder to be broken by hexane than that of the fungus.

3.7.1.3 Pre-treatment effect on lipid extraction

Organic solvent extraction combined with other technologies such as bead milling, grinding, and ultrasonication has also been reported (Table 3.4). The addition of other technologies is aim to disrupt the cells and enhance the extraction. The extraction can be performed in two separated steps with the first cell disruption and the second solvent extraction, or in one combined step with simultaneously cell disruption and solvent extraction.

Bead milling assisted solvent extraction: Bead milling is the process that bead mixes with cell suspension at high speed agitation. The mixing provides the contact and shearing between cells and beads, and thus achieves the disruption. After the disruption, solvent is generally used to recover the oil. Therefore, the milling is also considered as pre-treatment of solvent extraction. Size and shape of the bead, agitation, the strength of cell wall, and cell concentration of the suspension have great effect on the degree of the disruption (Klimek-Ochab et al. 2011).

Lipid extraction with chloroform and methanol (2:1 v/v) from *Chlorella* sp biomass with or without bead milling, showed significantly different results (Prabakaran and Ravindran 2011). Higher lipid was obtained from that with milling (0.15 g lipid/0.5 g dry biomass) than that

without milling (0.08 g lipid/0.5 g dry biomass). The similar results were obtained with the study on *Botryococcus* sp., *Chlorella vulgaris*, *Scenedesmus* sp., *Nostoc* sp., and *Tolypothrix* sp.(Lee et al. 2010). Apart from beads, sand has also been employed in cell wall disruption (Somashekar et al. 2001). However, sand self-breaking during homogenization in pestle and mortar is a great concern.

Ultrasonication assisted solvent extraction: similar as bead milling, ultrasonication is performed to disrupt the cell wall. The assistance on cell disruption can complete extraction in a few minutes instead of a few hours in conventional solvent extraction with high reproducibility (Wei et al. 2008). Several parameters including extraction time, solvents, and ultrasonication power, have been found associated with extraction efficiency (Metherel et al. 2009; Araujo et al. 2013). It was found that high power led to high lipid extraction efficiency. Normally, to obtain a similar extend of lipid recovery, ultrasonicaion assisted lipid extraction required 15 min but traditional chloroform and methanol extraction needs several hours (Metherel et al. 2009).

High-pressure homogenization assisted solvent extraction: applying high pressure in the cell induces high shear stress inside the orifice and creates a large pressure drop at the outlet, which results in the cell disruption. Study has showed that high-pressure homogenization (1200 psi 35 °C) could finish lipid extraction from microalga, *Scenedesmus* sp. within 30 min while traditional chloroform and methanol extraction demanded 5 h (Cho et al. 2012a).

Microwave assisted solvent extraction: microwave irradiation rapidly generates great heat and pressure in the extraction system and forces cell disruption. Pre-treating wet microorganism with microwave achieves water reduction as well as cell broken, which indicates that there would eliminate dewater process. It was reported that temperature has essential effect on the microwave extraction (Boldor et al. 2010). Every 10 °C increase in temperature from 50 to 70 °C could obtain around 6% (w/w) higher lipid recovery.

Enzyme lysis assisted solvent extraction: it has the potential to partially or fully disrupt cells with minimal damage to lipid. Appropriate enzyme selection is critical as the composition of cells largely varies (Mercer and Armenta 2011). Enzyme assisted lipid extraction is not widely

practiced mainly due to the high cost of enzyme production and the difficulty in enzyme recover and recycle. Enzyme is normally combined with other cell disruption such as microwave and ultrasonication as pre-treatment (Jin et al. 2012; Liang et al. 2012). Recombinant pIMAN5C was employed in lipid extraction from wet yeast *Rhodosporidium toruloides* and 94% of total lipid was obtained with enzyme dosage of 3 g/kg cells at 30 °C and pH 4.5 for 1.5 h (Jin et al. 2012).

Several studies have compared the difference pre-treatment effect on lipid extraction, and their performance varied from one to another (Lee et al. 2010; Cho et al. 2012a). There is no consistence on the report of optimal pre-treatment for lipid extraction from microorganisms. Lee et al. (2010) observed that the optimal pre-treatment for lipid extraction with chloroform and methanol for *Botryococcus* sp., *Chlorella vulgaris*, and *Scenedesmus* sp. were bead milling and microwave, heating, and microwave. Cho et al. (2010) addressed that high-pressure homogenization performed the best on lipid extraction from *Scenedesmus* sp. compared to microwave and ultrasonication. It was reported that ultrasonication was the best one for *Chlorella vulgaris* lipid extraction (Zheng et al. 2011). The diversity could be due to the difference of solvent selection, time, temperature, and so on. The assisting technologies of lipid extraction could be used as pre-treatment of solvent extraction as well as used during solvent extraction. However, safety issues are the main concern on simultaneously solvent extraction and cell disruption by ultrasonication, microwave, or high-pressure homogenization.

3.7.2 Supercritical fliud lipid extraction

In recent years, supercritical fluid extraction (SFE) has grabbed considerable attention due to its advantage of preserving the originality of the product, free of harmful solvent residues, easy in separation, and environmentally friendly (Sahena et al. 2009; Mercer and Armenta 2011). SFE achieves the lipid extraction by manipulating the chemicals which behave as both a liquid and a gas in their critical temperature and pressure. In critical stage, solvating power of the compound using in SFE is increased and then it plays as a solvent to extract the product from cells. The most remarkable point of using supercritical fluid method is that it is highly selective in extracting triglycerides (Cheng et al. 2011).

Several parameters such as viscosity, diffusivity, and critical temperature and pressure are considered on the selection of the chemical. Carbon dioxide is the most used one due to the low viscosity (<100 μPa.s), high diffusivity (<0.1 mm^2/s), and suitable critical temperature (31.1 °C) and pressure (72.8 atm). In an extraction vessel, oil-bearing substances contact with supercritical carbon dioxide for certain time (several hours). During the process, oil will be solubilized in CO_2 and extracted. CO_2 which contains oil is then collected and depressurized to allow the escape of CO_2, and finally oil is obtained.

Temperature, pressure, carbon dioxide flow rate, and moisture of the sample are significant factors in the extraction (Andrich et al. 2006; Spence et al. 2009). The impact of the factors is complicate. Low temperature leads to high density of supercritical fluid which results in the low mass transfer (Lou et al. 1995) and thus low lipid extraction efficiency. Increasing the temperature increases the diffusion rate and hence lifts the extraction rate. Rising the temperature from 50 to 200 °C could enhance the extraction efficiency from 66% to 99% (Langenfeld et al. 1993). High pressure provides high diffusion rate but when the pressure up to a level the extraction efficiency becomes constant, and to increase the efficiency requires the assistance of temperature. High carbon dioxide flow rate increases extraction efficiency as fresh flow enhances mass transfer. Moisture of samples has great influence in the extraction as it determines the contact time of supercritical fluid and lipid. Samples have the nature to keep their thick consistency in which moisture will be the barrier of the diffusion of any intruder (here refers to CO_2) to inside the cells and the diffusion of intercellular product (here refers to lipid) out of the cells (Mercer and Armenta 2011). Another significant parameter of extraction is the pre-treatment including the technologies discussed in organic solvent extraction. Normally, SFE requires the assistance of pre-treatment or addition of co-solvent such as ethanol and methanol, otherwise the extraction time would be high (more than 20 h) (Mouahid et al. 2012).

The application of supercritical CO_2 lipid extraction from microorganism has been extensively reported. The factors were evaluated in terms of the extraction efficiency for particular microorganisms. Some of the applications are summarized in Table 3.5. It was observed that high temperature provided high efficiency, and pressure wasn't impact much on the extraction. Large variation on carbon dioxide flow rate (from 0.2 to 10 kg/h) has just slightly influent in the

extraction efficiency (Table 3.5). Extraction time of SFE is generally similar as used in traditional organic solvent extraction; hence, it suggested that SFE with carbon dioxide is not prior to solvent extraction in terms of time.

3.7.3 Other chemical technologies of lipid extraction

New technologies such as switchable solvent extraction for lipid extraction have also been developed recently. In fact, these methods also count on solvent to dissolve the lipid and achieve the extraction. Their advantage is to utilize greener chemicals such as 1.8-diazabicyclo-[5.4.0]-undec-7-ene (French et al.) and ethanol. A study of lipid extraction with mixture of DBU and ethanol has successfully separated lipid from soy flakes to gain similar extraction efficiency as organic solvent extraction (Phan et al. 2009). The research took advantage of the polarity change in the presence and absence of carbon dioxide. The switch from low polarity to high polarity as environment alters makes the switchable solvent play similar role as chloroform and methanol mixture.

Nanomaterial lipid extraction has also been reported. It is stated that modified nanomaterial accomplished lipid extraction from live microalgae without harm on cells (Lin 2009). Nanomaterails as great carrier of immobilization due to the high surface area have been extensively applied. When organic solvent-like chemicals immobilized onto solid nanomaterials, it would achieve lipid extraction as well as avoid contamination of the solvent on lipid. Rare study has been conducted in this view of the point and effort is demanded.

3.8 Summary

Physical and chemical technologies have been developed in the lipid extraction. Physical extraction breaks the cells to release the lipid by providing force, while chemical extraction is to utilize solvent to pull out lipid from cells. Each of the method has its advantage and disadvantage. Physical method is clean but has low lipid extraction efficiency (around 70% of total lipid) and high energy consumption. Chemical extraction has high possibility on contamination of the lipid due to the presence of the residual solvents when toxic organic compounds are used. Clean solvent such as supercritical $CO_{2\,g}$ives high quality lipid but it normally depends on pre-treatment or co-solvent addition in order to achieve high extraction efficiency. New technologies such as switchable solvent and nanomaterial extraction have also been reported, but detailed information is not available as it could be still in the infant research stage.

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3.10 References

Adam, F., Abert-Vian, M., Peltier, G., and Chemat, F. (2012). ""Solvent-free" ultrasound-assisted extraction of lipids from fresh microalgae cells: A green, clean and scalable process." *Bioresour. Technol.*, 114, 457-465.

Adesoji, M. O., Kamaldeen, A. Y., Adebayo, L. W., and Kunle, O. A. (2012). "Design, development and testing of a screw press expeller for palm kernel and soybean oil extraction." *Post Harvest, Food and Process Engineering*.

Amalia Kartika, I., Pontalier, P. Y., and Rigal, L. (2010). "Twin-screw extruder for oil processing of sunflower seeds: Thermo-mechanical pressing and solvent extraction in a single step." *Industrial Crops and Products*, 32, 297-304.

Andrich, G., Nesti, U., Venturi, F., Zinnai, A., and Fiorentini, R. (2005). "Supercritical fluid extraction of bioactive lipids from the microalga Nannochloropsis sp." *European Journal of Lipid Science and Technology*, 107, 381-386.

Andrich, G., Zinnai, A., Nesti, U., and Venturi, F. (2006). "Supercritical fluid extraction of oil from microalga *Spirulina (arthrospira) platensis*." *Acta Alimentaria*, 35, 195-203.

Araujo, G. S., Matos, L. J. B. L., Fernandes, J. O., Cartaxo, S. J. M., Gonçalves, L. R. B., Fernandes, F. A. N., and Farias, W. R. L. (2013). "Extraction of lipids from microalgae by ultrasound application: Prospection of the optimal extraction method." *Ultrason. Sonochem.*, 20, 95-98.

Bachmann, J. (2001). "Small-scale oilseed processing." *Report of Appropriate Technology Transfer for Rural Areas*, Available at http://www.green-trust.org/2000/biofuel/oilseed.pdf accessed on 11/12/2012.

Bamgboye, A. I., and Adejumo, A. O. D. (2007). "Development of a sunflower oil expeller." *Int. Commis. Agric. Eng.*, 9, 1-7.

Becerra, M., Rodríguez-Belmonte, E., Cerdán, M. E., and Siso, M. I. G. (2001). "Extraction of intracellular proteins from Kluyveromyces lactis." *Food Technology and Biotechnology*, 39, 135-139.

Benov, L., and Al-Ibraheem, J. (2002). "Disrupting escherichia coli: A comparison of methods." *J. Biochem. Mol. Biol.*, 35, 428-431.

Bimbo, A. p. (2012). "The production and processing of marine oils." *Edible Oil Processing*, available at "http://lipidlibrary.aocs.org/processing/marine/index.htm" accessed on 12/12/2012.

Bligh, E. G., and Dyer, W. J. (1959). "A rapid method of total lipid extraction and purification." *Can.J.Biochem.Physiol.*, 37, 911-917.

Boldor, D., Kanitkar, A., Terigar, B. G., Leonardi, C., Lima, M., and Breitenbeck, G. A. (2010). "Microwave assisted extraction of biodiesel feedstock from the seeds of invasive Chinese tallow tree." *Environmental Science & Technology*, 44, 4019-4025.

Boyd, A. R., Champagne, P., McGinn, P. J., MacDougall, K. M., Melanson, J. E., and Jessop, P. G. (2012). "Switchable hydrophilicity solvents for lipid extraction from microalgae for biofuel production." *Bioresour. Technol.*, 118, 628-632.

Char, J.-M., Wang, J.-K., Chow, T.-J., and Chien, Q.-C. (2011). "Biodiesel production from microalgae through supercritical carbon dioxide extraction." *Journal of the Japan Insititute of Energy*, 90, 369-373.

Cheirsilp, B., Suwannarat, W., and Niyomdecha, R. (2011). "Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for lipid production from industrial wastes and its use as biodiesel feedstock." *New Biotechnol.*, 28, 362-368.

Chen, Y.-H., and Walker, T. H. (2012). "Fed-batch fermentation and supercritical fluid extraction of heterotrophic microalgal Chlorella protothecoides lipids." *Bioresource Technology*, 114, 512-517.

Cheng, C.-H., Du, T.-B., Pi, H.-C., Jang, S.-M., Lin, Y.-H., and Lee, H.-T. (2011). "Comparative study of lipid extraction from microalgae by organic solvent and supercritical CO_2." *Bioresour. Technol.*, 102, 10151-10153.

Cho, S.-C., Choi, W.-Y., Oh, S.-H., Lee, C.-G., Seo, Y.-C., Kim, J.-S., Song, C.-H., Kim, G.-V., Lee, S.-Y., Kang, D.-H., and Lee, H.-Y. (2012a). "Enhancement of lipid extraction from marine

microalga, scenedesmus associated with high-pressure homogenization process." *Journal of Biomedicine and Biotechnology*, 2012, 6.

Cho, S.-C., Choi, W.-Y., Oh, S.-H., Lee, C.-G., Seo, Y.-C., Kim, J.-S., Song, C.-H., Kim, G.-V., Lee, S.-Y., Kang, D.-H., and Lee, H.-Y. (2012b). "Enhancement of lipid extraction from marine microalga, *Scenedesmus* associated with high-pressure homogenization process." *Journal of Biomedicine and Biotechnology*, 2012, 1-6.

Choonia, H. S., and Lele, S. S. (2011). "β-Galactosidase release kinetics during ultrasonic disruption of Lactobacillus acidophilus isolated from fermented Eleusine coracana." *Food and Bioproducts Processing*, 89, 288-293.

Christiansen, K. L., Weller, C. L., Schlegel, V. L., and Dweikat, I. M. (2008). "Comparison of lipid extraction methods of food-grade Sorghum (Sorghum bicolor) using hexane." *Biological Engineering*, 1, 51-63.

Dejoye, C., Vian, M. A., Guy, L., Bouscarele, C., and Farid, C. (2011). "Combined extraction processes of lipid from *Chlorella vulgaris* microalgae: microwave prior to supercritical carbon dioxide extraction." *International Journal of Molecular Sciences*, 12, 9332-9341.

Dhanani, T., Shah, S., Gajbhiye, N. A., and Kumar, S. (2013). "Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera." *Arabian Journal of Chemistry*.

Dufreche, S., Hernandez, R., French, T., Sparks, D., Zappi, M., and Alley, E. (2007). "Extraction of lipids from municipal wastewater plant microorganisms for production of biodiesel." *J. Amer. Oil Chem. Soc.*, 84, 181–187.

Ferraz, T. P. L., Fiúza, M. C., dos Santos, M. L. A., Pontes de Carvalho, L., and Soares, N. M. (2004). "Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation." *J Biochem Bioph Methods*, 58, 187-193.

Folch, J., Lees, M., and G.H., S. S. (1957). "A simple method for the isolation and purification of total lipides from animal tissues." *J. Biol. Chem.*, 226, 497-509.

French, J. R. J., Turner, G. L., and Bradbury, J. F. (1976). "Nitrogen fixation by bacteria from the hindgut of termites." *Journal of General Microbiology*, 95, 202–206.

Galafassi, S., Cucchetti, D., Pizza, F., Franzosi, G., Bianchi, D., and Compagno, C. (2012). "Lipid production for second generation biodiesel by the oleaginous yeast Rhodotorula graminis." *Bioresource Technology*, 111, 398-403.

Grierson, S., Strezov, V., Bray, S., Mummacari, R., Danh, L. T., and Foster, N. (2011). "Assessment of bio-oil extraction from *Tetraselmis chui* microalgae comparing supercritical CO2, solvent extraction, and thermal processing." *Energy & Fuels*, 26, 248-255.

Hampson, J. W., and Ashby, R. D. (1999). "Extraction of lipid-grown bacterial cells by supercritical fluid and organic solvent to obtain pure medium chain-length polyhydroxyalkanoates." *Journal of the American Oil Chemists' Society*, 76, 1371-1374.

Hanif, M., Atsuta, Y., Fujie, K., and Daimon, H. (2012). "Supercritical fluid extraction of bacterial and archaeal lipid biomarkers from anaerobically digested sludge." *International Journal of Molecular Sciences*, 13, 3022-3037.

Hara, A., and Radin, N. (1978). "Lipid extraction of tissues with low-toxicity solvent." *Analytical Biochemistry*, 90, 420-426.

Jensen, S., Häggberg, L., Jörundsdóttir, H., and Odham, G. (2003). "A quantitative lipid extraction method for residue analysis of fish involving nonhalogenated solvents." *Journal of Agricultural and Food Chemistry*, 51, 5607-5611.

Jin, G., Yang, F., Hu, C., Shen, H., and Zhao, Z. K. (2012). "Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast Rhodosporidium toruloides." *Bioresource Technology*, 111, 378-382.

Khoomrung, S., Chumnanpuen, P., Jansa-Ard, S., Ståhlman, M., Nookaew, I., Borén, J., and Nielsen, J. (2013). "Rapid Quantification of Yeast Lipid using Microwave-Assisted Total Lipid Extraction and HPLC-CAD." *Analytical Chemistry*, 85, 4912-4919.

Klimek-Ochab, M., Brzezińska-Rodak, M., Żymańczyk-Duda, E., Lejczak, B., and Kafarski, P. (2011). "Comparative study of fungal cell disruption—scope and limitations of the methods." *Folia Microbiologica*, 56, 469-475.

Langenfeld, J. J., Hawthorne, S. B., Miller, D. J., and Pawliszyn, J. (1993). "Effects of temperature and pressure on supercritical fluid extraction efficiencies of polycyclic aromatic hydrocarbons and polychlorinated biphenyls." *Analytical Chemistry*, 65, 338-344.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., and Oh, H.-M. (2010). "Comparison of several methods for effective lipid extraction from microalgae." *Bioresource Technology*, 101, S75-S77.

Liang, K., Zhang, Q., and Cong, W. (2012). "Enzyme-Assisted Aqueous Extraction of Lipid from Microalgae." *Journal of Agricultural and Food Chemistry*, 60, 11771-11776.

Lin, V. (2009). "Nanofarming technology extracts biofuel oil without harming algae." *News released from Office of Public Affairs, April 14, 2009*.

Lou, X., Jamsen, H.-G., and Cramers, C. A. (1995). "Investigation of parameters affecting the supercritical fluid extraction of polymer additives from polyethylene " *Journal of Microcolumn Separations*, 7, 303-317.

Mercer, P., and Armenta, R. E. (2011). "Developments in oil extraction from microalgae." *European Journal of Lipid Science and Technology*, 113, 539-547.

Metherel, A. H., Taha, A. Y., Izadi, H., and Stark, K. D. (2009). "The application of ultrasound energy to increase lipid extraction throughput of solid matrix samples (flaxseed)." *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 81, 417-423.

Mitra, D., Rasmussen, M. L., Chand, P., Chintareddy, V. R., Yao, L., Grewell, D., Verkade, J. G., Wang, T., and van Leeuwen, J. (2012). "Value-added oil and animal feed production from corn-ethanol stillage using the oleaginous fungus Mucor circinelloides." *Bioresource Technology*, 107, 368-375.

Mouahid, A., Crampon, C., and Badens, E. (2012). "Supercritical CO2 extraction of lipids contained in three different microalgae (*Chlorella, Nannochloropsis oculata* and *Dunaliella salina*): study of the influence of water content and pretreatment on the

extraction kinetics and yields." *Avaiable at <http://issf2012.com/handouts/documents/257_004.pdf> (accessed May. 2013).*

Nikolić, N. Č., Cakić, S. M., Novaković, S. M., Cvetković, M. D., and Stanković, M. Z. (2009). "Effect of extraction techniques on yield and composition of soybean oil " *Macedonian Journal of Chemistry and Chemical Engineering*, 28 173-179.

Northcote, D. H., and Horne, R. W. (1952). "The chemical composition and structure of the yeast cell wall." *Biochem. J.*, 51, 232-236.

Olaniyan, A. M. (2010). "Development of a manually operated expeller for groundnut oil extraction in rural Nigerian communities." *Asia-Pacific Journal of Rural Development*, 20, 185-201.

Olaniyan, A. M., and Oje, K. (2007). "Development of mechanical expression rig for dry extraction of shea butter from shea kernel." *Journal of Food Science and Technology*, 44, 465-470.

Olaniyan, A. M., and Oje, K. (2011). "Development of model equations for selecting optimum parameters for dry process of shea butter extraction." *Journal of Cereals and Oilseeds*, 2, 47-56.

Phan, L., Brown, H., White, J., Hodgson, A., and Jessop, P. G. (2009). "Soybean oil extraction and separation using switchable or expanded solvents." *Green Chemistry*, 11, 53-59.

Prabakaran, P., and Ravindran, A. D. (2011). "A comparative study on effective cell disruption methods for lipid extraction from microalgae." *Lett. Appl. Microbiol.*, 53, 150-154.

Ranjan, A., Patil, C., and Moholkar, V. S. (2010). "Mechanistic assessment of microalgal lipid extraction." *Ind. Eng. Chem. Res.*, 49, 2979-2985.

Richter, B., Jones, B., Ezzell, J., Porter, N., Avdalovic, N., and Pohl, C. (1996). "Accelerated solvent extraction: a technique for sample preparation." *Analyst Chemistry*, 68, 1033-1039.

Sahena, F., Zaidul, I. S. M., Jinap, S., Karim, A. A., Abbas, K. A., Norulaini, N. A. N., and Omar, A. K. M. (2009). "Application of supercritical CO2 in lipid extraction – A review." *Journal of Food Engineering*, 95, 240-253.

Schwede, S., Kowalczyk, A., Gerber, M., and Span, R. (2011). "Influence of different cell disruption techniques on mono digestion of algal biomass." *World Renewable Energy Congress, Sweden, 8-13, May 2011, Linkoping, Sweden*.

Shin, J., Lee, G., and Kim, J. (1994). "Comparison of cell disruption methods for determining β-galactosidase activity expressed in animal cells." *Biotechnology Techniques*, 8, 425-430.

Sivakumaran, K., Goodrum, J. W., and Bradley, R. A. (1985). "Expeller optimization of peanut oil production." *Transactions of the American Society of Agricultural Engineers*, 1, 316-320.

Smedes, F. (1999). "Determination of total lipid using non-chlorinated solvents." *Analyst*, 124, 1711-1718.

Soh, L., and Zimmerman, J. (2011). "Biodiesel production: the potential of algal lipids extracted with supercritical carbon dioxide." *Green Chemistry*, 13, 1422-1429.

Somashekar, D., Venkateshwaran, G., Srividya, C., Krishnanand, Sambaiah, K., and Lokesh, B. R. (2001). "Efficacy of extraction methods for lipid and fatty acid composition from fungal cultures." *World Journal of Microbiology and Biotechnology*, 17, 317-320.

Soxhlet, F. (1879). "Die gewichtsanalytische Bestimmung des Milchfettes." *Polytechnisches J. (Dingler's)*, 232, 461.

Spence, A. J., Jimenez-Flores, R., Qian, M., and Goddik, L. (2009). "The influence of temperature and pressure factors in supercritical fluid extraction for optimizing nonpolar lipid extraction from buttermilk powder." *Journal of Dairy Science*, 92, 458-468.

Sree, R., Babu, N. S., Saiprasad, P., and Lingaiah, N. (2009). "Transesterification of edible and non-edible oils over basic solid Mg/Zr catalysts." *Fuel Processing Technology*, 90, 152-157.

Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., Sádaba, I., Ruiz-Vázquez, R. M., Torres-Martínez, S., and Garre, V. (2009). "Biodiesel production from biomass of an oleaginous fungus." *Biochem. Eng. J.*, 48, 22-27.

Walker, T., Cochran, H., and Hulbert, G. (1999). "Supercritical carbon dioxide extraction of lipids from Pythium irregulare." *Journal of the American Oil Chemists' Society*, 76, 595-602.

Wei, F., Gao, G.-Z., Wang, X.-F., Dong, X.-Y., Li, P.-P., Hua, W., Wang, X., Wu, X.-M., and Chen, H. (2008). "Quantitative determination of oil content in small quantity of oilseed rape by ultrasound-assisted extraction combined with gas chromatography." *Ultrasonics Sonochemistry*, 15, 938-942.

Zhao, X., Zheng, M., Liang, L., Zhang, Q., Wang, Y., and Jiang, G. (2005). "Assessment of PCBs and PCDD/Fs along the Chinese Bohai Sea coastline using molluscs as bioindicators." *Archives of Environmental Contamination and Toxicology*, 49, 178-185.

Zheng, H., Gao, Z., Yin, F., Ji, X., and Huang, H. (2012a). "Effect of CO2 supply conditions on lipid production of Chlorella vulgaris from enzymatic hydrolysates of lipid-extracted microalgal biomass residues." *Bioresource Technology*, 126, 24-30.

Zheng, H., Gao, Z., Yin, F., Ji, X., and Huang, H. (2012b). "Lipid production of Chlorella vulgaris from lipid-extracted microalgal biomass residues through two-step enzymatic hydrolysis." *Bioresource Technology*, 117, 1-6.

Zheng, H., Yin, J., Gao, Z., Huang, H., Ji, X., and Dou, C. (2011). "Disruption of *Chlorella vulgaris* cells for the release of biodiesel-producing lipids: A comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves." *Applied Biochemistry and Biotechnology*, 164, 1215-1224.

Zhou, R., Zhu, L., Chen, Y., and Kong, Q. (2008). "Concentrations and characteristics of organochlorine pesticides in aquatic biota from Qiantang River in China." *Environment Pollution*, 151, 190-199.

Table 3.1 Comparison of cell disruption technologies

Methods	Description	Advantages	Disadvantages
Blade homogenizers	a blender, using cutting blades to reduce size of substances	Easy to operate	Not efficient for disrupting microorganisms
Bead milling	Using glass, ceramic, or steel bead to crush the cells as they collide with agitation or stirring	Clean and suitable on cell disruption of spores, yeast and fungi, process is cheap	Not so efficient as high pressure and ultrasonification; heat generation
Pressure	Using pressure to produce shear to break the cells	Suitable for large scale production	High requirement on design
Ultrasonic	Forming micro-bubbles to vibrate the cells	Efficient	non-specific cell wall disruption; high heat generation; long operation time; generation of harmful free radicals
Freeze/thaw	Forming of ice crystal to break the cells	Easy to operate	Requiring several cycles; slow and high cost
Pressing	Compressing the cells and ultimately break the cells	Easy to operate and cheap	Low efficiency
Osmosis stock	Utilizing osmosis pressure resulted from the concentration difference between inside and outside of the cell membrane to break the cells	Cheap	Pre-treatment to weaken the cell wall for further disruption
Detergent	Using detergents to solubilize the phospholipid and disrupting the cells	Preserving the properties of the products	Pre-treatment to weaken the cell wall for further disruption; Requiring subsequently process to remove the detergent
Solvent	Using solvents to solubilize the phospholipid and disrupting the cells	Efficient	Requiring subsequently process to remove the detergent
Enzyme	Using lysozyme to disrupt cells	Selective	Need the addition of detergents to complete the disruption

Table 3.2 Oil extraction with expeller pressing

Technologies	Raw material	Lipid content (%)	Separation efficiency (%)	References
Expeller	Peanut	50	92	(Sivakumaran et al. 1985)
Ram press	Sunflower seed	25-40	50-56	(Bachmann 2001)
Mechanical expression rig	Shea kernel	34-44	58.5	(Olaniyan and Oje 2007)
Expeller	Sunflower seed	25-40	70	(Bamgboye and Adejumo 2007)
Screw press	Groundnut	35-50	75	(Olaniyan 2010)
Mechanical expression rig	Shea kernel	34-44	58.63	(Olaniyan and Oje 2011)
Screw press	Palm kernel	46-57	22.79	(Adesoji et al. 2012)
Screw press	Soybean	19-23	36.55	(Adesoji et al. 2012)

Table 3.3 Oil extraction with organic solvents

Solvent	Oleaginous substance	Character	Extraction conditions	Extraction efficiency (%)	References
Chloroform: methanol (2:1)	Animal tissue	non-polar and polar and	24h; 25 °C	96	(Folch et al. 1957)
Chloroform: methanol (2:1)	Human serum	non-polar and polar and	11 min; 20 °C	96	(Ferraz et al. 2004)
Chloroform: methanol (2:1)	*Mucor circinelloides* (fungus)	non-polar and polar and	1 h; 25 °C	94	(Vicente et al. 2009)
Chloroform: methanol (2:1)	*Rhodotorula glutinis* (yeast) and *Chlorella vulgaris* (microalga)	non-polar and polar and	1 h; 25 °C	95	(Cheirsilp et al. 2011)
Chloroform: methanol: water (2:2:1)	Fish	non-polar and polar and	A few miutes; 25 °C	94	(Bligh and Dyer 1959)
Chloroform: methanol: water (2:2:1)	*Mucor circinelloides* (fungus)	non-polar and polar and	1 h; 25 °C	89.6	(Vicente et al. 2009)
Hexane: isopropanol (2:1)	Serum	non-polar and polar and	11 min; 20 °C	88	(Ferraz et al. 2004)
Hexane: isopropanol (3:2)	*Rhodotorula graminis* (yeast)	non-polar and polar and	1 h; 25 °C	95	(Galafassi et al. 2012)
Hexane: methanol: acetone (3:1:1)	Sludge	Non-polar and polar	1h; 100 °C	97	(Dufreche et al. 2007)
Methanol followed by hexane	Sludge	Polar and non-polar	1h; 100 °C	78	(Dufreche et al. 2007)
Hexane	Serum	Non-polar	11 min; 20 °C	18	(Ferraz et al. 2004)
Hexane	Food-grade sorghum	Non-polar	30 min; 65 °C	10	(Christiansen et al. 2008)
Hexane	Sludge	Non-polar	1h; 100 °C	6.92	(Dufreche et al. 2007)
n-Hexane	*Mucor circinelloides* (fungus)	Non-polar	1 h; 25 °C	70.71	(Vicente et al. 2009)
n-Hexane	Soybean	Non-polar	2.5 h; 70 °C	68.7	(Nikolić et al. 2009)
Chloroform	Soybean	Non-polar	150 min; 61.2 °C;	75.7	(Nikolić et al. 2009)
Methanol	Sludge	Polar	1h; 100 °C	69	(Dufreche et al. 2007)
Methanol	*Mucor circinelloides* (fungus)	Polar	30 min, 25 °C	35.72	(Mitra et al. 2012)

Table 3.4 Assisted organic solvent lipid extraction

Oleaginous substances	Assistance technology	Solvents and conditions of the extraction	Extraction efficiency (%)	References
Botryococcus sp.	Bead Milling	chloroform–methanol (1 :1 v/v); 5min, 25 °C	94.2	(Lee et al. 2010)
Chlorella vulgaris		chloroform–methanol (1 :1 v/v); 5min, 25 °C	25.8	(Lee et al. 2010)
Chlorella vulgaris		chloroform–methanol (1 :1 v/v); 10 min, 30 °C	48.6	(Zheng et al. 2011)
Chlorella sp.		chloroform–methanol (2 :1 v/v); 10 min; 30 °C	83.2	(Prabakaran and Ravindran 2011)
Scenedesmus sp.		chloroform–methanol (1 :1 v/v); 5min, 25 °C	34.8%	(Lee et al. 2010)
Chlorella sp.		chloroform–methanol (2 :1 v/v) 5min, 25 °C	98.2	(Prabakaran and Ravindran 2011)
Nostoc sp.		chloroform–methanol (2 :1 v/v) 5min, 25 °C	97.1	(Prabakaran and Ravindran 2011)
Tolypothrix sp.		chloroform–methanol (2 :1 v/v) 5min, 25 °C	98.0	(Prabakaran and Ravindran 2011)
Mucor rouxii	Sand Milling	chloroform–methanol (2 :1 v/v)	96.23	(Somashekar et al. 2001)
Mucor hiemales		chloroform–methanol (2 :1 v/v)	92.6	(Somashekar et al. 2001)
Chlorella vulgaris		chloroform–methanol (1 :1 v/v); 10 min, 30 °C	0.06	(Zheng et al. 2011)
Scenedesmus sp.	High- pressure homogenization	chloroform–methanol (2 :1 v/v); 30 min; 35 °C	0.21	(Cho et al. 2012b)
Scenedesmus sp.	Microwave	chloroform–methanol (2 :1 v/v); 30 min; 35 °C	0.12	(Zheng et al. 2011)
Saccharomyces cerevisiae		chloroform–methanol (2 :1 v/v); 16 min; 60 °C	0.09	(Khoomrung et al. 2013)
Chlorella vulgaris		chloroform–methanol (2 :1 v/v); 10 min; 30 °C	0.18	(Zheng et al. 2011)
Chlorella sp.		chloroform–methanol (2 :1 v/v); 10 min; 30 °C	92.3	(Prabakaran and Ravindran 2011)
Nostoc sp.		chloroform–methanol (2 :1 v/v); 10 min; 30 °C	87.6	(Prabakaran and Ravindran 2011)
Tolypothrix sp.		chloroform–methanol (2 :1 v/v); 10 min; 30 °C	93.2	(Prabakaran and Ravindran 2011)
Chlorella sp.	Ultrasonication	chloroform–methanol (2 :1 v/v); 10 min; 30 °C	98.1	(Prabakaran and Ravindran 2011)
Nostoc sp.		chloroform–methanol (2 :1 v/v); 10 min; 30 °C	94.3	(Prabakaran and Ravindran

				2011)
Tolypothrix sp.		chloroform–methanol (2 :1 v/v); 10 min; 30 °C	82.7	(Prabakaran and Ravindran 2011)
Scenedesmus sp.		chloroform–methanol (2 :1 v/v); 30 min; 35 °C	0.16	(Cho et al. 2012b)
Chlorella vulgaris		chloroform–methanol (2 :1 v/v); 10 min; 30 °C	0.29	(Zheng et al. 2011)
Rhodosporidium toruloides	Enzyme	chloroform; 60 min; 30 °C	96.6	(Jin et al. 2012)
Chlorella vulgaris		Hexane-methanol (1:2 v/v); 30 min; 30 °C	93.4	(Zheng et al. 2012a)
Chlorella vulgaris		Hexane-methanol (1:2 v/v); 30 min; 30 °C	96.2	(Zheng et al. 2012b)
Chlorella vulgaris	Enzyme+sonication	Water; 10 min; 95 °C	49.82	(Liang et al. 2012)
Scenedesmus dimorphus		Water; 10 min; 95 °C	46.81	(Liang et al. 2012)
Nannochloropsis sp.		Water; 10 min; 95 °C	11.73	(Liang et al. 2012)

Table 3.5 Supercritical CO_2 lipid extraction

Microorganisms	Temp. (°C)	Pressure (atm)	CO_2 flow rate (kg/h)	Water content (%)	Time (h)	Extraction efficiency (%)	References
C. protothecoides	50	346	0.05	5	3	80	(Chen and Walker 2012)
Chlorella vulgaris	40	197	10	5	9	98.1	(Dejoye et al. 2011)
Chlorella sp.	40	296	0.9	5	3	92.2	(Char et al. 2011)
Mix culture (digested sludge)	77.6	233	NA	8	0.25	95.0	(Hanif et al. 2012)
Nannocloropsis sp	55	690	10	5	6	82.5	(Andrich et al. 2005)
Pavlova sp.	60	300	NA	NA	6	98.7	(Cheng et al. 2011)
Pseudomonas resinovorans	60	500	4	5	3	43	(Hampson and Ashby 1999)
Pythium irregular	60	271	NA	30	6	NA	(Walker et al. 1999)
Scenedesmus dimorphus	100	400	0.2	NA	1	98.5	(Soh and Zimmerman 2011)
Spirulina (Arthrospira) platensis	55	690	10	5	4	90.3	(Andrich et al. 2006)
Tetraselmis chui	60	246	NA	NA	1	5	(Grierson et al. 2011)

BIODIESEL PRODUCTION FROM TRANSESTERIFICATION

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CHAPTER 4 OF BOOK: BIODIESEL PRODUCTION: TECHNOLOGIES, CHALLENGES, AND FUTURE PROSPECTS (ACCEPTED)

4 BIODIESEL PRODUCTION FROM TRANSESTERIFICATION

4.1 Résumé

La trans-estérification est la méthode la plus courante de la synthèse de biodiesel. C'est une réaction qui se déroule entre l'huile/graisse et l'alcool. La réaction se produit à température et pression élevées (200 °C et 50 Mpa) en absence de catalyseur, alors qu'elle peut dérouler à des conditions modérées (50 à 60 °C et 0.101 MPa) en présence d'une base ou d'un acide fort comme catalyseur. Les paramètres, à titre d'exemple, le type de catalyseur, la propriété de l'huile des matières premières, et le ratio molaire de l'huile et du méthanol ont un grand impact sur la trans-estérification. La trans-estérification catalytique alcaline est largement appliquée dans la production industrielle de biodiesel en raison du court temps de réaction (moins de 2 h). Cette méthode n'est pas convenable car l'huile résultante contient une teneur élevée en acides gras libres supérieure à 2%. Pour cette raison, la trans-estérification acide est favorisée pour la synthèse de biodiesel. Les enzymes, libres ou immobilisées, peuvent être également utilisées dans la réaction offrant une grande efficacité mais le coût élevé entrave leur application. Les catalyseurs hétérogènes sont, soient l'acide solide, ou la base. Leurs inconvénients sont le transfert de masse. Un catalyseur hétérogène de taille nanométrique représente une solution au problème de transfert de masse et serait un catalyseur promoteur étant donné qu'il est facile à récupérer et pourrait être en contact direct avec le réactif. Les rapports molaires méthanol/pétrole (6:1 ou 9 :1) sont normalement suffisants pour atteindre la trans-estérification à haute efficacité, mais ils pourraient modifier les conditions de réaction (température et pression). Afin de réduire le temps de la réaction, des technologies telles que l'irradiation micro-ondes et les ultrasons ont été appliquées lors de la trans-estérification. Ils fournissent des taux de conversion élevés avec peu de temps (plusieurs minutes). La trans-estérification *in-situ* est d'un intérêt croissant en raison de la prévention de l'extraction pétrolière. La technologie convertit directement l'huile située dans les substances contenant de l'huile vers le biodiesel sans affecter le profil du biodiesel (composition d'ester d'acide gras). Le problème de ce procédé est le long temps de réaction et le rapport huile/alcool élevé. Pour résoudre le problème, l'ajout de solvant ou le recours à l'ultrasonication ou à l'irradiation micro-ondes peuvent être envisagés. Ces combinaisons technologiques seront sans doute largement appliquées dans la production de biodiesel dans le futur.

Mots clés: Trans-estérification; trans-estérification *in-situ*; catalyseur; enzyme

4.2 Abstract

Transetserification is the most common method of biodiesel synthesis. It is the reaction between oil/fat and alcohol. The reaction occurs at high temperature and pressure (200 °C and 50 Mpa) if catalyst is absence while it can take place at mild condition (50 to 60 °C, 0.101 Mpa) when base or strong acid is used as catalyst. Parameters including catalyst type, feedstock oil property, and methanol oil molar ratio have great impact on the transesterification. Alkali catalytic transesterification is widely applied in industrial biodiesel production due to the short reaction time required (less than 2 h). But the method is not suitable for tranesterification of oil contained FFA content greater than 2%, thus acidic catalyst transesterification should be used in biodiesel synthesis. Enzyme, free or immobilized, is also been employed in the reaction. It has high efficiency yet the high cost hampers its application. Heterogeneous catalysts are the solid acid or base. Its disadvantage is of mass transfer. Nano-sized heterogeneous catalyst is the solution of the problem and would be a promising catalyst as it is easy to be recovered and could be well contact with reactant. Methanol to oil molar ratio 6:1 or 9:1 is normally sufficient to achieve high efficiency tranesterification, while it could alter as the reaction condition (temperature and pressure) changes. In order to reduce the reaction time, assisting technologies such as microwave irradiation and ultrasonication have been applied in the transesterification. They provide high conversion rate with short time (several minute). In-situ tranesterification grabs growing interest due to the prevention of oil extraction. The technology directly converts oil located in oil bearing substances to biodiesel without affecting on biodiesel profile (fatty acid ester composition). The problem of the process is the long reaction time and high alcohol oil ratio. In order to solve problem, solvent, ultraosnication or microwave irradiation can be added. It would be widely applied in biodiesel production in future.

Keywords: Transesterification; *in-situ* transesterification; catalyst; enzyme

4.3 Introduction

Demand for alternative fuels has grown significantly due to the fact that traditional fuels are depleting, petroleum prices are rising, and the ever-growing importance on the control of greenhouse gas emissions (Koplow and Dernbach 2001; Vicente et al. 2009a). Biodiesel, fatty acid methyl esters (FAMEs), has captured interest as an alternative fuel due to the advantages that it is renewable, sustainable, environmentally friendly (burns much cleaner than petroleum diesel), compatible with current commercial diesel engines, as well as having excellent lubricity while providing similar energy density to diesel.

Several technologies have been developed to produce biodiesel including pyrolysis, microemulsions, and transesterification (Doll et al. 2008; Macala et al. 2008; Suarez et al. 2009). Microemulsions is the oldest method to produce biodiesel from oils or fats by blending with cosolvents, mostly short chain alcohol such as methanol and ethanol, and amphiphiles (ionic or nonionic). The addition of cosolvents reduces the viscosity of the oils/fats and hence the products (considered as biodiesel) are able to be directly used to power diesel engines. In early 1980's, the ethanol microemulsion in soybean oil has been investigated in short engine, and the blend biodiesel fuel showed excellent adaption with the engine (Goering et al. 1982b). Both soybean oil and triolein blending with amphiphile, 2-octanol, using methanol as immiscible liquid, gave good performance in diesel engine (Schwab et al. 1988; Ma and Hanna 1999). Recently, microemulsion frequently use in blend diesel with ethanol (Bilgin et al. 2002). Pyrolysis, also called "cracking", is a thermal decomposition process, which cracks long alkyl chains to small molecular at high temperature under oxygen free condition. Biodiesel production through pyrolysis can be simplified as showing in Equations 4.1 and 4.2.

Equation 4. 1 $$Feedstocks(oils/fats) \xrightarrow[(Catalyst)]{300-500^{\circ}C,atm} Gas + Mixture(liquid)$$

Equation 4. 2 $$Mixture(liquid) \xrightarrow{Separation(Distillation)} Biodiesel$$

Various feedstocks, such as plants oils (Fortes and Baugh 1999; Vitolo et al. 2001; Lima et al. 2004; Doll et al. 2008), and fats (Adebanjo et al. 2005), and waste oils (Nerín et al. 2000), were reported in biodiesel production through pyrolysis. However, the large amount energy consumption due to the high temperature requirement in the reaction becomes the concern (Nasikin et al. 2009).

Transesterification using oils to react with short chain alcohol (methanol or ethanol) to form biodiesel is the most common and vital process (Boz et al. 2009a; Dizge et al. 2009b; Singh and Singh 2010). There are catalytic and non-catalytic transesterification according to the presence and absence of catalyst. Non-catalyst takes advantage of high pressure or temperature to achieve high conversion rate, hence it is considered as high energy consumption approach. Generally, catalyst promotes the biodiesel conversion rate with short time (several hours), but downstream treatment is more complex than non-catalyst. The selection of catalyst in transesterification is determined by raw oil/fat properties. Free fatty acid (FFA) content in the oil/fat is the major factor as it causes soap formation in alkaline catalyst reaction which consumes catalyst as well as reduces biodiesel yield. Normally, alkaline catalyst reaction is not preferred when FFA content is higher than 2% of total oil/fat. Otherwise, two step transesterification with first step to convert FFA to biodiesel in acidic catalyst condition and second alkaline catalyst step can be employed (Sánchez et al. 2011; Chen et al. 2012). In catalytic transesterification, technologies such as ultrasonication and microwave have been applied to accelerate the conversion rate (Deng et al. 2010; Veljković et al. 2012). The addition of ultrasonication or microwave generates pressure and heat, and enhances mass transfer, thus the rate is increased.

Apart from biodiesel synthesis through transesterification of oil extracted from biomass, transesterification of oil-rich biomass to biodiesel, also called in-situ transesterification has been reported (Ehimen et al. 2010; Ehimen et al. 2012; Qian et al. 2013). It is getting more and more attention due to the prevention on oil extraction which is energy and cost consuming.

In this chapter, biodiesel production by transesterification has been addressed. Parameters which could effect on the transesterification have been discussed. The new technologies applications in transesterification have been reviewed.

4.4 Transesterification of oil/fat to biodiesel

Transesterification is known as the most popular approach for biodiesel manufacture. It uses oil/fat (triglyceride) to produce FAMEs and glycerol by reacting with alcohols. Among all alcohols, methanol is more preferable due to the cost consideration. This reaction can be described as Equation 4.3. Catalyst type, alcohol type, oil type, alcohol to oil ratio, and assistance technology addition are significant parameters of transetserification.

Equation 4.3

$$CH_2COOR_1 - CHCOOR_2 - CH_2COOR_3(triglyceride) + 3EtOH(methonal/ethanol) \xrightarrow{(Catalyst)} CH_3COOR_1 + CH_3COOR_2 + CH_3COOR_3 + CH_2OH - CHOH - CH_2OH(glycerol)$$

Where R_1, R_2, and R_3 are fatty acid chains. The products, CH_3COOR_1, CH_3COOR_2, and CH_3COOR_3 are representing alkyl (methyl, propyl, or ethyl) esters.

4.4.1 Catalyst effect transesterification

According to the absence or presence of catalyst in the process (Equation 4.3), biodiesel production can be divided as non-catalytic and catalytic biodiesel production.

Non-catalytic biodiesel synthesis, as the name suggests, is to produce biodiesel without catalyst addition. In late 1990's, it was reported that the conversion rate could be up to 85% after 10 h reaction when temperature was set at 235 °C (in catalytic system, temperature required to be around 50 °C) in biodiesel synthesizing with soybean oil and methanol in catalyst free system (Diasakou et al. 1998). When excess and supercritical methanol was used, the conversion rate was also increased up to 95% (Saka and Kusdiana 2001). In the study, the supercritical methanol was achieved by treating methanol with a period of 3 min under a pressure of 45-65 MPa at temperature of 350 °C to 400 °C. it was observed that excess alcohol and critical conditions such as high temperature (Diasakou et al. 1998), irradiation (Melo-Junior et al. 2009), supercritical treatment (Saka and Kusdiana 2001), were needed in the non-catalytic biodiesel production in order to achieve high conversion rate. However, it would lead to high synthesis cost (large amount addition of alcohol) and high energy input.

Catalytic biodiesel synthesis uses catalyst in the reaction to urge the conversion complete. To some extend it is considered superior to non-catalytic method because the reaction can occur in mild condition. The catalysts have been applied for biodiesel production is described below.

Homogeneous alkali catalyzed transesterification is the most common biodiesel production process due to the low cost by comparing with enzyme and heterogeneous catalysts, and high efficiency by comparing with acids (Grepen 2005). Generally, small amounts of water and FFA exist in oils and fats. As mentioned above, when alkalis are employed, soap can be formed due to the reaction occurring between alkalis and free fatty acids (Equation 4.4). Therefore, the required addition quantity of base will be more than theoretically required.

Equation 4.4 $$RCOOH + KOH / NaOH \rightarrow RCOOK / Na(soap) + H_2O$$

Where R represents fatty acid chains.

Homogeneous acid catalytic biodiesel synthesis requires strong acid (concentrated H_2SO_4), relative high temperature (around 65 °C), and long reaction time (> 24 h) in compared to base catalytic system (Canakci and Gerpen 2003; Vicente et al. 2009b). The use of aggressive reagent (strong acid) demands high attention on operation and high corrosive resistance material in reactor.

Enzymatic catalyst has attracted a growing attention in biodiesel production due to the fact that the process is more effective, selective, and environmental friendly (less by-products) than acidic or alkali catalyst (Shaw et al. 1991; Du et al. 2004; Park et al. 2008; Chen et al. 2011). Enzyme (lipase) using in biodiesel synthesis process can be generated by all living organisms, such as microorganism, animal, and plant (Akoh et al. 2007). Among all the lipase sources, microorganism has shown a great advantage because of the high lipase yield. So far, many microorganisms such as *Candida antarctica* (Watanabe et al. 2007), *Thermomyces lanuginose* (Xie and Ma 2009), *Chromobacterium viscosum* (Shah et al. 2004), *Penicillium* sp. *Pseudomonas* sp. and *Rhizopus* sp. (Sellappan and Akoh 2005), have been investigated to produce lipase.

The mechanism of lipase catalytic transesterification process is predicted as presented in Figure 4.1. Lipase (a polar substance), which can be activated in water, catalyzes the reaction by enter the substrates from the liquid to liquid interface formed between lipid (insoluble in water) and alcohol (soluble in water) in water. It reflects that enzymatic synthesis allows water presence in the reaction which is an inhibition in acid or base catalytic synthesis system. Lipase catalytic biodiesel synthesis has been widely studied, but the high lipase cost hampers its industrial application.

In order to reduce the production cost, two solution have been reported, microbe whole cell utilization and immobilized lipase utilization (Li et al. 2007a; Salis et al. 2008; Xie and Ma 2009). Microbe whole cell utilization is a method to use the whole cells from microbe which contains large amount lipase in the cells instead of using pure lipase as catalyst; therefore, the production cost would be reduced because of the avoidance of lipase separation and purification which are the major causes of high lipase cost. Li et al. (2007a) found that above 90% oil conversion is accomplished by employing *Rhizopus oryzae* whole cells as catalyst. It indicates that the lipase efficiency is comparable to free lipase. Immobilized lipase catalytic process offers a cost-effective way for biodiesel production by developing lipase reuse capacity. A number of carriers, such as fiber cloth, acrylic resin, silica gel, hydrotalcite, nanoparticles, and macroporous and microporous materials, have been reported for lipase immobilization (Noureddini et al. 2005; Bai et al. 2006; Gao et al. 2006; Dizge et al. 2009b). Some studies showed that the reused lipase could perform as stable and active as the initial (Noureddini et al. 2005; Jegannathan et al. 2008; Salis et al. 2008). Comparison of biodiesel synthesis with free lipase, whole cell lipase, and immobilized lipase is exhibited in Table 4.1. The immobilized lipase shows a comparable performance in biodiesel yield.

Apart from whole cell catalytic or immobilized catalytic biodiesel synthesis, the combination technology which is to immobilize whole cell catalyst onto carriers, has also been investigated (Zeng et al. 2006; Fukuda et al. 2008; He et al. 2008). This method could be more cost-efficient in biodiesel production with comparison to the production using whole cell or free lipase immobilized onto carriers alone as catalyst.

Heterogeneous catalytic transesterification is another efficient method of biodiesel production, which requires no neutralization in the end of the process as well as could keep the catalyst remaining in the reaction system by filtration. Heterogeneous catalysts are usually alkaline; therefore, it is also called solid base catalysts, such as calcined Li-CaO (Watkins et al. 2004), Mg-Al hydrotalcites (Xie et al. 2006), magnesia-rich magnesium aluminate spinel (Wang et al. 2008), Mg/Zr (Sree et al. 2009), and so on. CaO and MgO are the most often investigated catalyst in transesterification (Table 4.2). Based on the studies, nanoparticle sized catalysts have shown enormous advantage because of their high efficiency. 99% biodiesel conversion using nanocrystalline calcium oxides with 0.6% wt addition (based on the oil weight) was obtained (Reddy et al. 2006). Boz et al. (2009) reported that 99.84% biodiesel yield was achieved using nano-γ-Al_2O_3 catalyst particles (<50 nm) with only 3% wt addition (based on the oil weight). It is evident that the use of nanoparticle catalysts has dramatically reduced the catalyst addition quantity with even higher biodiesel yield (10% wt addition is needed for normal size catalysts) (Veljković et al. 2009).

Overall, base catalytic synthesis is the major source of biodiesel in market due to the low price and developed technology. However, the drawbacks, such as soap formation, large base consumption, and complicated separation and purification of biodiesel, require the development of alternative approaches. Immobilized lipase enzymatic synthesis and heterogeneous catalyst synthesis have great potential in industrial production of biodiesel when nanotechnology is employed (Table 4.2).

4.4.2 Alcohol type effect on transesterification

Short chain alcohol such as methanol and ethanol are used in the transesterification as increase in chain length of alcohol lowers the biodiesel formation rate (Hanh et al. 2009). The biodiesel produced by transesterification with methanol and ethanol are called methylesters and ethylesters, respectively. Methanol is the most applied alcohol in current biodiesel production due to that it is cheaper, has smaller polarity, and provides high conversion rate (Kulkarni et al. 2007). Ethanol is getting increasing attention as it is non-poisoning and results in higher biodiesel lubricity compared with methanol (Peterson et al. 1992).

To compromise the advantage and disadvantage of methanol and ethanol, their mixture (1:1 mol/mol) has been used in the transesterification (Kulkarni et al. 2007; Kim et al. 2010). The studies showed that no difference had been observed in conversion rate with the use of the mixture of methanol and ethanol, single methanol, or single ethanol. But, the lubricity of the produced biodiesel with the mixture of methanol and ethanol was improved compare with that with single methanol.

4.4.3 Oil/fat type effect on transesterification

Vegetable oils and animal fats are the current biodiesel production feedstock. Increase price of vegetable oils and animal fat shifts the focus on biodiesel production from cooking oils and microbial oils. The suitability of these oils has to be evaluated in terms of the property of the feedstock (oil or fat) which includes fatty acid composition, free fatty acid content, water sulfur, phosphorus content as they determine the transesterification catalyst selection and the product properties.

Fatty acid composition plays important role in biodiesel qualities because of the fact that it is related to the viscosity, oxidation stability, cetane number (CN) (indicator of ignition quality), cold flow property, flash point, calorific value (also called heat content or energy density), and density of biodiesel. Viscosity indicates the fuel features of spray, mixture formation, and combustion process. High viscosity can cause early injection and increase combustion chamber temperature. Normally, viscosity increases with the increase in the chain length and with the increase of fatty acid saturation level, while better oxidation stability requires high level of fatty acid saturation (Goering et al. 1982a; Graboski et al. 1998b; Içingür and Altiparmak 2003). Cetane number shows the similar trend as viscosity, which implies that CN increases as the increase in chain length and saturation of fatty acid (Içingür and Altiparmak 2003; Knothe 2005). Cold flow properties depend on the saturation level of the feedstock oil. The higher of the saturation level is, the poorer cold flow properties is (Chapagain and Wiesman 2009; Ramos et al. 2009). The flash point will be low when the chain length is short (Karmakar et al. 2010). It is predicted that greater saturation gave higher calorific value (Karmakar et al. 2010). Polyunsaturation level seems to be proportion to the density according to the report (Karmakar et al. 2010).

As discussed before, alkaline catalytic transesterification is not suitable for feedstock with high FFA content (over 2%) such as animal fat or used cooking oils due to the concern of soap formation. Generally, biodiesel production from oil or fat with high FFA content requires two steps conversion with the first step of esterification (FFA to biodiesel with acidic catalysis) and the second step to finally complete the transesterification with alkali catalysis.

Water can cause triglyceride hydrolyzing to FFA, and hence results in soap formation (Anderson et al. 2003; Sanford et al. 2009). Moreover, the presence of water could also cause emulsions. Therefore, when water content is greater than 0.05% (w/w), water removal step should be performed (Sanford et al. 2009).

Phosphorus can damage catalytic converters used in emissions control systems of the vehicles (del Río 2007). Phosphorus content in biodiesel from feedstock should be controlled to conserve the functionality of the exhaust gas treatment systems during their operational life in the vehicles, and thereby reduce emission pollutants level of the environment.

Similarly, sulfur presence can choke catalytic converter up and harm the emission control systems of vehicles. Generally speaking, sulfur content of biodiesel production feedstock is nearly zero, which is the reason that normally in order to decrease the sulfur content in petrodiesel, biodiesel is used to blend with petrodiesel (Sanford et al. 2009).

4.4.4 Alcohol to oil/fat molar ratio effect on transesterification

It shows that 1 molar of triglyceride requires 3 molar alcohol in transesterification (Equation 4.3). To drive the reaction to proceed to the right, excess alcohol is required. It is the reason of that normally 6 to 1 molar ratio of alcohol to oil is used in industrial biodiesel production from transesterification (Boz et al. 2009b; Dizge et al. 2009a). However, it isn't true that the higher the molar ratio is the better conversion rate is. High alcohol to oil molar ratio could increase the solubility of biodiesel and results in the difficulty on the separation of glycerol and biodiesel. When glycerol remains in the system it would lead the reaction to go towards the left (dissociation of biodiesel). Researchers have investigated the effect of molar ratio of methanol to oil from 3:1 to 12:1 on transesterification of Jatropha oil to biodiesel and observed that 9:1 was the best one in which the conversion rate was above 93% (Vyas et al. 2011). The

highest conversion rate (93.5%) was obtained in the methanol to oil molar ratio of 6:1 among 3:1, 6:1, and 8:1 in the transesterification of duck oil to biodiesel (Liu and Wang 2013). The investigation showed that methanol to waste cooking oil molar ratio at around 6:1 provided the better conversion rate that 3:1, 9:1, and 12:1 (Kawentar and Budiman 2013).

4.4.5 Other technology addition effect on transesterification

Some have reported that ultrasonic cavitation or hydrodynamic cavitation, for enhancing transeterification (Stavarache et al. 2005; Ji et al. 2006; Gogate 2008). In these studies, high conversion biodiesel yield (98 - 99%) was obtained within short period time of 10 to 30 min, and only half quantity of catalyst (0.5% (w/w)) was required compared with conversional base catalyzed process (several hours reaction time). Cavitation caused by ultrasound or flow, microscopically generates high temperatures (227-14727 ^{o}C) and pressures (100-5000 atm) in local, but the overall system keeps atmospheric conditions (T: 25 ^{o}C, P: 1 atm). The locally high temperature and pressure enhance the transesterification biodiesel synthesis (Suslick 1989). Therefore, ultrasonic or hydrodynamic cavitation can be used in biodiesel production industry to reduce the reaction time.

Microwave assisted transesterification has also been reported. To achieve similar conversion rate, microwave assistance could significantly reduce the reaction time from several hours to several minutes (Azcan and Danisman 2008; Azcan and Yilmaz 2013). In fact, it is the process to utilize microwave irradiation to rapidly heat up the system and accomplish the conversion. However, the effect of the high temperature on feedstock oil should be studied.

4.5 Transesterification of oil bearing substances to biodiesel

The general transesterification is to convert oil/fat to biodiesel. Recently, directly transferring oil-bearing substance to biodiesel without the step of oil separation/extraction from the substance has been studied. The process is also called in-situ transesterification. The technology becomes promising due to the avoidance of oil extraction which is high energy and cost requiring process.

The difference of the in-situ one from the normal one is to perform the transesterification by using oil-bearing substances instead of oil. In normal transesterification, oil directly contacts with methanol and catalyst, hence the reaction is easier than in-situ one. Either long reaction time or large amount methanol addition have to be provided or cell disruption technologies have to be added simultaneously in in-situ transesterification in order to achieve the transesterification. Using soy flakes to produce biodiesel through alkali transesterification at 60 °C (8 h) required three times higher time to achieve similar conversion rate as using normal transetserification oil to biodiesel (less than 2 h) in the same condition (Haas et al. 2004). Some study even reported that the time would be 16 h (Haas and Scott 2007). In addition, review on in-situ transesterification summarized and showed that very large methanol to oil molar ratio (300:1 to 900:1) was required to obtain high conversion rate (Samuel and Dairo 2012).

The high reaction time as well as large amount of alcohol addition, requires improvement of the current in-situ transesterification. Treatment for cell disruption which enhances contact between oil and reactant (alcohol) would assist the process. There are many methods (homogenization, ultrasonication, microwave, etc.) for cell disruption. The suitable ones for in-situ transesterification are solvent addition, bead milling, ultrasonication, and microwave. In fact, methanol is a reactant as well as a solvent in in-situ tranesterification. Methanol is polar (weak the cell wall) and oil is non-polar, thus methanol cannot pull out oil from cells. Solvent addition can be used to enhance oil transfer from cell to outer environment, and thus achieve high biodiesel yield in in-situ transesterification (Mondala et al. 2009). Hexane and toluene can be used. It was reported that toluene addition highly improved the biodiesel yield to 86% from 27% and reduced the reaction time to 1 h from 4 h compared to that without solvent addition (Xu and Mi 2011).

Ultrasonication addition in the in-situ tranesterification is to create vigorous mixing and enhances mass transfer. The micro bubbles formation and collapse causes rapidly pressure and temperature variation in microscopic local and enhance the mass transfer. Study found that 93% conversion rate was achieved in 15 min at 60 °C with methanol oil molar ratio of 315: 1 under ultrasonication in in-situ transesterification of microalgae biomass to biodiesel (24 kHz, 200 W) and only 32% conversion was obtained for normal in-situ transesterification (Ehimen et al.

2012). The combination of ultrasonication and co-solvent addition could further increase conversion rate to 99% with much less methanol addition (methanol oil molar ratio 79:1) (Ehimen et al. 2012).

It was observed that the biodiesel from in-situ tranesterification had similar profile as the biodiesel obtained from traditional transesterification of oil (Haas et al. 2004; Samuel and Dairo 2012). The remarkable advantage of the technology is the simplification of the process while the disadvantage is the large amount excess alcohol demand (79:1 for in situ one and 6:1 for normal one). Great effort is required to reduce the addition of alcohol as it is associated with the energy and cost consumption of the process.

4.6 Summary

Transesterification is the most applied biodiesel synthesis route. It is the process that 1 molar triglyceride reacts with 3 molar alcohol to form 3 molar biodiesel (FAMEs) and 1 molar glycerol. In the presence of catalyst (homogeneous acid or base, enzyme, and heterogeneous catalyst), the reaction is faster and conversion rate is higher in mild condition (50 to 60 °C, 0.101 Mpa) compared to non-catalyst reaction which requires high temperature (around 200 °C) and pressure (50 Mpa).

Parameters including catalyst type, feedstock oil property, and methanol oil molar ratio have great impact on the transesterification. Alkali catalytic transesterification is widely applied in industrial biodiesel production due to the short reaction time required (less than 2 h). But the method is not suitable for tranesterification of oil contained FFA content greater than 2%, thus acidic catalyst transesterification should be used in biodiesel synthesis. Enzyme, free or immobilized, is also been employed in the reaction. It has high efficiency yet the high cost hampers its application. Heterogeneous catalysts are the solid acid or base. Its disadvantage is of mass transfer. Nano-sized heterogeneous catalyst is the solution of the problem and would be a promising catalyst as it is easy to be recovered and could be well contact with reactant. Methanol to oil molar ratio 6:1 or 9:1 is normally sufficient to achieve high efficiency tranesterification, while it could alter as the reaction condition (temperature and pressure)

changes. In order to reduce the reaction time, assisting technologies such as microwave irradiation and ultrasonication have been applied in the transesterification. They provide high conversion rate with short time (several minute).

In-situ tranesterification grabs growing interest due to the prevention of oil extraction. The technology directly converts oil located in oil bearing substances to biodiesel without affecting on biodiesel profile (fatty acid ester composition). The problem of the process is the long reaction time and high alcohol oil ratio. In order to solve problem, solvent, ultraosnication or microwave irradiation can be added. It would be widely applied in biodiesel production in future.

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4.8 References

Abigor, R. D., Uaudia, P. O., Foglia, T. A., Haas, M. J., Jones, K. C., Okpefa, E., Obibuzor, J. U., and Bafor, M. E. (2000). "Lipase-catalyzed production of biodiesel fuel from some Nigerian lauric oils." *Biochemical Society Transactions*, 28, 979-981.

Adachi, D., Hama, S., Numata, T., Nakashima, K., Ogino, C., Fukuda, H., and Kondo, A. (2011). "Development of an Aspergillus oryzae whole-cell biocatalyst coexpressing triglyceride and partial glyceride lipases for biodiesel production." *Bioresource Technology*, 102, 6723-6729.

Adebanjo, A. O., Dalai, A. K., and Bakhshi, N. N. (2005). "Production of Diesel-Like Fuel and Other Value-Added Chemicals from Pyrolysis of Animal Fat." *Energy & Fuels*, 19, 1735-1741.

Akoh, C. C., Chang, S.-W., Lee, G.-C., and Shaw, J.-F. (2007). "Enzymatic Approach to Biodiesel Production." *Journal of Agricultural and Food Chemistry*, 55, 8995-9005.

Anderson, D., Masterson, D., McDonald, B., and Sullivan, L. (2003). "Industrial Biodiesel Plant Design and Engineering: Practical Experience." *the Chemistry and Technology Conference, Session Seven: Renewable Energy Management*, International Palm Oil Conference (PIPOC), 24-28 August 2003, Putrajaya Marriot Hotel, Putrajaya, MALAYSIA.

Azcan, N., and Danisman, A. (2008). "Microwave assisted transesterification of rapeseed oil." *Fuel*, 87, 1781-1788.

Azcan, N., and Yilmaz, O. (2013). "Microwave assisted transesterification of waste frying oil and concentrate methyl ester content of biodiesel by molecular distillation." *Fuel*, 104, 614-619.

Bai, Y.-X., Li, Y.-F., Yang, Y., and Yi, L.-X. (2006). "Covalent immobilization of triacylglycerol lipase onto functionalized novel mesoporous silica supports." *Journal of Biotechnology*, 125, 574-582.

Ban, K., Kaieda, M., Matsumoto, T., Kondo, A., and Fukuda, H. (2001). "Whole cell biocatalyst for biodiesel fuel production utilizing Rhizopus oryzae cells immobilized within biomass support particles." *Biochemical Engineering Journal*, 8, 39-43.

Bilgin, A., Durgun, O., and Ahin, Z. (2002). "The effects of Diesel-ethanol blends on Diesel engine performance." *Energy Sources*, 24.

Boz, N., Degirmenbasi, N., and Kalyon, D. M. (2009a). "Conversion of biomass to fuel: Transesterification of vegetable oil to biodiesel using KF loaded nano-gamma-Al_2O_3 as catalyst." *Applied Catalysis B-Environmental*, 89, 590-596.

Boz, N., Degirmenbasi, N., and Kalyon, D. M. (2009b). "Conversion of biomass to fuel: Transesterification of vegetable oil to biodiesel using KF loaded nano-gamma-Al_2O_3 as catalyst." *Appl. Catal., B*, 89, 590-596.

Canakci, M., and Gerpen, J. V. (2003). "A Pilot Plant to Produce Biodiesel from High Free Fatty Acid Feedstocks." *ASAE Transactions*, 46, 945-954

Carmo Jr, A. C., de Souza, L. K. C., da Costa, C. E. F., Longo, E., Zamian, J. R., and da Rocha Filho, G. N. (2009). "Production of biodiesel by esterification of palmitic acid over mesoporous aluminosilicate Al-MCM-41." *Fuel*, 88, 461-468.

Chapagain, B. P., and Wiesman, Z. (2009). "MALDI-TOF/MS Fingerprinting of Triacylglycerols (TAGs) in Olive Oils Produced in the Israeli Negev Desert." *Journal of Agricultural and Food Chemistry*, 57, 1135-1142.

Chen, H.-C., Ju, H.-Y., Wu, T.-T., Liu, Y.-C., Lee, C.-C., Chang, C., Chung, Y.-L., and Shieh, C.-J. (2011). "Continuous Production of Lipase-Catalyzed Biodiesel in a Packed-Bed Reactor: Optimization and Enzyme Reuse Study." *Journal of Biomedicine and Biotechnology*, 2011.

Chen, L., Liu, T., Zhang, W., Chen, X., and Wang, J. (2012). "Biodiesel production from algae oil high in free fatty acids by two-step catalytic conversion." *Bioresource Technology*, 111, 208-214.

Dalai, A. K., Kulkarni, M. G., and Meher, L. C. (2006). "Biodiesel Productions from Vegetable Oils Using Heterogeneous Catalysts and Their Applications as Lubricity Additives " *EIC Climate Change Technology, 2006 IEEE*, 1-8.

del Río, M. I. T. (2007). "An analysis of the influence of phosphorus poisoning on the exhaust emission aftertreatment systems of light-duty diesel vehicles." *M.S. thesis of the Faculty of Science Nelson Mandela Metropolitan University, Germany*, 1-167.

Deng, L., Tan, T., Wang, F., and Xu, X. (2003).Enzymatic production of fatty acid alkyl esters with a lipase preparation from Candida sp. 99-125. Vol. 105, 727-734: WILEY-VCH Verlag.

Deng, X., Fang, Z., and Liu, Y.-h. (2010). "Ultrasonic transesterification of Jatropha curcas L. oil to biodiesel by a two-step process." *Energy Conversion and Management*, 51, 2802-2807.

Di Serio, M., Ledda, M., Cozzolino, M., Minutillo, G., Tesser, R., and Santacesaria, E. (2006). "Transesterification of Soybean Oil to Biodiesel by Using Heterogeneous Basic Catalysts." *Industrial & Engineering Chemistry Research*, 45, 3009-3014.

Diasakou, M., Louloudi, A., and Papayannakos, N. (1998). "Kinetics of the non-catalytic transesterification of soybean oil." *Fuel*, 77, 1297-1302.

Dizge, N., Keskinler, B., and Tanriseven, A. (2009a). "Biodiesel production from canola oil by using lipase immobilized onto hydrophobic microporous styrene-divinylbenzene copolymer." *Biochem. Eng. J.*, 44, 220-225.

Dizge, N., Keskinler, B., and Tanriseven, A. (2009b). "Biodiesel production from canola oil by using lipase immobilized onto hydrophobic microporous styrene-divinylbenzene copolymer." *Biochemical Engineering Journal*, 44, 220-225.

Doll, K. M., Sharma, B. K., Suarez, P. A. Z., and Erhan, S. Z. (2008). "Comparing Biofuels Obtained from Pyrolysis, of Soybean Oil or Soapstock, with Traditional Soybean Biodiesel: Density, Kinematic Viscosity, and Surface Tensionsâ€ " *Energy & Fuels*, 22, 2061-2066.

Du, W., Xu, Y.-Y., Liu, D.-H., and Li, Z.-B. (2005). "Study on acyl migration in immobilized lipozyme TL-catalyzed transesterification of soybean oil for biodiesel production." *Journal of Molecular Catalysis B: Enzymatic*, 37, 68-71.

Du, W., Xu, Y., and Liu, D. (2003). "Lipase-catalysed transesterification of soya bean oil for biodiesel production during continuous batch operation." *Biotechnology and Applied Biochemistry*, 38, 103-106.

Du, W., Xu, Y., Liu, D., and Zeng, J. (2004). "Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors." *Journal of Molecular Catalysis B: Enzymatic*, 30, 125-129.

Ehimen, E. A., Sun, Z., and Carrington, G. C. (2012). "Use of Ultrasound and Co-Solvents to Improve the In-Situ Transesterification of Microalgae Biomass." *Procedia Environmental Sciences*, 15, 47-55.

Ehimen, E. A., Sun, Z. F., and Carrington, C. G. (2010). "Variables affecting the in situ transesterification of microalgae lipids." *Fuel*, 89, 677-684.

Fortes, I. C. P., and Baugh, P. J. (1999). "Study of analytical on-line pyrolysis of oils from macauba fruit (Acrocomia sclerocarpa M) via GC-MS." *J. Braz. Chem. Soc.*, 10, 469-477.

Fukuda, H., Hama, S., Tamalampudi, S., and Noda, H. (2008). "Whole-cell biocatalysts for biodiesel fuel production." *Trends in Biotechnology*, 26, 668-673.

Gao, Y., Tan, T.-W., Nie, K.-L., and Wang, F. (2006). "Immobilization of Lipase on Macroporous Resin and Its Application in Synthesis of Biodiesel in Low Aqueous Media." *Chinese Journal of Biotechnology*, 22, 114-118.

Goering, C., Schwab, A., Daugherty, M., Pryde, E., and Heakin, A. (1982a). "Fuel properties of eleven vegetable oils." *Trans ASAE*, 25, 1472-1477.

Goering, C. E., Camppion, R. N., Schwab, A. W., and Pryde, E. H. (1982b). "Vegetable oil fuels." *Proceeding of the International Conference on Plant and Vegetable Oils as Fuels, Fargo, North Dakota. American Society of Agricultural Engineers, St Joseph*, MI 4 279-286.

Gogate, P. R. (2008). "Cavitational reactors for process intensification of chemical processing applications: A critical review." *Chemical Engineering and Processing: Process Intensification*, 47, 515-527.

Graboski, M., McCormick, R. s. P. E. C., and 24:125–164, S. (1998a). "Combustion of fat and vegetable oil derived fuels in diesel engines." *Progress in Energy and Combustion Science*, 24, 125-164.

Graboski, M., McCormick, R. s. P. E. C., and 24:125–164, S. (1998b). "Combustion of fat and vegetable oil derived fuels in diesel engines." *Progress in Energy and Combustion Science*, 24, 125-164.

Grepen, J. v. (2005). "Biodiesel processing and production." *Fuel Process Technology*, 86, 1097-1107.

Guan, F., Peng, P., Wang, G., Yin, T., Peng, Q., Huang, J., Guan, G., and Li, Y. (2010). "Combination of two lipases more efficiently catalyzes methanolysis of soybean oil for biodiesel production in aqueous medium." *Process Biochemistry*, 45, 1677-1682.

Ha, S. H., Lan, M. N., Lee, S. H., Hwang, S. M., and Koo, Y.-M. (2007). "Lipase-catalyzed biodiesel production from soybean oil in ionic liquids." *Enzyme and Microbial Technology*, 41, 480-483.

Haas, M., and Scott, K. (2007). "Moisture Removal Substantially Improves the Efficiency of in Situ Biodiesel Production from Soybeans." *Journal of the American Oil Chemists' Society*, 84, 197-204.

Haas, M., Scott, K., Marmer, W., and Foglia, T. (2004). "In situ alkaline transesterification: An effective method for the production of fatty acid esters from vegetable oils." *Journal of the American Oil Chemists' Society*, 81, 83-89.

Hanh, H. D., Dong, N. T., Okitsu, K., Nishimura, R., and Maeda, Y. (2009). "Biodiesel production through transesterification of triolein with various alcohols in an ultrasonic field." *Renewable Energy*, 34, 766-768.

He, Q., Xu, Y., Teng, Y., and Wang, D. (2008). "Biodiesel Production Catalyzed by Whole-Cell Lipase from Rhizopus chinensis." *Chinese Journal of Catalysis*, 29, 41-46.

Hu, S., Guan, Y., Wang, Y., and Han, H. (2011). "Nano-magnetic catalyst KF/CaO-Fe3O4 for biodiesel production." *Applied Energy*, 88, 2685-2690.

Içingür, Y., and Altiparmak, D. (2003). "Effect of fuel cetane number and injection pressure on a DI Diesel engine performance and emissions." *Energy Conversion and Management*, 44, 389-397.

Iso, M., Chen, B., Eguchi, M., Kudo, T., and Shrestha, S. (2001). "Production of biodiesel fuel from triglycerides and alcohol using immobilized lipase." *Journal of Molecular Catalysis B: Enzymatic*, 16, 53-58.

Jegannathan, K. R., Abang, S., Poncelet, D., Chan, E. S., and Ravindra, P. (2008). "Production of Biodiesel Using Immobilized Lipaseâ€"A Critical Review." *Critical Reviews in Biotechnology*, 28, 253-264.

Jeong, G.-T., and Park, D.-H. (2008). "Lipase-Catalyzed Transesterification of Rapeseed Oil for Biodiesel Production with tert-Butanol." *Applied Biochemistry and Biotechnology*, 148, 131-139.

Ji, J., Wang, J., Li, Y., Yu, Y., and Xu, Z. (2006). "Preparation of biodiesel with the help of ultrasonic and hydrodynamic cavitation." *Ultrasonics*, 44, e411-e414.

Jin, G., Bierma, T. J., Hamaker, C. G., Mucha, R., Schola, V., Stewart, J., and Wade, C. (2009). "Use of a whole-cell biocatalyst to produce biodiesel in a water-containing system." *Journal of Environmental Science and Health, Part A*, 44, 21-28.

Kaieda, M., Samukawa, T., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., Nomoto, F., Ohtsuka, K., Izumoto, E., and Fukuda, H. (1999). "Biodiesel fuel production from plant oil catalyzed by Rhizopus oryzae lipase in a water-containing system without an organic solvent." *Journal of Bioscience and Bioengineering*, 88, 627-631.

Kamini, N. R., and Iefuji, H. (2001). "Lipase catalyzed methanolysis of vegetable oils in aqueous medium by Cryptococcus spp. S-2." *Process Biochemistry*, 37, 405-410.

Karmakar, A., Karmakar, S., and Mukherjee, S. (2010). "Properties of various plants and animals feedstocks for biodiesel production." *Bioresource Technology*, 101, 7201-7210.

Kaur, M., and Ali, A. (2011). "Lithium ion impregnated calcium oxide as nano catalyst for the biodiesel production from karanja and jatropha oils." *Renewable Energy*, 36, 2866-2871.

Kawashima, A., Matsubara, K., and Honda, K. (2009). "Acceleration of catalytic activity of calcium oxide for biodiesel production." *Bioresource Technology*, 100, 696-700.

Kawentar, W. A., and Budiman, A. (2013). "Synthesis of biodiesel from second-used cooking oil." *Energy Procedia*, 32, 190-199.

Kim, M., Yan, S., Salley, S. O., and Ng, K. Y. S. (2010). "Competitive transesterification of soybean oil with mixed methanol/ethanol over heterogeneous catalysts." *Bioresource Technology*, 101, 4409-4414.

Knothe, G. (2005). "Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters." *Fuel Processing Technology*, 86, 1059-1070.

Kojima, S., Du, D., Sato, M., and Park, E. Y. (2004). "Efficient production of fatty acid methyl ester from waste activated bleaching earth using diesel oil as organic solvent." *Journal of Bioscience and Bioengineering*, 98, 420-424.

Koplow, D., and Dernbach, J. (2001). "FEDERAL FOSSIL FUEL SUBSIDIES AND GREENHOUSE GAS EMISSIONS: A Case Study of Increasing Transparency for Fiscal Policy." *Annual Review of Energy and the Environment*, 26, 361-389.

Kulkarni, M. G., Dalai, A. K., and Bakhshi, N. N. (2007). "Transesterification of canola oil in mixed methanol/ethanol system and use of esters as lubricity additive." *Bioresource Technology*, 98, 2027-2033.

Lara Pizarro, A. V., and Park, E. Y. (2003). "Lipase-catalyzed production of biodiesel fuel from vegetable oils contained in waste activated bleaching earth." *Process Biochemistry*, 38, 1077-1082.

Lee, J. H., Kim, S. B., Kang, S. W., Song, Y. S., Park, C., Han, S. O., and Kim, S. W. (2011). "Biodiesel production by a mixture of Candida rugosa and Rhizopus oryzae lipases using a supercritical carbon dioxide process." *Bioresource Technology*, 102, 2105-2108.

Li, Q., and Yan, Y. (2010). "Production of biodiesel catalyzed by immobilized Pseudomonas cepacia lipase from Sapium sebiferum oil in micro-aqueous phase." *Applied Energy*, 87, 3148-3154.

Li, W., Du, W., and Liu, D. (2007a). "Rhizopus oryzae Whole-Cell-Catalyzed Biodiesel Production from Oleic Acid in tert-Butanol Medium." *Energy & Fuels*, 22, 155-158.

Li, W., Du, W., and Liu, D. (2007b). "Rhizopus oryzae Whole-Cell-Catalyzed Biodiesel Production from Oleic Acid in tert-Butanol Mediumâ€ " *Energy & Fuels*, 22, 155-158.

Lima, D. G., Soares, V. C. D., Ribeiro, E. B., Carvalho, D. A., Cardoso, É. C. V., Rassi, F. C., Mundim, K. C., Rubim, J. C., and Suarez, P. A. Z. (2004). "Diesel-like fuel obtained by pyrolysis of vegetable oils." *Journal of Analytical and Applied Pyrolysis*, 71, 987-996.

Linko, Y.-Y., Lämsä, M., Wu, X., Uosukainen, E., Seppälä, J., and Linko, P. (1998). "Biodegradable products by lipase biocatalysis." *Journal of Biotechnology*, 66, 41-50.

Liu, K., and Wang, R. (2013). "Biodiesel production by transesterification of duck oil with methanol in the presence of alkali catalyst." *Petroleum & Coal*, 55, 68-72.

Liu, X., He, H., Wang, Y., and Zhu, S. (2007). "Transesterification of soybean oil to biodiesel using SrO as a solid base catalyst." *Catalysis Communications*, 8, 1107-1111.

Luz Martiìnez, S., Romero, R., Loìpez, J. C., Romero, A., Saìnchez Mendieta, V., and Natividad, R. (2011). "Preparation and Characterization of CaO Nanoparticles/NaX Zeolite Catalysts for the Transesterification of Sunflower Oil." *Industrial & Engineering Chemistry Research*, 50, 2665-2670.

Ma, F., and Hanna, A. M. (1999). "Biodiesel production: a review." *Bioresource Technology*, 70, 1-15.

Macala, G., Robertson, A., Johnson, C., Day, Z., Lewis, R., White, M., Iretskii, A., and Ford, P. (2008).Transesterification Catalysts from Iron Doped Hydrotalcite-like Precursors: Solid

Bases for Biodiesel Production. In *Catalysis Letters*, Vol. 122, 205-209: Springer Netherlands.

Melo-Junior, C. A. R., Albuquerque, C. E. R., Fortuny, M., Dariva, C., Egues, S., Santos, A. F., and Ramos, A. L. D. (2009). "Use of Microwave Irradiation in the Noncatalytic Esterification of C18 Fatty Acids." *Energy & Fuels*, 23, 580-585.

Modi, M. K., Reddy, J. R. C., Rao, B. V. S. K., and Prasad, R. B. N. (2007). "Lipase-mediated conversion of vegetable oils into biodiesel using ethyl acetate as acyl acceptor." *Bioresource Technology*, 98, 1260-1264.

Mondala, A., Liang, K., Toghiani, H., Hernandez, R., and French, T. (2009). "Biodiesel production by in situ transesterification of municipal primary and secondary sludges." *Bioresour. Technol.*, 100, 1203-1210.

Moreno-Pirajàn, J. C., and Giraldo, L. (2011). "Study of immobilized candida rugosa lipase for biodiesel fuel production from palm oil by flow microcalorimetry." *Arabian Journal of Chemistry*, 4, 55-62.

Nakagaki, S., Bail, A., Santos, V. C. d., Souza, V. H. R. d., Vrubel, H., Nunes, F. S., and Ramos, L. P. (2008). "Use of anhydrous sodium molybdate as an efficient heterogeneous catalyst for soybean oil methanolysis." *Applied Catalysis A: General*, 351, 267-274.

Nasikin, M., Susanto, B. H., Hirsaman, M. A., and Wijanarko, A. (2009). "Biogasoline from Palm Oil by Simultaneous Cracking and hydrogenation Reaction over Nimo/zeolite Catalyst." *World Applied Sciences (Special Issue for Environment)*, 5.

Nerín, C., Domeño, C., Moliner, R., Lázaro, M. J., Suelves, I., and Valderrama, J. (2000). "Behaviour of different industrial waste oils in a pyrolysis process: metals distribution and valuable products." *Journal of Analytical and Applied Pyrolysis*, 55, 171-183.

Nie, K., Xie, F., Wang, F., and Tan, T. (2006). "Lipase catalyzed methanolysis to produce biodiesel: Optimization of the biodiesel production." *Journal of Molecular Catalysis B: Enzymatic*, 43, 142-147.

Noureddini, H., Gao, X., and Philkana, R. S. (2005). "Immobilized Pseudomonas cepacia lipase for biodiesel fuel production from soybean oil." *Bioresource Technology*, 96, 769-777.

Park, E. Y., Sato, M., and Kojima, S. (2008). "Lipase-catalyzed biodiesel production from waste activated bleaching earth as raw material in a pilot plant." *Bioresource Technology*, 99, 3130-3135.

Park, Y.-M., Lee, J. Y., Chung, S.-H., Park, I. S., Lee, S.-Y., Kim, D.-K., Lee, J.-S., and Lee, K.-Y. (2010). "Esterification of used vegetable oils using the heterogeneous WO3/ZrO2 catalyst for production of biodiesel." *Bioresource Technology*, 101, S59-S61.

Peterson, C. L., Reece, D. L., Cruz, R., , and Thompson, J. (1992). "A comparison of ethyl and methyl esters of vegetable oil and diesel fuel substitutes." *In: Proceedings of the Alternative Energy Conference of the American Society of Agricultural Engineers*, 99-110.

Qian, J., Yang, Q., Sun, F., He, M., Chen, Q., Yun, Z., and Qin, L. (2013). "Cogeneration of biodiesel and nontoxic rapeseed meal from rapeseed through in-situ alkaline transesterification." *Bioresource Technology*, 128, 8-13.

Ramos, M. J., Fernández, C. M., Casas, A., Rodríguez, L., and Pérez, Á. (2009). "Influence of fatty acid composition of raw materials on biodiesel properties." *Bioresource Technology*, 100, 261-268.

Reddy, C., Oshel, R., and Verkade, J. G. (2006). "Room-Temperature Conversion of Soybean Oil and Poultry Fat to Biodiesel Catalyzed by Nanocrystalline Calcium Oxides." *Energy & Fuels*, 20, 1310-1314.

Results from GHGenius "Feedstocks, Power, Fuels, Fertilizers and Materials." *Report by (S&T)2 Consultants, January 2006*.

Royon, D., Daz, M., Ellenrieder, G., and Locatelli, S. (2007). "Enzymatic production of biodiesel from cotton seed oil using t-butanol as a solvent." *Bioresource Technology*, 98, 648-653.

Sánchez, E., Ojeda, K., El-Halwagi, M., and Kafarov, V. (2011). "Biodiesel from microalgae oil production in two sequential esterification/transesterification reactors: Pinch analysis of heat integration." *Chemical Engineering Journal*, 176–177, 211-216.

Saka, S., and Kusdiana, D. (2001). "Biodiesel fuel from rapeseed oil as prepared in supercritical methanol." *Fuel*, 80, 225-231.

Salis, A., Pinna, M., Monduzzi, M., and Solinas, V. (2008). "Comparison among immobilised lipases on macroporous polypropylene toward biodiesel synthesis." *Journal of Molecular Catalysis B: Enzymatic*, 54, 19-26.

Samart, C., Sreetongkittikul, P., and Sookman, C. (2009). "Heterogeneous catalysis of transesterification of soybean oil using KI/mesoporous silica." *Fuel Processing Technology*, 90, 922-925.

Samuel, O. D., and Dairo, O. U. (2012). "A critical review of in-situ transesterification process for biodiesel production." *The Pacific Journal of Science and Technology*, 13, 72-79.

Sanford, S. D., White, J. M., Shah, P. S., Wee, C., Valverde, M. A., and Meier, G. R. (2009). "Feedstock and Biodiesel Characteristics Report." *Renewable Energy Group Report*.

Schwab, A., Dykstra, G., Selke, E., Sorenson, S., and Pryde, E. (1988). "Diesel fuel from thermal decomposition of soybean oil." *Journal of the American Oil Chemists' Society*, 65, 1781-1786.

Sellappan, S., and Akoh, C. C. (2005). "Applications of lipases in modification of food lipids." *In Handbook of Industrial Catalysis Hou, C. T., Ed.; Taylor and Francis: Boca Raton, FL*, 9, 1-39.

Shah, S., and Gupta, M. N. (2007). "Lipase catalyzed preparation of biodiesel from Jatropha oil in a solvent free system." *Process Biochemistry*, 42, 409-414.

Shah, S., Sharma, S., and Gupta, M. N. (2004). "Biodiesel Preparation by Lipase-Catalyzed Transesterification of Jatropha Oil." *Energy & Fuels*, 18, 154-159.

Shaw, J.-F., Wang, D.-L., and Wang, Y. J. (1991). "Lipase-catalysed ethanolysis and isopropanolysis of triglycerides with long-chain fatty acids." *Enzyme and Microbial Technology*, 13, 544-546.

Shieh, C. J., Liao, H. F., and Lee, C. C. (2003). "Optimization of lipase-catalyzed biodiesel by response surface methodology." *Bioresource Technology*, 88, 103-106.

Singh, S. P., and Singh, D. (2010). "Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: A review." *Renewable & Sustainable Energy Reviews*, 14, 200-216.

Sree, R., Seshu Babu, N., Sai Prasad, P. S., and Lingaiah, N. (2009). "Transesterification of edible and non-edible oils over basic solid Mg/Zr catalysts." *Fuel Processing Technology*, 90, 152-157.

Stavarache, C., Vinatoru, M., Nishimura, R., and Maeda, Y. (2005). "Fatty acids methyl esters from vegetable oil by means of ultrasonic energy." *Ultrasonics Sonochemistry*, 12, 367-372.

Suarez, P. A. Z., Moser, B. R., Sharma, B. K., and Erhan, S. Z. (2009). "Comparing the lubricity of biofuels obtained from pyrolysis and alcoholysis of soybean oil and their blends with petroleum diesel." *Fuel*, 88, 1143-1147.

Suslick, K. S. (1989). "The chemical effects of ultrasound." *Scientific American*, Feb., 80-86.

Veljković, V. B., Avramović, J. M., and Stamenković, O. S. (2012). "Biodiesel production by ultrasound-assisted transesterification: State of the art and the perspectives." *Renewable and Sustainable Energy Reviews*, 16, 1193-1209.

Veljković, V. B., Stamenković, O. S., Todorović, Z. B., Lazić, M. L., and Skala, D. U. (2009). "Kinetics of sunflower oil methanolysis catalyzed by calcium oxide." *Fuel*, 88, 1554-1562.

Verziu, M., Cojocaru, B., Hu, J., Richards, R., Ciuculescu, C., Filip, P., and Parvulescu, V. (2008). "Sunflower and rapeseed oil transesterification to biodiesel over different nanocrystalline MgO catalysts." *Green Chemistry*, 10, 373-381.

Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., Sádaba, I., Ruiz-Vázquez, R. M., Torres-Martínez, S., and Garre, V. (2009a). "Biodiesel production from biomass of an oleaginous fungus." *Biochemical Engineering Journal*, 48, 22-27.

Vicente, G., Bautista, L. F., Rodriguez, R., Gutierrez, F. J., Sadaba, I., Ruiz-Vazquez, R. M., Torres-Martinez, S., and Garre, V. (2009b). "Biodiesel production from biomass of an oleaginous fungus." *Biochemical Engineering Journal*, 48, 22-27.

Vitolo, S., Bresci, B., Seggiani, M., and Gallo, M. G. (2001). "Catalytic upgrading of pyrolytic oils over HZSM-5 zeolite: behaviour of the catalyst when used in repeated upgrading-regenerating cycles." *Fuel*, 80, 17-26.

Vyas, A. P., Verma, J. L., and Subrahmanyam, N. (2011). "Effects of molar ratio, akali catalyst concentration and temperature on transesterification of Jatropha oil with methanol under ultrasonic irradiation." *Advances in Chemical Engineering and Science*, 1, 45-50.

Wang, Y., Zhang, F., Xu, S., Yang, L., Li, D., Evans, D. G., and Duan, X. (2008). "Preparation of macrospherical magnesia-rich magnesium aluminate spinel catalysts for methanolysis of soybean oil." *Chemical Engineering Science*, 63, 4306-4312.

Watanabe, Y., Pinsirodom, P., Nagao, T., Yamauchi, A., Kobayashi, T., Nishida, Y., Takagi, Y., and Shimada, Y. (2007). "Conversion of acid oil by-produced in vegetable oil refining to biodiesel fuel by immobilized Candida antarctica lipase." *Journal of Molecular Catalysis B: Enzymatic*, 44, 99-105.

Watanabe, Y., Shimada, Y., Sugihara, A., and Tominaga, Y. (2002). "Conversion of degummed soybean oil to biodiesel fuel with immobilized Candida antarctica lipase." *Journal of Molecular Catalysis B: Enzymatic*, 17, 151-155.

Watkins, R., Lee, A., and Wilson, K. (2004). "Li-CaO catalysed tri-glyceride transesterification for biodiesel applications." *Green Chemistry*, 6, 335-340.

Xie, W., and Chen, L. H. (2006). "Transesterification of soybean oil catalyzed by potassium loaded on alumina as a solid-base catalyst." *Applied Catalysis A: General*, 300, 67-74.

Xie, W., and Ma, N. (2009). "Immobilized Lipase on Fe3O4 Nanoparticles as Biocatalyst for Biodiesel Production." *Energy & Fuels*, 23, 1347-1353.

Xie, W., Peng, H., and Chen, L. (2006). "Calcined Mg-Al hydrotalcites as solid base catalysts for methanolysis of soybean oil." *Journal of Molecular Catalysis A: Chemical*, 246, 24-32.

Xu, C., Sun, J., Zhao, B., and Liu, Q. (2010). "On the study of KF/Zn(Al)O catalyst for biodiesel production from vegetable oil." *Applied Catalysis B: Environmental*, 99, 111-117.

Xu, R., and Mi, Y. (2011). "Simplifying the process of microalgal biodiesel production through in situ transesterification technology." *Journal of the American Oil Chemists' Society*, 88, 91-99.

Yücel, Y. (2011). "Biodiesel production from pomace oil by using lipase immobilized onto olive pomace." *Bioresource Technology*, 102, 3977-3980.

Yan, S., Lu, H., and Liang, B. (2007). "Supported CaO Catalysts Used in the Transesterification of Rapeseed Oil for the Purpose of Biodiesel Production." *Energy & Fuels*, 22, 646-651.

Yoosuk, B., Udomsap, P., Puttasawat, B., and Krasae, P. (2010). "Improving transesterification acitvity of CaO with hydration technique." *Bioresource Technology*, 101, 3784-3786.

Zeng, J., Du, W., Liu, X., Liu, D., and Dai, L. (2006). "Study on the effect of cultivation parameters and pretreatment on Rhizopus oryzae cell-catalyzed transesterification of vegetable oils for biodiesel production." *Journal of Molecular Catalysis B: Enzymatic*, 43, 15-18.

Table 4.1 Summary of biodiesel production from enzymes

Catalyst Type	Lipase source/Name	Feedstock	Catalyst addition C/O ratio (% wt/wt)	Temperature (ºC)	Time (h)	Yield (%)	Reference
Free lipase	*Candida antarctica*	Rapeseed oil	5	40	24	76.1	(Jeong and Park 2008)
	Candida rugosa	Rapeseed oil	40	45	24	97	(Linko et al. 1998)
	Candida cylindracea	Waste-activated bleaching earth	10	37	3	<100	(Kojima et al. 2004)
	Chromobacterium Viscosum	Jatropha Oil	10	40	8	62	(Shah et al. 2004)
	Cryptococcus spp	Rich bran oil	10	30	96	80	(Kamini and Iefuji 2001)
	Pseudomonas cepacia	Palm kemel oil	10	40	8	72	(Abigor et al. 2000)
	Rhizomucor miehei+ Penicillium cyclopium	Soybean oil	NA	30	12	95	(Guan et al. 2010)
	Rhizopus oryzae	Soybean oil	4-30	35	72	80-90	(Kaieda et al. 1999)
	Rhizopus oryzae	Palm oil	13	37	72	55	(Lara Pizarro and Park 2003)
Whole cell lipase	*Fusarium heterosporum*	Palm oil	3.1	30	96	98	(Adachi et al. 2011)
	Rhizopus oryzae	Soybean	3.6	35	72	90	(Ban et al. 2001)
	Rhizopus oryzae	Rapeseed oils	10	35	48	90	(Li et al. 2007b)
	Rhizopus oryzae	Waste vegetable oil	4.5	30	72	80	(Jin et al. 2009)
		Virgin canola oil				75	
		Brown grease				55	
Immobilized lipase	*Canadida antarctica*	Soybean oil	4	30	24	93.8	(Watanabe et al. 2002)
	Canadida antarctica	Cottonseed oil	1.7	50	24	97	(Royon et al. 2007)
	Candida antarctica	Jatropha oil	10	50	12	91.3	(Modi et al. 2007)
		Karanj oil				90	
		Sunflower oil				92.7	
	Candida antarctica	Acid oil	1	30	24	98	(Watanabe et al. 2007)

Candida antarctica	Soybean oil	2	50	12	80	(Ha et al. 2007)
Candida rugosa + *Rhizopus oryzae*	Soybean oil	20	45	3	99.13	(Lee et al. 2011)
Candida rugosa	Palm oil	1	35	2	85	(Moreno-Pirajàn and Giraldo 2011)
Candida sp.	Rapeseed oil	5	40	36	98	(Deng et al. 2003)
Candida sp.	Salad oil	20	40	6	96	(Nie et al. 2006)
Lipozyme	Soybean oil	-	30	-	95	(Du et al. 2003)
Lipozyme	Soybean oil,	10	40	36	90	(Du et al. 2005)
Pseudomonas cepacia	Soybean oil	4.75	35	1	67	(Noureddini et al. 2005)
Pseudomonas cepacia	Jatropha oil	10	50	8	93	(Shah and Gupta 2007)
Pseudomonas cepacia	Tallow tree oil	2.7	41	24	97	(Li and Yan 2010)
Pseudomonas fluorescens	Safflower oil,	0.3	50	25	99	(Iso et al. 2001)
Rhizopus miehei	Soybean oil	25	36.5	6.3	92.2	(Shieh et al. 2003)
Thermomyces lanuginosus	Canola oil	1	50	24	97	(Dizge et al. 2009b)
Thermomyces lanuginosus	Soybean oil	60	50	30	90	(Xie and Ma 2009)
Thermomyces lanuginosus	Pomace oil	5	25	24	93	(Yücel 2011)

Table 4.2 **Summary of biodiesel synthesis catalyzed by heterogeneous catalysts**

Lipase source/ Name	Feedstock	Catalyst addition C/O ratio (% wt/wt)	Temperature (ºC)	Time (h)	Yield (%)	Reference
Al-MCM-41	Palmitic acid	0.6	130	2	79	(Carmo Jr et al. 2009)
Ba(OH)	Canola oil	NA	90	8	90	(Dalai et al. 2006)
CaO (nano sized)	Poultry fat	0.6	25	12	99	(Reddy et al. 2006)
CaO	Rapeseed oil	0.8	60	3	90	(Kawashima et al. 2009)
CaO	Sunflower oil	1	60	2	98	(Veljković et al. 2009)
CaO	Palm oil	7	60	1	94	(Yoosuk et al. 2010)
CaO (nano sized)	Soybean oil	16	60	6	93.5	(Luz Martìnez et al. 2011)
CaO/MgO	Rapeseed oil	2	64.5	8	92	(Yan et al. 2007)
KI/mesoporous silica	Soybean oil	15	70	8	90.09	(Samart et al. 2009)
KF/Al_2O_3	Canola oil	6.5	60	8	87	(Xie and Chen 2006)
KF/Al_2O_3 (nano sized)	Vegetable oil	3	NA	8	99.84	(Boz et al. 2009a)
$KF/CaO–Fe_3O_4$ (nano sized)	Stillingia oil	4	65	3	95	(Hu et al. 2011)
KF/Zn(Al)O	Vegetable oil	3	65	3	95	(Xu et al. 2010)
Li-CaO (nano sized)	Karanja oil	5	65	1	100	(Kaur and Ali 2011)
	Jatropha oil			2	100	
MgO	Soybean oil	5	180	2	72	(Di Serio et al. 2006)
MgO (nano sized)	Soybean oil	1.5	70	6	90	(Verziu et al. 2008)
Na_2MoO_4	Soybean oil	5	65	3	95	(Nakagaki et al. 2008)
SrO	Soybean oil	3	70	0.5	95	(Liu et al. 2007)
WO_3/ZrO_2	Soybean oil	20	75	3	93	(Park et al. 2010)

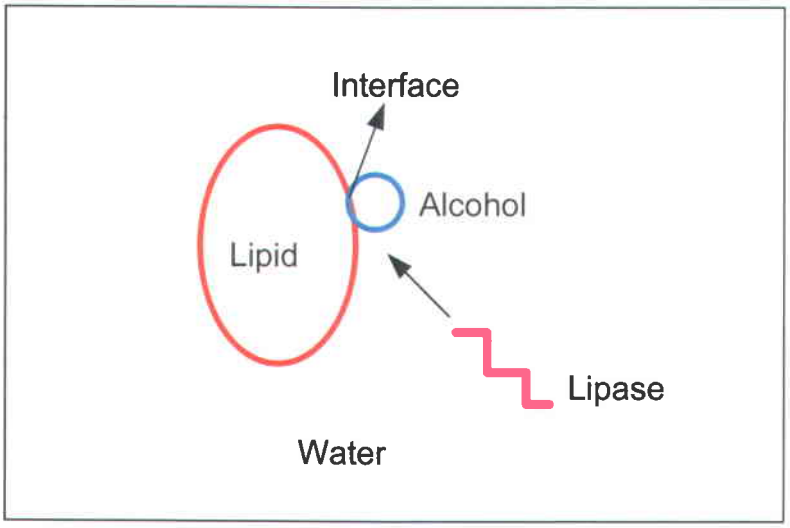

Figure 4.1 **The mechanism of enzymatic biodiesel synthesis**

PARTIE III : PRODUCTION DE BIODIESEL À PARTIR DE LIPIDES DE BOUE ET DE GLYCÉROL BRUT

BIODIESEL PRODUCTION FROM SLUDGE DERIVED OIL

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BIORESOURCE TECHNOLOGY (UNDER REVIEW)

5 BIODIESEL PRODUCTION FROM SLUDGE DERIVED OIL

5.1 Résumé

La hausse des prix des matières premières traditionnelles (huiles végétales et graisses animales) pour la production de biodiesel à pousser les chercheurs et les ingénieurs à chercher des sources alternatives de pétrole. Les boues sont produites naturellement, largement et en abondance partout dans le monde, et contiennent des lipides. Dans cette étude, les huiles dérivées de boues ont été utilisées pour la production de biodiesel. Différents types de boues, y compris municipale primaire, secondaire, mixte, et les boues secondaires de pâte à papier collectées de la ville de Québec ont été utilisées comme sources de lipides et milieux de culture des microorganismes oléagineux. Il a été constaté que les boues d'origine avait une teneur en lipides allant de 5% à 11% (p/p). La teneur en huile dans les différents types de boues suivait l'ordre suivant: boues secondaires de pâtes à papiers > boues municipales primaires > boues municipales mixtes > boues municipales secondaires. Différents types de boues ont été également utilisés pour l'accumulation de lipides par *Pichia amethionina* sp., *Galactomyces* sp. et *Trichosporon oleaginosus*. Les résultats ont montré que la teneur maximale en lipides a été obtenue dans les boues secondaires municipales. De plus, l'effet de la concentration initiale en matières en suspension des boues (10 à 30 g/L) sur l'accumulation de lipides a été étudiée. Des teneurs maximales en lipides de 30.2 et 32.4% p/p du poids sec, ont été obtenues par *Pichia amethionina* sp. et *Galactomyces* sp., respectivement, à une concentration en matières solides en suspension de 25 g/L. Par contre, une teneur maximale en lipides de 37.7% p/p sec a été accumulée par *Trichosporon oleaginosus* à une concentration en matières solides en suspension de 30 g/L. L'effet du rapport carbone-azote (C/N) sur l'accumulation de lipides dans les trois souches a été étudié et les résultats montrent que l'effet du rapport C/N dans le cas d'une concentration de matières en suspension (10 g/L) est plus élevé que celui relatif à une concentration de 30 g/L. De plus, plus le ratio C/N est élevé, plus l'accumulation de lipides est élevée aussi.

Mots clés : Biodiesel; boues; microorganismes oléagineux; matières premières; huile dérivée de boues

5.2 Abstract

The rising price of traditional feedstock including vegetable oils and animal fats for biodiesel production urges researchers and engineers to seek alternative oil sources. Sludge is naturally, widely, and abundantly produced all over the world, and contains lipid. This study deals with biodiesel production from sludge derived oil. Primary, secondary, and mixed sludge from municipal and secondary sludge from pulp and paper industry wastewater treatment collected in Québec City, Canada has 5% to 11% oil content (based on weight) in initial sludge. The oil content is in the order of pulp and paper secondary sludge > municipal primary sludge > municipal mixed sludge > municipal secondary sludge. Different types of sludge were also used for lipid production by *Pichia amethionina sp.*, *Galactomyces sp.*, and *Trichosporon oleaginosus*. The results showed that maximum lipid content was obtained in municipal secondary sludge among all types. Further, effect of initial sludge suspended solids concentration (10 to 30 g/L) on lipid accumulation was investigated. The maximum lipid content of 30.2 and 32.4 % w/w dry weight, was reached by *Pichia amethionina* sp. and *Galactomyces* sp., respectively, at 25 g/L suspended solids concentration, and that of 37.7% w/w dry weight by *Trichosporon oleaginosus* at 30 g/L suspended solids concentration. Carbon to nitrogen ratio studied showed that C/N ratio impact on lipid accumulation in the three strains is more obvious in lower suspended solids concentration (10 g/L) than in the higher one (30 g/L).

Keywords: Biodiesel; wastewater sludge; oleaginous microorganism; feedstock; sludge derived oil

5.3 Introduction

Feedstock is the key of successful biodiesel production as it determines renewable or finite issue and the production cost, which contributes more than 80% of the production cost (Haas et al., 2006; Kargbo, 2010). Vegetable oils and animal fats are the main source of biodiesel production in industry. The cost of these two types of oils is gradually growing due to the competition with food industry and kitchen. Moreover, the long life cycle (at most twice a year) and large land taken make the production unfavorable and inefficient. The fact forces to seek replacements.

Oleaginous microorganisms such as *Lipomyces starkeyi* and *Cryptococcus curvatus*, have been found to be very comparable alternative due to their fast growth rate (several hours to several days), large lipid contents (up to 80% on dry microorganism weight basis), more amenable to genetic manipulation for further improvement of lipid profiles, and less land requirement as compared to oilseed crops and animals (Sergeeva et al., 2008; Meng et al., 2009a; Cheirsilp et al., 2011; Chi et al., 2011; Galafassi et al., 2012). In addition, the feedstock oil properties are critical in biodiesel production as it determines the biodiesel properties. The properties of feedstock oil that has been used in biodiesel production are provided in Table 5.1. By comparing the feedstock properties, it can be learnt that microbial oil has similar properties as traditional feedstock oil (plant oil and animal fat). In addition, as mentioned previously, microbial oil is abundant and sustainable. Therefore, using microorganisms as oil/fat source would be a favorable way in biodiesel production.

Glucose is generally used as carbon source for growing oleaginous microorganisms which results in high lipid production cost and consequently high biodiesel production cost. Wastewater sludge contains abundant nutrients that are essential for the growth of microorganism (Zhuang et al., 2011; Su et al., 2012). Studies have revealed that wastewater sludge could be used as medium for the growth of microorganisms such as *Acidithiobacillus ferrooxidans, Lipomyces starkeyi, Sinorhizobium meliloti*, and *Bacillus thuringiensis* (Picher et al., 2002; Vidyarthi et al., 2002; Angerbauer et al., 2008a; Zhao et al., 2009). The use of wastewater

sludge as nutrient medium to cultivate oleaginous microorganisms would reduce the cost of lipid production and mitigate the sludge disposal pressure.

The aim of the work is to investigate lipid content in original wastewater sludge and the ability of wastewater sludge as culture medium to produce microbial oil. Sludge type and initial suspended solids concentration of sludge impact on lipid accumulation in microorganism were demonstrated. Glucose and glycerol was utilized to study lipid accumulation enhancement with the addition of carbon source supplement.

5.4 Materials and methods

5.4.1 Strains

Oleaginous yeast *Pichia amethionina* sp. SLY, *Trichosporon oleaginosus* ATCC 20509, and fungus *Galactomyces* sp. SOF were used for lipid accumulation study. *Pichia amethionina* sp. and *Galactomyces* sp. were isolated from municipal secondary sludge and soil in our lab. Biolog system (BIOLOG Inc., Hayward, USA) was used for investigating metabolic potential of the strains according to their ability to utilize different carbon sources. Identified strains were grown on the tryptic soy agar plates for 24 h at 30 ± 1 °C and then preserved at 4 °C for further study. *Trichosporon oleaginosus* ATCC 20509 was subcultured and streaked on malt extract agar plates, incubated for 24 h at 30 ± 1 °C and then preserved at 4 °C for further study.

5.4.2 Basic medium

Pichia amethionina sp. SLY and *Galactomyces* sp. SOF were cultivation with N-limit synthetic medium (C/N ratio of 50) 40 g/L glucose, 1.0 g/L $(NH_4)_2SO_4$, 7 g/L KH_2PO_4, 2 g/L NaH_2PO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, and 1.0 g/L yeast extract. *Trichosporon oleaginosus* was grown in a basic medium containing (gram per liter) 40 glucose, 2.7 KH_2PO_4, 0.95 Na_2HPO_4, 0.404 NH_4Cl, 0.2 $MgSO_4 \cdot 7H_2O$, 0.1 yeast extract, EDTA 0.1, 0.04 $CaCl_2 \cdot 2H_2O$, 0.0055 $FeSO_4 \cdot 7H_2O$, 0.0052 citric acid$\cdot H_2O$, 0.001 $ZnSO_4 \cdot 7H_2O$, and 0.00076 $MnSO_4 \cdot H_2O$ (Zheng et al., 2012).

5.4.3 Wastewater sludge as medium

Different types of wastewater sludge were used for microbial oil production study. The primary (PWS), secondary (SWS), and mixed (MWS) wastewater sludge samples were obtained from a municipal wastewater treatment plant, Communauté Urbain de Québec (CUQ), and the pulp & paper secondary wastewater sludge (PPSWS) was collected from White Birch Paper Industry located in Québec. The sludge was first concentrated by allowing it to undergo gravity settling at 4 ºC for 24 h. The resulting solution had a suspended solids concentration (SS) around 20 g/L. To achieve high SS concentrations (up to 30 g/L), the sludge was centrifuged with SORVALL RC 5C Plus centrifugation at 5000 rpm for 15 min. The supernatant and concentrated sludge were stored for further utilization.

To study different types of sludge effect on the lipid accumulation, sludge with 30 g/L SS concentrations was employed. Municipal secondary sludge was utilized to investigate suspended solids concentration effect on lipid accumulation. The suspended solids concentrations, 10 to 30 g/L, were obtained by mixing the supernatant and concentrated sludge.

5.4.4 Carbon source supplement for lipid accumulation enhancement

Municipal secondary sludge with 10 to 30 g/L SS concentration was used in the experiment. Glucose and glycerol were added to the sludge to achieve the C/N ratio of 50 to 200.

5.4.5 Inoculum and culture conditions

A loopful of any strain *Pichia amethionina* sp. SLY, *Galactomyces* sp. SOF, or *Trichosporon oleaginosus* from tryptic soy agar or malt extract agar plates was used to inoculate a 500-mL Erlenmeyer flask containing 150 mL of sterilized YPD (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) medium. The flasks were incubated on a rotary shaker at 170 rpm and 28 °C for 24 h. The cells in exponential phase from these flasks were used as pre-culture.

5.4.6 Lipid accumulation in basic and sludge mediums

Basic and sludge mediums were adjusted to pH 6.5 and sterilized at 121°C for 15 min prior to use. Then the mediums were inoculated with 10% v/v of pre-cultured of *Pichia amethionina* sp.

SLY, *Galactomyces* sp. SOF, and *Trichosporon oleaginosus*. The experiment was performed in shake flasks. The fermentation occurred at 28 ºC at 170 rpm. The samples were withdrawn with interval of 12 h.

5.4.7 Analytical techniques

5.4.7.1 Sludge characteristics

The sludge employed was characterized with standard methods (APHA, 2005), and the results in shown in Table 5.2.

5.4.7.2 Cell dry weight

Taking 5 mL of the fermentation broth from each sample and then centrifuged at 5000 rpm for 15 min. The pellets were then washed with distilled water twice, and dried till the weight constant to get cell dry weight.

5.4.7.3 Lipid content

The standard chloroform and methanol extraction procedure with minor modification was employed to determine the lipid content in the raw sludge and sludge-biomass (Folch et al., 1957; Vicente et al., 2009a). Samples of raw sludge and sludge-biomass were dewatered by centrifugation at 5000 rpm for 15 min, washed 2 times with distilled water, and then dried by lyophilisation. 200 mg dry matters were mixed with 4 ml solvent mixture of chloroform and methanol (2:1 v/v), and then subjected to 60 °C for 4 h. The mixture was then centrifuged at 5000 rpm for 15 min and the supernatant solvent phase was withdrawn and transferred into a pre-weighed glass vial (W1). The extraction procedure was repeated two times. Afterwards, the vial containing the total volume of the supernatant collected from each extraction was put under evaporation and then weighed (W2). The lipid amount was calculated by the difference of W1 and W2. The lipid content in the biomass is (W2-W1)/200 mg ×100%. The obtained lipid was stored in dark at 4 °C for further study.

5.4.7.4 Free fatty acid content in the lipid

The titration method was used to determine the Free Fatty Acid (FFA) content in the lipid (Woyewoda et al., 1976). Samples taken at 48 h were used for determining FFA content in lipid.

The extraction method is the same as descripted in Section of Lipid content and composition. Lipid obtained from extraction in vials was dissolved with 5 mL hexane and transferred to a 100-mL conical flask. Hexane was then evaporated at 60 °C. 10 mL of mixture of chloroform: methanol 2:1 v/v was added to the lipid in conical flask and then put two drops of phenolphthalein. 10 mL of mixture of chloroform: methanol 2:1 v/v with two drops phenolphthalein was added to a dry conical flask used as blank. 0.01 N KOH filled in 25 mL burette was then added to the conical flask drop by drop with gently shaking the flask in a swirling manner. The titration was ended when a pink colour was observed and persisting at least 5 second. Thereafter, the volume of 0.01 N KOH used was taken down to calculated the FFA content by utilizing the equation as shown in Equation 5.1.

Equation 5.1 **FFA content in oleic acid= $28.2 \times N \times (V-B)/W_{lipid} \times 100\%$**

Where V = the volume in ml of titration solution; B = the volume in ml of the blank; N = the normality of the titration solution; W = the weight of the sample of oil in grams.

5.4.7.5 *Fatty acids composition*

Transesterification was carried out through acid catalysis. Lipid obtained from solvent extraction in vials was first dissolved in hexane (20 ml hexane for per gram lipid), and then 1% sulfuric acid in methanol (40 ml acidic methanol for per gram lipid) was added. The mixture was subjected to 50 °C for 12 hours. 1.3-dichlorobenzene was used as internal standard. After reaction, 5% NaCl was added with the amount of 100 ml per gram lipid, and then biodiesel (fatty acid methyl esters, FAMEs) was extracted by two times washing with hexane (100 ml per gram lipid), then the hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% sodium bicarbonate (20 ml per gram lipid), and the top layer was then dried over 60 °C oven (Halim et al., 2011), and then redissolved in hexane for analysis.

The FAMEs in hexane were analyzed using a Gas Chromatography Linked to Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500). The dimensions of the column used were 30 m × 0.25 mm, with a phase thickness of 0.25 µm. The calibration curve was prepared with a mixture

comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1.3-dichlorobenzene was used as internal standard for preparing calibration curve.

5.5 Results and discussion

5.5.1 Strain identification and characterization

The Biolog study showed that the two isolated strains SLY and SOF belong to *Pichia amethionina* sp. and *Galactomyces* sp., respectively. *Pichia amethionina* sp. SLY can oxidize most of the carbon source but cannot utilize them as solo carbon source, such as succinic acid and glycerol (Table 5.3 and 5.4). However, inulin, cellobiose, gentiobiose, a-D-glucose, and sucrose can be utilized as solo carbon source. In addition, it showed that *Pichia amethionina* sp. SLY can co-utilize dextrin, acetoin, and 1.2-propanediol with D-xylose. *Galactomyces* sp. SOF can oxidize few carbon source including inulin, a-D-glucose, D-galactose, D-sorbose, and glycerol, and can utilize them as solo carbon source. Glycerol as a by-product of biodiesel production has become a concern due to the increase of the production. The results indicate that the glycerol can be used as carbon source to grow *Galactomyces* sp. SOF. It is also seen that *Galactomyces* sp. SOF can assimilate almost all the carbon while *Pichia amethionina* sp. SLY can utilize only few (Table 5.4). It suggests that *Galactomyces* sp. SOF can grow better in complex carbon based medium than *Pichia amethionina* sp. SLY.

5.5.2 Raw sludge oil content

The lipid contents were 6.81%, 5.33%, 6.42%, and 10.95% w/w for municipal primary sludge, secondary sludge, mixed sludge, and pulp & paper secondary wastewater sludge, respectively. The lipid of primary sludge is considered mainly from human waste and kitchen discharge. The lipid in sewage wastewater can adsorb on the suspended solids and then settle in primary sludge. Lipid content in primary sludge has a range from 6 to 35 % of dry sludge based on the variation of the wastewater (Turovskiy and Mathai, 2006). Secondary sludge mainly contains biomass; therefore, the lipid in secondary sludge is considered from cells. Mixed sludge is the mixture of primary and secondary (1:1 v/v), thus, the lipid is a mixture of that in primary and secondary sludge. Similarly as secondary sludge, lipid existing in pulp & paper secondary sludge is mainly from cells. The higher lipid content was observed in PPSWS than in SWS, which would

be due to that pulp & paper wastewater (C/N: 50) has a higher C/N ratio than municipal wastewater (C/N: 5) (Dobrzynska et al., 2004; Pokhrel and Viraraghavan, 2004). The high C/N ratio medium which is played by wastewater in the treatment tends to cultivate the microorganisms with high lipid content (Li et al., 2006; Isleten-Hosoglu et al., 2012).

5.5.3 Lipid accumulation with sludge medium cultivation

Suspended solids are the total of cells and sludge suspended solids. It was observed that suspended solids concentration increased from 0 to 12 h and then gradually decreased (Figure 5.1). The dissolved nutrients are easy to be utilized by cells; therefore, the suspended solids concentration increased due to biomass increase. Thereafter, suspended solids concentration showed a decrease trend. This would be due to that readily taken nutrients was consumed by 12 h, and then microorganisms started to attack the carbon source in complex form such as cellulose which requires high energy consumption. In this period, cells increased and nutrients in suspended solids form of the sludge decreased; however, the increase was smaller than decrease as the nutrients were consumed for cell concentration increase as well as cell life activities. Thus it resulted in the observation of a decrease of the suspended solid concentration.

From 0 to 48 h, lipid in the strains rapidly accumulated (Figure 5.1), and from 48 to 72 h, it was observed that the lipid content was reduced. Similarly as interpreted previously, this would be due to that available nutrients in the sludge mediums were completed by 48 h, and then microorganisms had to consume the body fat for maintaining the life activities, thus lipid content reduced.

At 0 h, it can be considered that there wasn't growth of microorganism; however, lipid presence was observed (Figure 5.1). It is due to the natural lipid existing in raw sludge (Section of Raw sludge lipid content). Municipal and pulp & paper secondary sludge as medium provides higher lipid content in strains which would be due to the higher biodegradability of secondary wastewater sludge than other sludge (Girault et al., 2012).

The lipid accumulation in different sludge is summarized in Table 5.5. The lipid accumulation in basic medium was up to 58.56, 53.26, and 61.66% of cell dry weight at 48 h for *Pichia amethionina* sp. SLY, *Galactomyces* sp. SOF, and *Trichosporon oleaginosus*. In sludge medium,

the lipid contents were lower than in basic medium, but the lipid concentration was comparable (Table 5.5). In sludge, non-degradable substances such as fibers are present, which is considered constant through the fermentation (Angerbauer et al., 2008b). Lipid content was calculated by lipid amount in the total dry suspended solids which contains biomass as well as fibers; therefore, the lipid content was low. When comparing lipid concentration (g/L), it was observed that the municipal and pulp & paper secondary sludge provide higher production than basic medium. It would be due to that secondary sludge didn't inhibit the three microorganism growth and the lipid presence in raw sludge wasn't utilized by the microorganisms, which was finally extracted along with the cell lipid. Trace element presence in the sludge enhanced lipid accumulation as well, it would be also a reason of the high lipid concentration in sludge medium than that in basic medium (Li et al., 2006; Muhid et al., 2008).

With basic medium, *Pichia amethionina* sp. SLY (58.56% w/w) accumulated higher lipid content than *Galactomyces* sp. SOF (53.26% w/w); however, *Galactomyces* sp. SOF gave higher lipid content in sludge mediums than *Pichia amethionina* sp. SLY (Table 5.5). It indicates that *Galactomyces* sp. SOF is more suitable for lipid accumulation with sludge as mediums, and it consists with Biolog results that *Galactomyces* sp. SOF could better use complex carbon as mediums than *Pichia amethionina* sp. SLY.

5.5.4 Sludge concentration effect on lipid accumulation

Municipal secondary sludge with various SS concentration (10, 15, 20, 25, and 30 g/L) were used to investigate the SS impact on lipid accumulation. The lipid accumulation in *Pichia amethionina* sp. SLY, *Galactomyces* sp. SOF, and *Trichosporon oleaginosus* against time was measured and shown in Figure 5.2. It revealed that *Pichia amethionina* sp. SLY (30.17% w/w dry weight) and *Galactomyces* sp. SOF (32.42% w/w dry weight) accumulated higher lipid in 25 g/L of SS concentration than in other concentration, but the difference of lipid accumulation in strains with 30 g/L SS concentration was slight. For *Trichosporon oleaginosus*, the highest lipid accumulation (37.69% w/w dry weight) was at SS concentration of 30 g/L; however, there wasn't distinguish difference from that in 25 g/L SS. It was reported that lipid accumulation was mainly affected by C/N ratio (Karatay and Dönmez, 2010; Kraisintu et al., 2010), while the C/N was the same in the different SS concentration medium. The available amounts of nutrients are

higher in higher SS concentration medium; therefore, the lipid accumulation increased with the increase of the SS concentration. When the concentration of the nutrient is greater than a certain value, it becomes inhibition, which is considered the concentration of nutrient in 25 g/L SS concentration in this study. Hence, when SS concentration is greater than 25 g/L, the lipid accumulation in strain didn't increase as SS concentration increase. Apart from the important nutrients such as carbon and nitrogen, wastewater sludge contains other substances which inhibit microorganism growth. For instance, free fatty acid (FFA) has been reported having negative impact on cell growth due to the ability of disrupting electron transport chain and oxidative phosphorylation in cell membrane, as well as inactivity enzymes, even directly breaking down the cells (Shin et al., 2007; Desbois and Smith, 2010). As the SS concentration increase, the FFA amount presence in the medium increased as well, which would lead to the inhibition on lipid accumulation.

5.5.5 C/N ratio impact on lipid accumulation

At different SS concentration, glucose or glycerol was added to give a final C/N ratio of 50, 100, 150, and 200. At 10 g/L SS concentration, addition of glucose made great difference on lipid accumulation of *Pichia amethionina* sp. SLY (Figure 5.3). The highest lipid of was obtained at C/N ratio of 100 (around 36% w/w dry weight), which was almost one time higher than that in C/N ratio of 50. For other SS concentration, C/N ratio of 100 was also higher than other C/N ratio, but the different wasn't remarkable. Too high C/N ratios, 150 and 200, inhibited the lipid accumulation (Figure 5.3). The addition of glycerol didn't impact on the lipid accumulation of *Pichia amethionina* sp. SLY, which would be due to that the strain couldn't utilize glycerol.

Unlike *Pichia amethionina* sp. SLY, *Galactomyces* sp. SOF and *Trichosporon oleaginosus* could use glucose as well as glycerol as carbon source. For *Galactomyces* sp. SOF, C/N ratio of 100 led to better lipid accumulation than other ratio, and the impact was significant at lower SS concentration than the higher. For *Trichosporon oleaginous*, C/N ratio has affected on low SS concentration medium, but no impact was observed on high SS concentration for C/N ratio of 50 and 100. The greater impact on low SS concentration than in the high one indicates that certain substances in sludge inhibit the lipid accumulation, and the inhibition was more profound than the enhancement of C/N ratio on lipid accumulation.

5.5.6 Free fatty acid content in sludge derived lipid

Free fatty acid content in the lipid determines the selection of transesterification catalyst. The biodiesel yield will be significantly reduced in alkaline transesterification when FFA content was higher than 0.5% w/w the feedstock lipid (Berrios et al., 2007). The free fatty acid contents in the sludge and sludge-biomass samples are given in Table 5.6. *Pichia amethionina* sp. SLY, *Galactomyces* sp. SOF, and *Trichosporon oleaginosus* harvested from basic medium has a free fatty acid content 0.41, 0.84, and 0.39% w/w total lipid, respectively. It indicates that, the lipid extracted from the strains can be converted to biodiesel through alkaline catalytic transesterification. Raw sludge lipid has high FFA content from 1.26 to 3.12% w/w total lipid. Raw primary sludge and its cultivated microorganisms contain high FFA more than 2%. FFA content in lipid from other raw sludge (SWS, MWS, and PPWS) and their cultivated microorganisms is greater than 0.5%. It suggested that acidic catalytic transesterification should be employed for biodiesel synthesis.

5.5.7 Biodiesel profile of sludge derived lipid

In general, fatty acids include unsaturated (with double bonds), which includes mono-unsaturated (one double bond, Cn:1) and polyunsaturated (more than one double bonds, Cn:2.3), and saturated (no double bond, Cn:0) fatty acids. The fatty acid composition plays important role in biodiesel qualities as it determines the viscosity, oxidation stability, cold flow property, flash point, calorific value (also called heat content or energy density), and density of biodiesel. Viscosity indicates the fuel features of spray, mixture formation, and combustion process. High viscosity can cause early injection and increase combustion chamber temperature. Normally, viscosity increases with the increase in the chain length and with the increase of fatty acid saturation level, while better oxidation stability requires high level of fatty acid saturation (Goering et al., 1982; Graboski et al., 1998; Içingür and Altiparmak, 2003). Cold flow properties depend on the saturation level of the feedstock oil. The higher of the saturation level is, the poorer cold flow properties is (Chapagain and Wiesman, 2009; Ramos et al., 2009). The flash point will be low when the chain length is short (Karmakar et al., 2010). It is predicted that greater saturation gave higher calorific value (Karmakar et al., 2010). Polyunsaturation level seems to be proportion to the density according to the report (Karmakar et al., 2010).

The fatty acid profile shows that C16 and C18 are the major composition of sludge (primary, secondary, mixed and pulp & paper sludge) derived oil which are also richly presenting in vegetable oil (Figure 5.4). But the saturated and unsaturated fatty acid fractions in sludge and its cultivated *Pichia amethionina* sp. SLY, *Galactomyces* sp. SOF, and *Trichosporon oleaginosus* are difference from soybean oil (Figure 5.5). Fatty acid composition of secondary sludge is shown in Figures 5.4 and 5.5 as example, and that of primary, mixed, and pulp & paper sludge is similar as secondary ones. Sludge derived oil are rich in the saturated but soybean oil has bigger fraction in unsaturated fatty acid. This suggested that sludge derived biodiesel has greater oxidation stability and density (chamber volume can be reduced in the vehicles) than soybean biodiesel. Soybean biodiesel has smaller viscosity than sludge derived biodiesel.

5.6 Conclusions

Sludge lipid content depends on the sludge types and wastewater treatment plant location. Compared to secondary and mixed sludge, primary sludge has the highest lipid content. While among all, pulp & paper wastewater sludge showed the highest lipid content (around 11% w/w dry sludge).

In comparison with primary and mixed sludge, secondary and pulp & paper sludge are more suitable for lipid accumulation of *Pichia amethionina* sp. SLY, *Galactomyces* sp. SOF, and *Trichosporon oleaginosus*, which is probably due to that there is more available carbon in the sludge. With sludge suspended solid concentration of 25 and 30 g/L, lipid was accumulated most in the strains. The impact of carbon to nitrogen ratio showed that C/N ratio of 100 with addition of glucose led to high lipid accumulation, while C/N ratio of 100 with addition of glycerol provided high lipid accumulation in *Galactomyces* sp. SOF and *Trichosporon oleaginosus*.

Sludge derived oil synthesized biodiesel has similar major fatty acid chain length (C16 to C18), but the saturation degree is different from soybean oil biodiesel. Biodiesel produced from sludge derived oil has higher density, oxidation stability, and viscosity. The study indicates that sludge is a promising feedstock of biodiesel production.

5.7 Acknowledgements

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5.8 References

Ahmad, M., Rashid, S., Khan, M. A., Zafar, M., Sultana, S., and Gulzar, S., 2009. Optimization of base catalyzed transesterification of peanut oil biodiesel. *African Journal of Biotechnology* 8, 441-446.

Alcantara, R., Amores, J., Canoira, L., Fidalgo, E., Franco, M. J., and Navarro, A., 2000. Catalytic production of biodiesel from soy-bean oil, used frying oil and tallow. *Biomass and Bioenergy* 18, 515-527.

Alvarez, and Steinbüchel, 2002. Triacylglycerols in prokaryotic microorganisms. *Applied Microbiology and Biotechnology* 60, 367-376.

Angerbauer, C., Siebenhofer, M., Mittelbach, M., and Guebitz, G. M., 2008a. Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production. *Bioresource Technology* 99, 3051-3056.

Angerbauer, C., Siebenhofer, M., Mittelbach, M., and Guebitz, G. M., 2008b. Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production. *Bioresour. Technol.* 99, 3051-3056.

APHA, 2005. Standard methods for examination of waters and wastewaters. *Washington, DC: American Public Health Association Publications. ISBN 0875531547.*

Barnwal, B. K., and Sharma, M. P., 2005. Prospects of biodiesel production from vegetable oils in India. *Renewable and Sustainable Energy Reviews* 9, 363-378.

Berrios, M., Siles, J., Martín, M. A., and Martín, A., 2007. A kinetic study of the esterification of free fatty acids (FFA) in sunflower oil. *Fuel* 86, 2383-2388.

Boocock, D. G. B., Konar, S. K., Leung, A., and Ly, L. D., 1992. Fuels and chemicals from sewage sludge: 1. The solvent extraction and composition of a lipid from raw sewage sludge. *Fuel* 71, 1283-1289

Canakci, M., and Sanli, H., 2008. Biodiesel production from various feedstocks and their effects on the fuel properties. *Journal of Industrial Microbiology & Biotechnology* 35, 431-441.

Canakci, M., and Van Gerpen, 2001. Biodiesel production from oils and fats with high free fatty acids. *Transaction of the ASAE* 44, 1429-1436.

Chapagain, B. P., and Wiesman, Z., 2009. MALDI-TOF/MS Fingerprinting of Triacylglycerols (TAGs) in Olive Oils Produced in the Israeli Negev Desert. *Journal of Agricultural and Food Chemistry* 57, 1135-1142.

Cheirsilp, B., Suwannarat, W., and Niyomdecha, R., 2011. Mixed culture of oleaginous yeast Rhodotorula glutinis and microalga Chlorella vulgaris for lipid production from industrial wastes and its use as biodiesel feedstock. *New Biotechnol.* 28, 362-368.

Chi, Z., Zheng, Y., Ma, J., and Chen, S., 2011. Oleaginous yeast Cryptococcus curvatus culture with dark fermentation hydrogen production effluent as feedstock for microbial lipid production. *International Journal of Hydrogen Energy* 36, 9542-9550.

Demirbas, A., 2003. Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey. *Energy Conversion and Management* 44, 2093-2109.

Desbois, A., and Smith, V., 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Applied Microbiology and Biotechnology* 85, 1629-1642.

Dobrzynska, A., Wojnowska-Baryła, I., and Bernat, K., 2004. Carbon removal by activated sludge under fully aerobic conditions at different COD/N ratio. *Polish Journal of Environmental Studies* 13, 33-40.

El-sharkawyL, S. H., Dostal, L., and Rosazza, J. P. N., 1993. Microbiological transformations of lipids: acyl-specific hydrolysis of lard by yeasts. *Applied and Environmental Microbiology* 59, 725-728.

Elvin-Lewis, M., 1988. Non-Traditional Oilseeds and Oils of India. *Economic Botany* 42, 540-540.

Exler, J., Lemar, L., and Smith, J., 1995. Fat and Fatty Acid Content of Selected Foods Containing Trans-Fatty Acids. *U.S. Department of Agriculture*, 1-38.

Folch, J., Lees, M., and G.H., S. S., 1957. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* 226, 497-509.

Galafassi, S., Cucchetti, D., Pizza, F., Franzosi, G., Bianchi, D., and Compagno, C., 2012. Lipid production for second generation biodiesel by the oleaginous yeast Rhodotorula graminis. *Bioresource Technology* 111, 398-403.

Girault, R., Bridoux, G., Nauleau, F., Poullain, C., Buffet, J., Peu, P., Sadowski, A. G., and Béline, F., 2012. Anaerobic co-digestion of waste activated sludge and greasy sludge from flotation process: Batch versus CSTR experiments to investigate optimal design. *Bioresource Technology* 105, 1-8.

Goering, C., Schwab, A., Daugherty, M., Pryde, E., and Heakin, A., 1982. Fuel properties of eleven vegetable oils. *Trans ASAE* 25, 1472-1477.

Graboski, M., McCormick, R. s. P. E. C., and 24:125–164, S., 1998. Combustion of fat and vegetable oil derived fuels in diesel engines. *Progress in Energy and Combustion Science* 24, 125-164.

Haas, M. J., McAloon, A. J., Yee, W. C., and Foglia, T. A., 2006. A process model to estimate biodiesel production costs. *Bioresour. Technol.* 97, 671-678.

Halim, R., Gladman, B., Danquah, M. K., and Webley, P. A., 2011. Oil extraction from microalgae for biodiesel production. *Bioresource Technology* 102, 178-185.

http://www.extension.org/pages/30256/animal-fats-for-biodiesel-production.

Içingür, Y., and Altiparmak, D., 2003. Effect of fuel cetane number and injection pressure on a DI Diesel engine performance and emissions. *Energy Conversion and Management* 44, 389-397.

Isleten-Hosoglu, M., Gultepe, I., and Elibol, M., 2012. Optimization of carbon and nitrogen sources for biomass and lipid production by Chlorella saccharophila under heterotrophic

conditions and development of Nile red fluorescence based method for quantification of its neutral lipid content. *Biochemical Engineering Journal* 61, 11-19.

Issariyakul, T., Kulkarni, M., Dalai, A., and Bakhshi, N., 2007. Production of biodiesel from waste fryer grease using mixed methanol/ethanol system. *Fuel Processing Technology* 88, 429-436.

Karatay, S. E., and Dönmez, G., 2010. Improving the lipid accumulation properties of the yeast cells for biodiesel production using molasses. *Bioresource Technology* 101, 7988-7990.

Kargbo, D. M., 2010. Biodiesel production from municipal sewage sludges. *Energy Fuels* 24, 2791-2794.

Karmakar, A., Karmakar, S., and Mukherjee, S., 2010. Properties of various plants and animals feedstocks for biodiesel production. *Bioresource Technology* 101, 7201-7210.

Kraisintu, P., Yongmanitchai, W., and Limtong, S., 2010. Selection and Optimization for Lipid Production of a Newly Isolated Oleaginous Yeast , Rhodosporidium toruloides DMKU3-TK16. *Production* 445, 436 - 445.

Lee, I., Johnson, L., and Hammond, E., 1995. Use of branched-chain esters to reduce the crystallization temperature of biodiesel. *Journal of the American Oil Chemists' Society* 72, 1155-1160.

Li, Y.-H., Liu, B., Zhao, Z.-B., and Bai, F.-W., 2006. Optimization of Culture Conditions for Lipid Production by Rhodosporidium toruloides. *Chinese Journal of Biotechnology* 22, 650-656.

Lips, H. J., 1950. FURTHER STUDIES ON CANADIAN LARD: PHOSPHORUS CONTENT, COLOR, AND STABILITY. *Canadian Journal of Research* 28f, 21-30.

Meng, X., Chen, G., and Wang, Y., 2008. Biodiesel production from waste cooking oil via alkali catalyst and its engine test. *Fuel Processing Technology* 89, 851-857.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., and Xian, M., 2009a. Biodiesel production from oleaginous microorganisms. *Renewable Energy* 34, 1–5.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., and Xian, M., 2009b. Biodiesel production from oleaginous microorganisms. *Renewable Energy* 34, 1-5.

Muhid, F., Wan Nawi, W. N. N., Kader, A. J. A., Wan Yusoff, W. M., and Hamid, A. A., 2008. Effects of Metal Ion Concentrations on Lipid and Gamma Linolenic Acid Production by Cunninghamella sp. 2A1. *OnLine Journal of Biological Sciences* 8, 62-67.

Ngo, H. L., Zafiropoulos, N. A., Foglia, T. A., Samulski, E. T., and Lin, W., 2007. Efficient Two-Step Synthesis of Biodiesel from Greasesâ€ *Energy & Fuels* 22, 626-634.

Papanikolaou, S., and Aggelis, G. (2011).Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. n/a-n/a: WILEY-VCH Verlag.

Papanikolaou, S., Sarantou, S., Komaitis, M., and Aggelis, G. (2004).Repression of reserve lipid turnover in Cunninghamella echinulata and Mortierella isabellina cultivated in multiple-limited media. Vol. 97, 867-875: Blackwell Science Ltd.

Pasias, S. A., Barakos, N. K., and Papayannakos, N. G., 2009. Catalytic Effect of Free Fatty Acids on Cotton Seed Oil Thermal Transesterification. *Industrial & Engineering Chemistry Research* 48, 4266-4273.

Picher, S., Drogui, P., Guay, R., and Blais, J. F., 2002. Wastewater sludge and pig manure used as culture media for bioleaching of metal sulphides. *Hydrometallurgy* 65, 177-186.

Pokhrel, D., and Viraraghavan, T., 2004. Treatment of pulp and paper mill wastewater—a review. *Science of The Total Environment* 333, 37-58.

Ramos, M. J., Fernández, C. M., Casas, A., Rodríguez, L., and Pérez, Á., 2009. Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource Technology* 100, 261-268.

Rice, B., Frohlich, A., and and Leonard, R., 1997. Bio-diesel Production Based on Waste Cooking Oil: Promotion of the Establishment of an Industry in Ireland. *Teagasc, Final Report Sept, 1997.*

Sanford, S. D., White, J. M., Shah, P. S., Wee, C., Valverde, M. A., and Meier, G. R., 2009. Feedstock and Biodiesel Characteristics Report. *Renewable Energy Group Report*.

Sergeeva, Y., Galanina, L., Andrianova, D., and Feofilova, E., 2008. Lipids of filamentous fungi as a material for producing biodiesel fuel. *Appl. Biochem. Microbiol.* 44, 523-527.

Shin, S. Y., Bajpai, V. K., Kim, H. R., and Kang, S. C., 2007. Antibacterial activity of eicosapentaenoic acid (EPA) against foodborne and food spoilage microorganisms. *LWT - Food Science and Technology* 40, 1515-1519.

Singh, S. P., and Singh, D., 2010. Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: A review. *Renewable & Sustainable Energy Reviews* 14, 200-216.

Su, Y., Mennerich, A., and Urban, B., 2012. Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios. *Bioresource Technology* 105, 67-73.

Turovskiy, I. S., and Mathai, P. K., 2006. Wastewater sludge processing: Chapter 2. Sludge quantities and characteristics. *Wiley-Interscience; 1 edition (Jun 2 2006)*, 30-59.

Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., Sádaba, I., Ruiz-Vázquez, R. M., Torres-Martínez, S., and Garre, V., 2009a. Biodiesel production from biomass of an oleaginous fungus. *Biochemical Engineering Journal* 48, 22-27.

Vicente, G., Bautista, L. F., Rodriguez, R., Gutierrez, F. J., Sadaba, I., Ruiz-Vazquez, R. M., Torres-Martinez, S., and Garre, V., 2009b. Biodiesel production from biomass of an oleaginous fungus. *Biochemical Engineering Journal* 48, 22-27.

Vidyarthi, A. S., Tyagi, R. D., Valero, J. R., and Surampalli, R. Y., 2002. Studies on the production of B. thuringiensis based biopesticides using wastewater sludge as a raw material. *Water Research* 36, 4850-4860.

Willson, R. M., Wiesman, Z., and Brenner, A., 2010. Analyzing alternative bio-waste feedstocks for potential biodiesel production using time domain (TD)-NMR. *Waste Management* 30, 1881-1888.

Woyewoda, P. J. K., A.D. , Regier, L. W., and Ackman, R. G., 1976. An improved titrametric method for determination of free fatty acid in fish oils. *Environment Canada Fisheries*

and Marine Service Technology Branch Halifax- Nova Scotia New Series Circular No. 61, November 1976.

Zhao, Y. Q., Zhao, X. H., and Babatunde, A. O., 2009. Use of dewatered alum sludge as main substrate in treatment reed bed receiving agricultural wastewater: Long-term trial. *Bioresource Technology* 100, 644-648.

Zheng, Y., Chi, Z., Ahring, B. K., and Chen, S., 2012. Oleaginous yeast *Cryptococcus curvatus* for biofuel production: Ammonia's effect. *Biomass and Bioenergy* 37, 114-121.

Zhuang, L., Zhou, S., Wang, Y., Liu, Z., and Xu, R., 2011. Cost-effective production of Bacillus thuringiensis biopesticides by solid-state fermentation using wastewater sludge: Effects of heavy metals. *Bioresource Technology* 102, 4820-4826.

Table 5.1 ***The biodiesel production feedstock properties***

Feedstock	SL (%)	FFA (%)	WC (%)	PC (ppm)	SC (ppm)	SV (mgKOH/g)	References
Soybean oil	15.34	0.07	0.029	3.7	0.8	195.3	(Canakci and Van Gerpen, 2001; Sanford et al., 2009)
Sunflower oil	9.34	0.04	0.02	<0.1	0.1	193.14	(Goering et al., 1982; Sanford et al., 2009)
Palm oil	47.3	0.54	0.049	7.3	1.0	208.62	(Demirbas, 2003; Sanford et al., 2009)
Canola oil	4.34	0.34	0.085	17.9	5.7	189.80	(Goering et al., 1982; Sanford et al., 2009)
Corn oil	14	12.22	0.153	<0.1	10.5	183.06	(Goering et al., 1982; Demirbas, 2003; Sanford et al., 2009)
Peanut oil	16	<2	<0.5	NA	10	191.50	(Demirbas, 2003; Barnwal and Sharma, 2005; Ahmad et al., 2009)
Cottonseed oil	30.6-29	9.8	0.05	0.5	10	194	(Goering et al., 1982; Pasias et al., 2009; Singh and Singh, 2010)
Coconut oil	68.7	0.07	0.027	2.0	2.7	267.56	(Demirbas, 2003; Sanford et al., 2009)
Jatropha curcas oil	27.1	1.17	0.073	322.9	3.5	200.80	(Elvin-Lewis, 1988; Sanford et al., 2009)
Poultry fat	29.69	1.7	0.065	209.3	27.2	188.08	(Exler et al., 1995; Sanford et al., 2009)
Lard	41-50	<18	0.048	<10	100	195	(http://www.extension.org/pages/30256/animal-fats-for-biodiesel-production; Lips, 1950; El-sharkawyL et al., 1993; Lee et al., 1995)
Beef tallow	47-63	1.61	0.051	270.8	25.2	198.00	(Lee et al., 1995; Canakci and Sanli, 2008; Sanford et al., 2009)
Brown grease	37.03	7.38-40	0.485	132.1	30.7	198.36	(Ngo et al., 2007; Sanford et al., 2009)
Waste cooking oil	55-90	2.72-7.25	0.242	27.0	3.4	198.50	(Rice et al., 1997; Meng et al., 2008; Sanford et al., 2009)
Waste fryer grease	87.8	5.60	7.3	NA	NA	177.87	(Alcantara et al., 2000; Issariyakul et al., 2007)
Microalgae	12-21	0.45-1.75	0.014-0.021	286.2-339.7	15.4-28.1	160.6-185.82	(Meng et al., 2009b; Sanford et al., 2009)
Bacteria	19-22	<1	De	Tr	Tr	NA	(Alvarez and Steinbüchel, 2002)
Yeast	12-47	<1	De	Tr	Tr	NA	(Alvarez and Steinbüchel, 2002; Papanikolaou and Aggelis, 2011)
Fungi	9-29	0.5-31.6	De	Tr	Tr	NA	(Alvarez and Steinbüchel, 2002; Papanikolaou et al., 2004; Vicente et al., 2009b)
Wastewater sludge oil	75	65	De	NA	NA	NA	(Boocock et al., 1992; Willson et al., 2010)

SL: saturation level; FFA: free fatty acid; WC: water content; PC: phosphorus content; SC: sulfur content; SV: saponification value; De: depends on the drying; Tr: trace amount; NA: not available.

Table 5.2 Characterization of the sludge

Properties	Primary wastewater sludge	Secondary wastewater sludge	Mixed wastewater sludge	Pulp & paper secondary wastewater sludge
TS (g/L)	27.58 ± 1.18	25.36 ± 0.95	25.65 ± 0.56	26.02 ± 1.48
TSS (g/L)	24.2 ± 1.02	20.7 ± 0.39	22.1 ± 1.37	22.9 ± 0.93
VSS (mg/L)	18.8 ± 0.24	15.5 ± 1.02	16.4 ± 0.47	20.5 ± 1.03
TC (g/ kg TS)	483 ± 12.01	421 ± 15.19	451 ± 9.60	567 ± 14.61
TN (g/ kg TS)	33.82 ± 0.69	49.91 ± 2.38	37.14 ± 1.11	15.14 ± 1.82
TP (g/kg TS)	34.13 ± 1.47	28.76 ± 0.83	31.59 ± 0.66	17.26 ± 0.29
pH	5.61 ± 0.01	6.42 ± 0.01	5.89 ± 0.03	6.52 ± 0.01

TP: total phosphorus; TN: total nitrogen; TC: total carbon; TS: total solid; TSS: total suspended solid; VSS: volatile suspended solid.

Table 5.3 Carbon source oxidation by SLY and SOF

Carbon source	*Pichia amethionina var amethonina* SLY	*Galactomyces geothrichum* SOF
Acetic acid	-	-
Formic acid	-	-
Propionic acid	-	-
Succinic acid	+	-
Methyl succinate	-	-
L-aspartic acid	-	-
L-glutamic acid	-	-
L-proline	-	-
D-gluconic acid	+	-
Dextrin	+	-
Inulin	+	+
Cellobiose	+	-
Gentiobiose	+	-
Maltose	-	-
Maltotriose	-	-
Melezitose	-	-
Melibiose	-	-
Palatinose	+	-
Raffinose	-	-
Stachyose	-	-
Sucrose	+	-
Trehalose	-	-
Turanose	+	-
N-ACETYL-D-glucosamine	-	-
a-D-glucose	+	+
D-galactose	-	+
D-psicose	+	-
L-sorbose	+	+
Salicin	+	-
D-mannitol	+	-
D-sorbitol	+	-
D-arabitol	-	-
Xylitol	-	-
Glycerol	+	+
Tween 80	-	-

Table 5.4 Carbon source assimilation by SLY and SOF

Carbon source	*Pichia amethionina var amethonina* SLY	*Galactomyces geothrichum* SOF
Fumaric acid	-	+
L-malic acid	+	+
Methyl succinate	-	+
Bromo succinic acid	-	+
L-glutamic acid	-	+
g-amino butyric acid	-	+
a-keto-glutaric acid	-	+
2-keto-D-gluconic acid	-	-
D-gluconic acid	-	-
Dextrin	-	+
Inulin	+	+
Cellobiose	+	-
Gentiobiose	+	-
Maltose	-	+
Maltotriose	-	+
D-melezitose	-	+
D-melibiose	-	+
Palatinose	-	-
D-raffinose	-	+
Stachyose	-	-
Sucrose	+	-
D-trehalose	-	-
Turanose	+	+
N-acetyl-D-glucosamine	-	-
D-glucosamine	-	-
a-D-glucose	+	+
D-galactose	-	+
D-psicose	-	+
L-rhamnose	-	-
L-sorbose	-	+
a-methyl-D-glucoside	-	-
b-methyl-D-glucoside	-	+
Amygdalin	-	+
Arbutin	-	+
Salicin	-	+
Maltitol	-	-
D-mannitol	-	+
D-sorbitol	-	+
Adonitol	-	-
D-arabitol	-	+

Xylitol	-	-
i-erythritol	-	-
Glycerol	-	+
Tween 80	-	+
L-arabinose	-	+
D-arabinose	-	+
D-ribose	-	+
D-xylose	-	+
Methyl succinate + D-xylose	-	+
N-acetyl-L-glutamic acid + D- xylose	-	+
Quinic acid + D- xylose	-	+
D-glucuronic + D- xylose	-	+
Dextrin + D- xylose	+	+
a-D-lactose + D- xylose	-	+
D-melibiose + D- xylose	-	+
D-galactose + D- xylose	-	+
m-inositol + D- xylose	-	+
1.2-propanediol + D- xylose	+	+
Acetoin + D- xylose	+	+

Table 5.5 Lipid accumulation in different medium

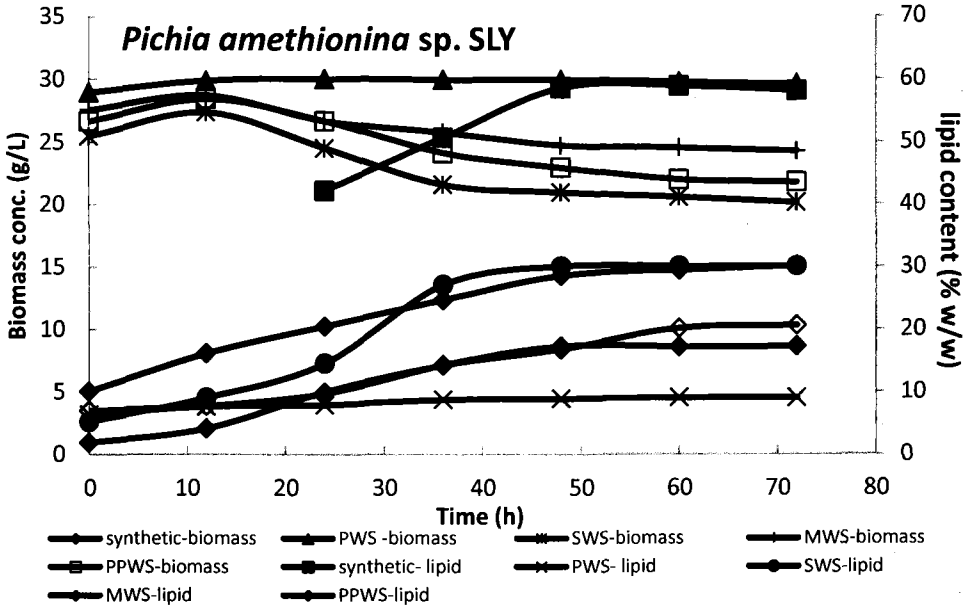
Microorganisms	Medium	Lipid content (% w/w dry SS)	Lipid concentration (g/L)
Pichia amethionina sp. SLY	Basic medium	58.56 ± 2.09	5.04 ± 0.26
	PWS	8.87 ± 0.70	2.65 ± 0.13
	SWS	30.05 ± 1.04	6.29 ± 0.39
	MWS	16.77 ± 0.48	4.14 ± 0.22
	PPWS	28.55 ± 1.30	6.54 ± 0.31
Galactomyces sp. SOF,	Basic medium	53.26 ± 1.24	5.36 ± 0.16
	PWS	15.65 ± 0.77	3.49 ± 0.05
	SWS	31.61 ± 1.02	5.77 ± 0.27
	MWS	21.65 ± 1.31	5.35 ± 0.19
	PPWS	31.19 ± 0.84	6.03 ± 0.28
Trichosporon oleaginosus	Basic medium	61.66 ± 2.19	6.51 ± 0.31
	PWS	16.48 ± 0.46	4.73 ± 0.09
	SWS	35.99 ± 1.68	7.48 ± 0.21
	MWS	24.16 ± 0.92	5.68 ± 0.19
	PPWS	33.29 ± 0.60	7.08 ± 0.51

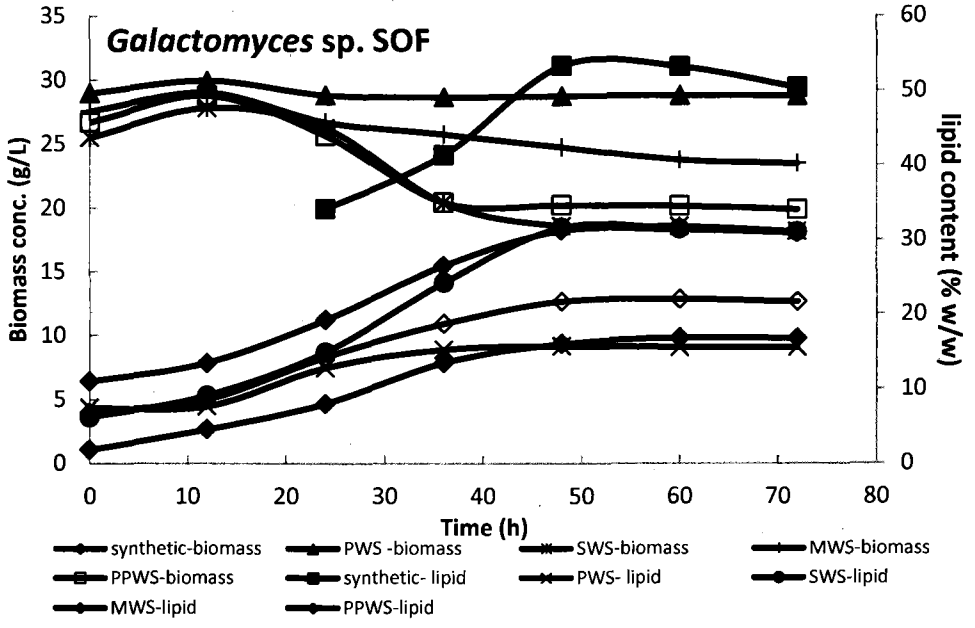
Harvested at 48 h fermentation.

Table 5.6 Free fatty acid content in sludge derived lipid

Lipid source		FFA content (% w/w lipid)
Municipal primary sludge		3.12 ± 0.08
Municipal secondary sludge		1.26 ± 0.02
Municipal mixed sludge		2.77 ± 0.13
Pulp &paper secondary sludge		1.86 ± 0.02
Pichia amethionina sp. SLY	in basic medium	0.41 ± 0.00
	in municipal primary sludge	2.33 ± 0.08
	in municipal secondary sludge	0.84 ± 0.02
	in municipal mixed sludge	1.43 ± 0.06
	in pulp & paper secondary sludge	1.16 ± 0.02
Galactomyces sp. SOF	in basic medium	0.84 ± 0.03
	in municipal primary sludge	2.76 ± 0.15
	in municipal secondary sludge	1.24 ± 0.02
	in municipal mixed sludge	1.72 ± 0.01
	in pulp & paper secondary sludge	1.41 ± 0.03
Trichosporon oleaginosus	in basic medium	0.39 ± 0.02
	in municipal primary sludge	2.22 ± 0.02
	in municipal secondary sludge	0.53 ± 0.02
	in municipal mixed sludge	1.52 ± 0.08
	in in pulp & paper secondary sludge	0.71 ± 0.01

The experiment was done with duplicates.


Pichia amethionina sp. SLY
Biomass conc. (g/L)
lipid content (% w/w)
Time (h)
synthetic-biomass
PWS -biomass
SWS-biomass
MWS-biomass
PPWS-biomass
synthetic- lipid
PWS- lipid
SWS-lipid
MWS-lipid
PPWS-lipid


Galactomyces sp. SOF
Biomass conc. (g/L)
lipid content (% w/w)
Time (h)
synthetic-biomass
PWS -biomass
SWS-biomass
MWS-biomass
PPWS-biomass
synthetic- lipid
PWS- lipid
SWS-lipid
MWS-lipid
PPWS-lipid

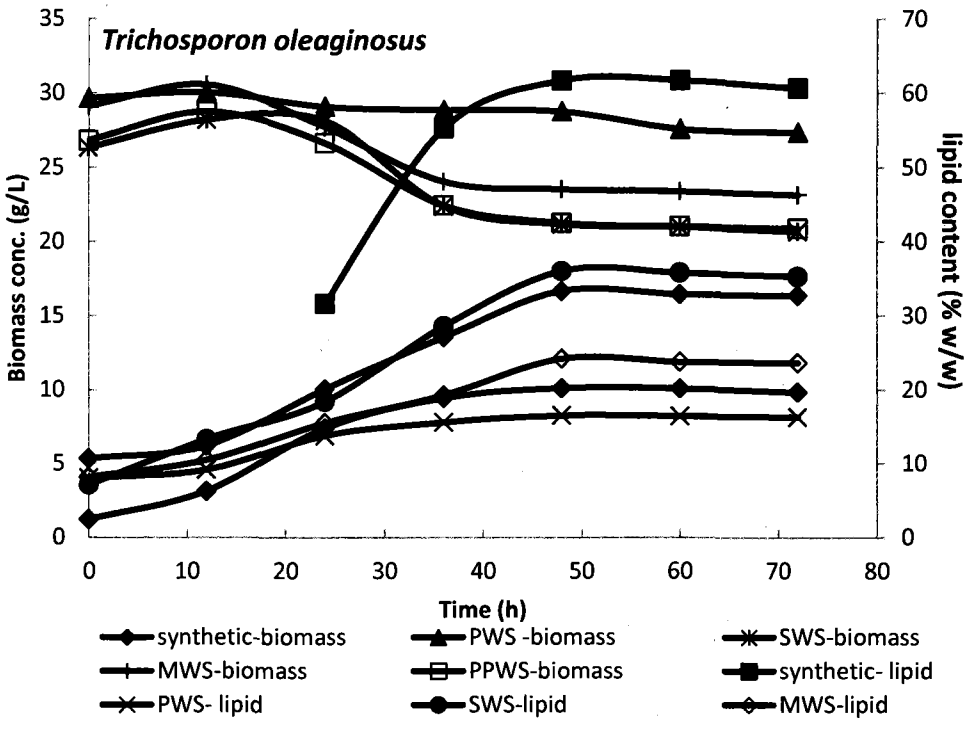
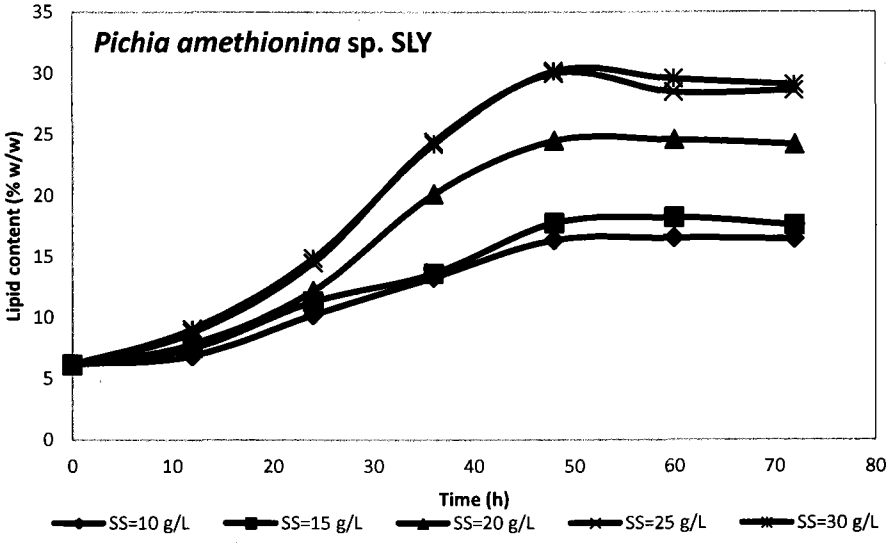
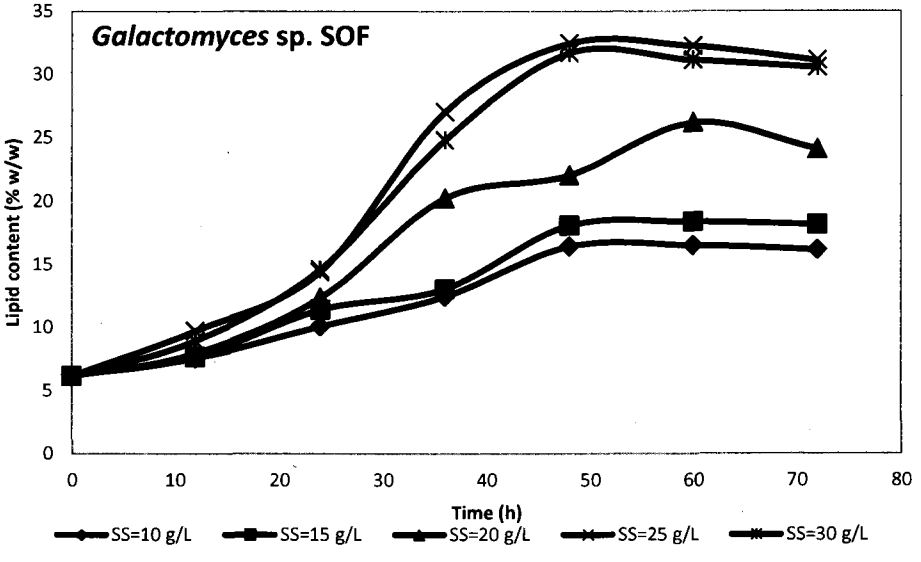


Figure 5.1 **Lipid accumulation of *Pichia amethionina* sp. SLY, *Galactomyces* sp. SOF, and *Trichosporon oleaginosus* in different types of sludge; The standard deviation is less than 5% (PWS=primary wastewater sludge; SWS=secondary wastewater sludge; MWS=mixed wastewater sludge; PPWS=pulp&paper wastewater sludge)**


Pichia amethionina sp. SLY
Lipid content (% w/w)
Time (h)
SS=10 g/L
SS=15 g/L
SS=20 g/L
SS=25 g/L
SS=30 g/L


Galactomyces sp. SOF
Lipid content (% w/w)
Time (h)
SS=10 g/L
SS=15 g/L
SS=20 g/L
SS=25 g/L
SS=30 g/L

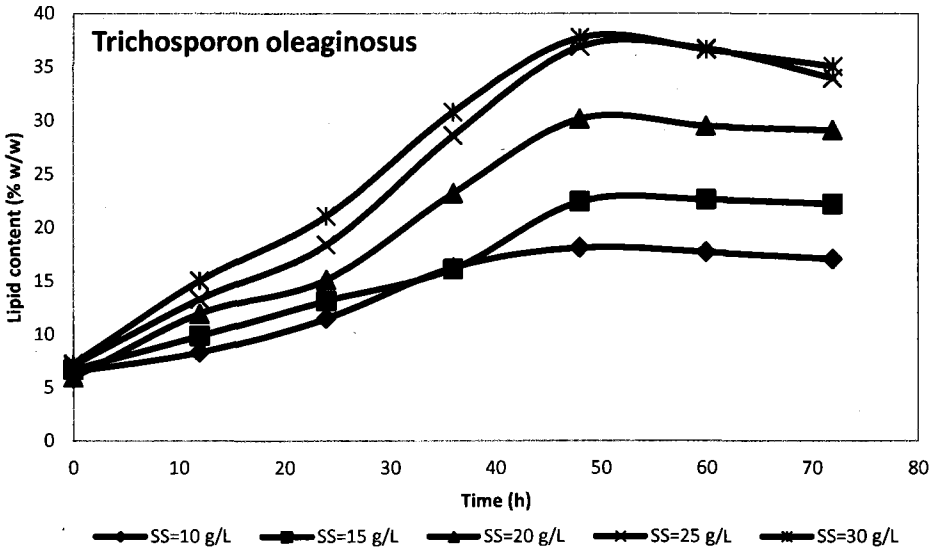
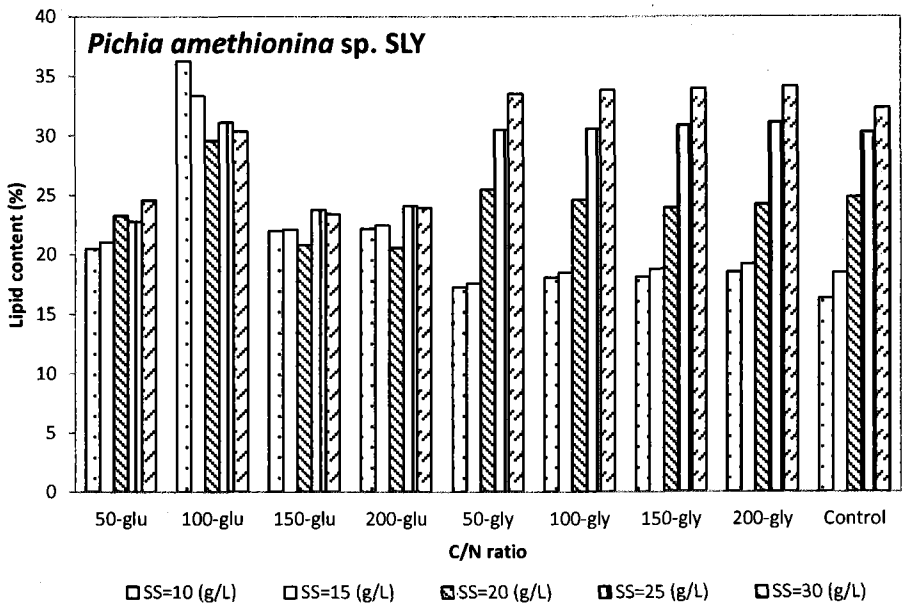
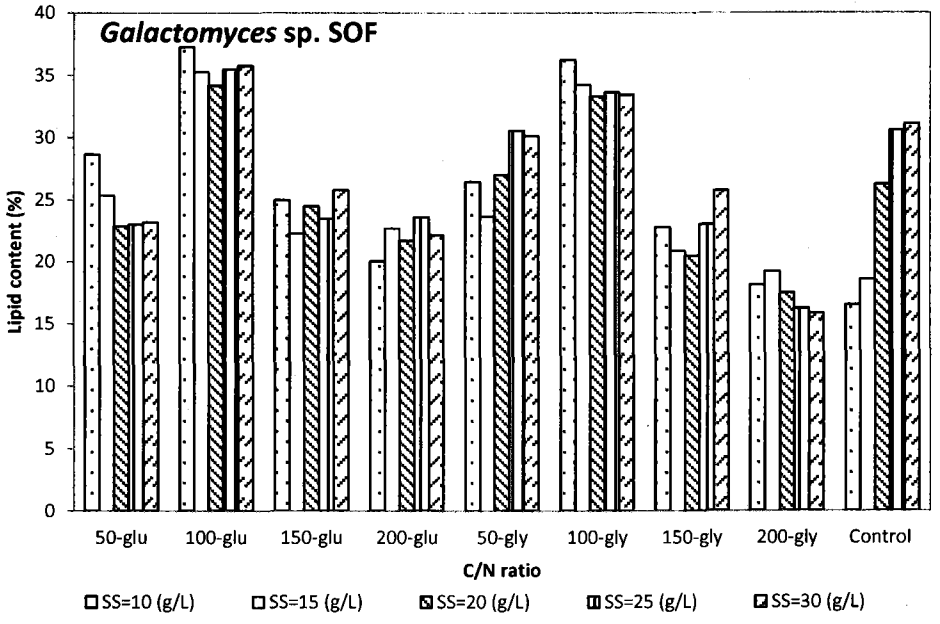


Figure 5.2 **Suspended solids concentration effect on lipid accumulation in the strains; The standard deviation is less than 5%**


Pichia amethionina sp. SLY
Lipid content (%)
0
5
10
15
20
25
30
35
40
50-glu
100-glu
150-glu
200-glu
50-gly
100-gly
150-gly
200-gly
Control
C/N ratio
SS=10 (g/L)
SS=15 (g/L)
SS=20 (g/L)
SS=25 (g/L)
SS=30 (g/L)


Galactomyces sp. SOF
Lipid content (%)
0
5
10
15
20
25
30
35
40
50-glu
100-glu
150-glu
200-glu
50-gly
100-gly
150-gly
200-gly
Control
C/N ratio
SS=10 (g/L)
SS=15 (g/L)
SS=20 (g/L)
SS=25 (g/L)
SS=30 (g/L)

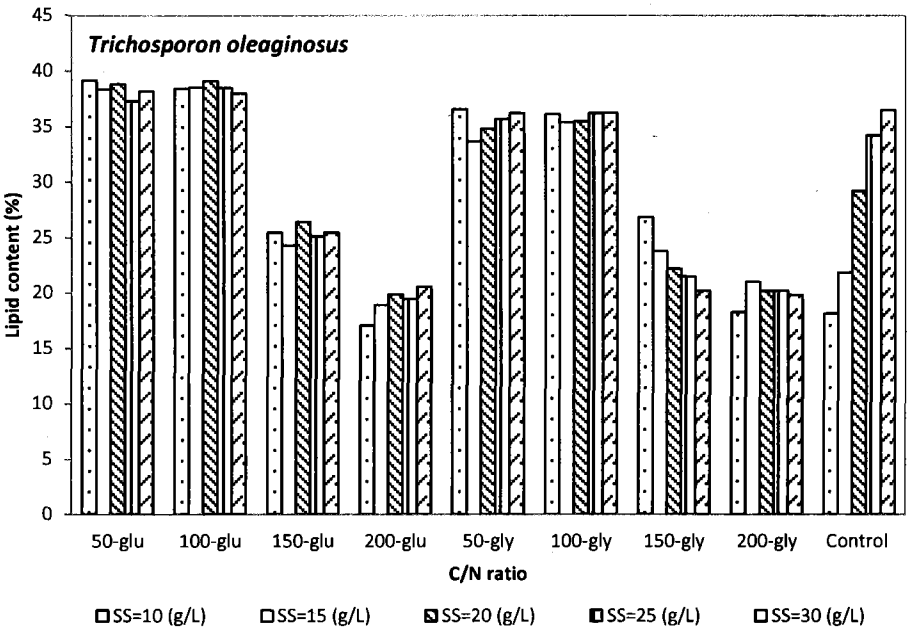


Figure 5.3 **C/N ratio effect on lipid accumulation in the strains, the standard deviation is less than 5%**

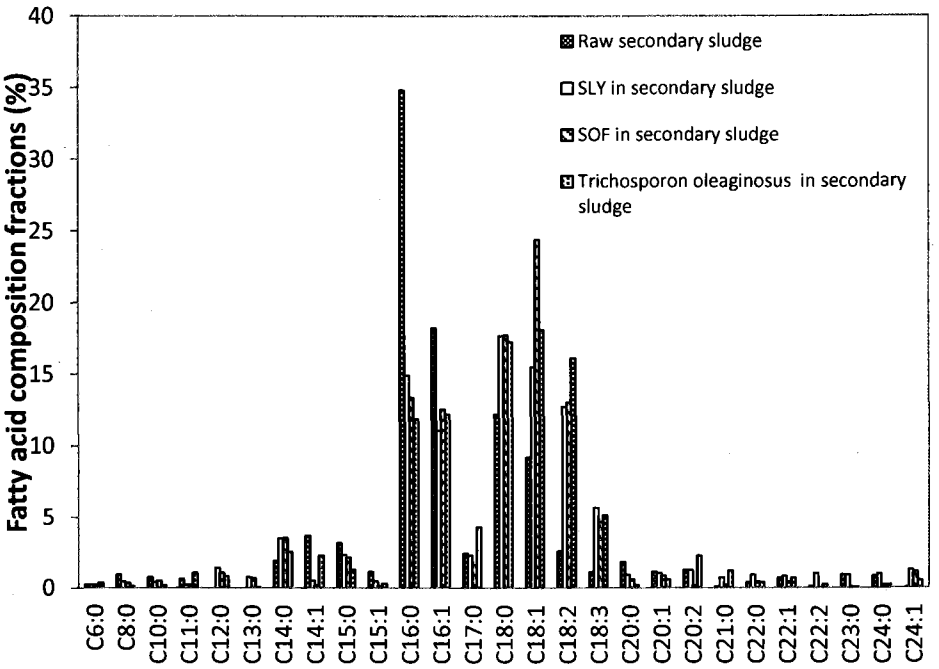


Figure 5.4 **Fatty acid profile of sludge cultivated *Pichia amethionina* sp. SLY, *Galactomyces* sp. SOF, and *Trichosporon oleaginosus*; the standard deviation is less than 5%**

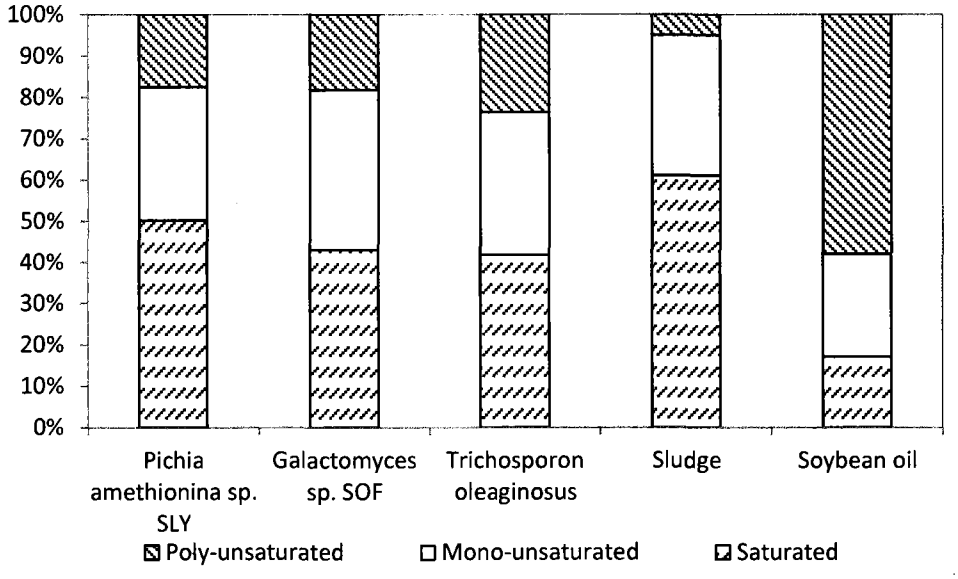


Figure 5.5 **Comparison of the saturation of biodiesel synthesized with sludge derived oil and soybean oil (sludge is secondary sludge)**

LIPID PRODUCTION FROM MICROORGANISM CULTIVATED WITH PRE-TREATED WASTEWATER SLUDGE

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BIORESOURCE TECHNOLOGY (UNDER REVIEW)

6 LIPID PRODUCTION FROM MICROORGANISM CULTIVATED WITH PRE-TREATED WASTEWATER SLUDGE

6.1 Résumé

Les boues d'épuration contiennent la plupart des éléments nutritifs nécessaires pour la croissance des microorganismes. Dans cette étude, les traitements thermiques et chimiques (traitements thermo-(acide et alcalin)) ont été appliqués à des boues contenant différentes concentrations de matières en suspension (10 à 30 g/L) et les boues prétraitées ont été utilisées comme matière première pour la production de lipides par *Trichosporon oleaginosus*. Les résultats ont montré que les prétraitements chimiques et thermiques conduisent à une forte augmentation de la concentration de carbone organique dissous (de 1.5 à 6.0 g/L) et de l'azote dissous (0.5 à 1.8 g/L) de la boue. Le temps de fermentation par *Trichosporon oleaginosus* pour obtenir une teneur maximale en lipides (environ 39% p/p) était de 48 h et de 42 h dans les boues qui ont subit un prétraitement thermique et dans les boues qui ont subit un prétraitement thermochimique, respectivement. Le biodiesel produit par les boues prétraitées contiennent principalement les acides gras C14, C16, C18, lesquels sont également présents en grande quantité dans le biodiesel commercial actuellement produit à partir de graines de soja, de canola et d'huiles de graines de tournesol. Les résultats de cette étude offre une nouvelle approche qui se base sur l'utilisation des boues d'épuration comme matière première pour la production de biodiesel.

Mots clés : Boues ; accumulation des lipides ; prétraitement des boues ; biodiesel

6.2 Abstract

Wastewater sludge contains most of the nutrients required for growth of microorganism. In this study, thermal and chemical (acidic and alkaline)-thermal treatments were applied to different sludge suspended solids concentrations (10 to 30 g/L) and the pre-treated sludge was used as raw material for lipid production by *Trichosporon oleaginosus*. The results showed that chemical-thermal pre-treatment led to a large increase in the concentration of dissolved organic carbon (from 1.5 to 6.0 g/L) and dissolved nitrogen (from 0.5 to 1.8 g/L) of the sludge. The fermentation time was 48 h in thermal pre-treated sludge, while only 42 h in chemical-thermal pre-treated sludge to obtain maximum lipid content (around 39% w/w) by *Trichosporon oleaginosus*. The biodiesel produced by pre-treated sludge in the study mainly contains C16 and C18, which are also rich in currently commercial biodiesel produced from soybean seed, canola, and sunflower seed oils. It shows the new approach that wastewater sludge as a feedstock for the production of biodiesel.

Keywords: Wastewater sludge; lipid accumulation; sludge pre-treatment; biodiesel

6.3 Introduction

Biodiesel which is renewable, sustainable, and environmentally friendly has been a favorable alternative fuel. The current biodiesel is synthesized with feedstocks such as plant seed oil and animal fat which are also food source. Due to the high cost of the feedstock, alternative is required. Microbial oil derived from oleaginous microorganisms has been reported suitable for biodiesel synthesis (Meng et al., 2009; Cao et al., 2010; Cheirsilp et al., 2011). Glucose is the most utilized carbon source for growth of oleaginous microorganisms, yet it leads to high cost of lipid accumulation and sequentially expensive biodiesel production. Therefore, it is becoming a major interest in the search for cost-effective biodiesel feedstocks, or raw materials.

Wastewater sludge is an attractive alternative as it contains nutrients that are essential for the growth of microorganism; however, they are normally in large molecules form and difficult to be utilized (Angerbauer et al., 2008; Su et al., 2012). Pre-treatment (acidic- or alkaline- thermal) with low or high pH, subjected to high temperature for certain of time to allow the hydrolysis is mostly applied, it breaks down the larger molecules into smaller ones which could be better assimilated by microorganisms (Brar et al., 2004; Yezza et al., 2005). Pre-treatment on sludge was found to enhance the growth of microorganism such as *Bacillus sp. Methanosarcina sp. Methanosaeta sp.* and *Clostridium sp.* (Yezza et al., 2005; Tommasi et al., 2008; Zhang et al., 2010). The main objective of the study was to investigate thermal and chemical-thermal pre-treatment impact on sludge for production of lipid by *Trichosporon oleaginosus*. The sludge characteristics were analyzed before and after pre-treatment. The obtained sludge after treatment was demonstrated on lipid accumulation in *Trichosporon oleaginosus*. Struvite formation was conducted on the pre-treated sludge to evaluate the increase of carbon to nitrogen ratio enhancement on lipid accumulation in the strain.

6.4 Materials and methods

6.4.1 Strain

Trichosporon oleaginosus (ATCC 20509) was used in this study. *Trichosporon oleaginosus* was subcultured and streaked on malt extract agar plates, incubated for 24 h at 30 ± 1 °C and then preserved at 4 °C for further study.

6.4.2 Medium

6.4.2.1 Basic medium

Basic medium is prepared by the mineral medium (pH 6.5) containing (g/L) 20 glucose, 0.189 NH_4Cl, 2.7 KH_2PO_4, 0.95 Na_2HPO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.1 yeast extract, 0.1 EDTA, 0.04 $CaCl_2 \cdot 2H_2O$, 0.0055 $FeSO_4 \cdot 7H_2O$, 0.0052 citric acid$\cdot H_2O$, 0.001 $ZnSO_4 \cdot 7H_2O$, and 0.00076 $MnSO_4 \cdot H_2O$. The medium was sterilized at 121 ° for 15 min prior to use.

6.4.2.2 Sludge

Secondary wastewater sludge (SWS) was collected from a municipal wastewater treatment plant, Communauté Urbain de Québec (CUQ) in Quebec, Canada. The sludge was first concentrated by allowing it to undergo gravity settling at 4 ºC for 24 h, and the supernatant and concentrated sludge with suspended solids (SS) concentration around 20 g/L was collected. To achieve high SS concentrations (up to 30 g/L), the sludge was centrifuged with SORVALL RC 5C Plus centrifuge at 5000 rpm for 15 min. The desired SS concentration (10 to 30 g/L) was obtained by mixing the concentrated solid and the supernatant collected from gravity settling and centrifugation.

6.4.2.3 Chemical-thermal pre-treated sludge medium

Acidic- thermal treatment: sludge with different SS concentration was added 2 M HCl to adjust the pH to 2. Thereafter, the sludge was subjected to 121 °C for 30 min. The resulting solution was used to analyze the characteristics of the sludge (100 mL), for struvite formation (2000 mL), and as medium for lipid production (2000 mL). The pH of sludge solution was adjusted to 6.5 and sterilized at 121 °C for 15 min prior to using as medium.

Alkaline-thermal treatment: sludge with different SS concentration was added 2 M NaOH to adjust the pH to 12. Thereafter, the sludge was subjected to 121 °C for 30 min. The resulting solution was used to analyze the characteristics of the sludge (100 mL), for struvite formation (2000 mL), and as medium for lipid production (2000 mL). The pH of sludge solution was adjusted to 6.5 and sterilized at 121 °C for 15 min prior to using as medium.

Thermal treatment: sludge with different SS concentration was subjected to 121 °C for 30 min. The resulting solution was used to analyze the characteristics of the sludge (100 mL), for struvite formation (2000 mL), and as medium for lipid production (2000 mL). The pH of the sludge solution was adjusted to 6.5 and sterilized at 121 °C for 15 min prior to using as medium.

6.4.2.4 Medium with struvite formation

The sludge obtained from pre-treatment was used in struvite formation. The pre-treated (acidic-, alkaline- thermal and thermal) sludge solution was centrifuged at 5000 rpm for 15 min after cooling down to room temperature. The supernatants were adjusted to pH 9 and then mixed with 0.1 M $MgSO_4 \cdot 7H_2O$ and 0.1 M Na_3PO_4. The mixtures were then passed through filter paper (pre-weighted) to remove the solids and then the filter paper was dried under at 105 °C till weight constant. The filtrates obtained from filtration were mixed with the solid obtained from centrifugation, and then were used as medium for lipid accumulation in *Trichosporon oleaginosus* after adjusting pH to 6.5 and sterilization at 121 °C for 15 min. The blanks were done by allowing the supernatant after adjusting to pH 9 to pass through pre-weighed filter paper, and dried at 105 °C till weight constant.

6.4.3 Inoculum and culture conditions

A loopful of *Trichosporon oleaginosus* from malt extract agar plate was used to inoculate a 500-mL Erlenmeyer flask containing 150 mL of sterilized YPD (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) medium. The flasks were incubated on a rotary shaker at 170 rpm and 28 °C for 24 h. The cells in exponential phase from these flasks were used to inoculate basic and sludge mediums with a volume ratio of 10% (v/v). The fermentation occurred at 170 rpm and 28 °C.

6.4.4 Analytical techniques

6.4.4.1 Sludge characterization

Total sludge solids (TS), volatile solids (VS), and SS were determined according to the standard methods (APHA, 2005). Ammonia nitrogen and total nitrogen in wastewater sludge were analysed with Quikchem FIA+ 8000 Series (Lachat, Zellweger Analytics). Total phosphorus was analysed by Inductively Coupled Plasma (VISTA AX CCD Simultaneous ICP-AES). Total carbon was measured with NA 1500-NCS analyser (Carlo Erba Instrument). Dissolved organic carbon (DOC), total dissolved nitrogen (TDN), dissolved ammonia nitrogen (DAN), total dissolved phosphorus (TDP), PO_4^{3-}, and metals was measured as following described. The samples were centrifuged at 5000 rpm for 15 min and decanted the supernatant. Then the residue was washed twice with distilled water and united the supernatants. One portion of the supernatant was filtrated with Whatman filter paper (Ø 4 cm) and then the liquid was used to measure DOC with Total Organic Carbon Analyzer (TOC-V_{CPH}, SHIMADZU) and TDN and DAN with Quikchem FIA+ 8000 Series. The other portion of the supernatant was filter with Whatman Cellulose Nitrate Membrane filters (pore size 0.45 µm, Ø 25 mm) and the liquid was used to measure TDP, PO_4^{3-} and metals with Inductively Coupled Plasma (VISTA AX CCD Simultaneous ICP-AES).

6.4.4.2 Struvite

Struvite formation amount was determined based on a mass balance. The dry weight (105 °C drying) difference of filter paper before and after filtration is considered as the weight of struvite formed.

6.4.4.3 Cell dry weight

Taking 5 mL of the fermentation broth from each sample and then centrifuged at 5000 rpm for 15 min. The pellets were then washed with distilled water twice, and dried till the weight constant to get cell dry weight.

6.4.4.4 Lipid content and composition

The standard chloroform and methanol extraction procedure with minor modification was employed to determine the lipid content in the raw sludge and sludge-biomass (Folch et al.,

1957; Vicente et al., 2009). Samples of raw sludge and sludge-biomass were dewatered by centrifugation at 5000 rpm for 15 min, washed 2 times with distilled water, and then dried by lyophilisation. 200 mg dry matters were mixed with 4 ml solvent mixture of chloroform and methanol (2:1 v/v), and then subjected to 60 °C for 4 h. The mixture was then centrifuged at 5000 rpm for 15 min and the supernatant solvent phase was withdrawn and transferred into a pre-weighed glass vial (W1). The extraction procedure was repeated two times. Afterwards, the vial containing the total volume of the supernatant collected from each extraction was put under evaporation and then weighed (W2). The lipid amount was calculated by the difference of W1 and W2. The lipid content in the biomass is (W2-W1)/200 mg ×100%. The obtained lipid was stored in dark at 4 °C for further study.

6.4.4.5 Lipid profile

Transesterification was carried out through acid catalysis. Lipid obtained from solvent extraction in vials was first dissolved in hexane (20 ml hexane for per gram lipid), and then 1% sulfuric acid in methanol (40 ml acidic methanol for per gram lipid) was added. The mixture was subjected to 50 °C for 12 h. After reaction, 5% NaCl was added with the amount of 100 ml per gram lipid, and then biodiesel (fatty acid methyl esters, FAMEs) was extracted by two times washing with hexane (100 ml per gram lipid), then the hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% sodium bicarbonate (20 mL per gram lipid), and the top layer was then dried over 60 °C oven (Halim et al., 2011), and then redissolved in hexane for analysis.

The FAMEs in hexane were analyzed using a Gas Chromatography Linked to Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500). The dimensions of the column used were 30 m × 0.25 mm, with a phase thickness of 0.25 µm. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1.3-dichlorobenzene was used as internal standard for preparing calibration curve.

The experiment results are the average of the triplicates.

6.5 Results and discussion

6.5.1 Chemical-thermal pre-treatment on sludge

Chemical-thermal treatment on sludge has been used to enhance sludge dewaterability and biodegradability since sixties. It has been reported that the treatment on biological sludge such as secondary sludge was more efficient than that containing tightly bonded network such as primary and mixed sludge (Eskicioglu et al., 2008). It is found that treatment with temperature of 150 °C to 170 °C gave a better digestion performance and dewaterability, respectively (Camacho et al., 2008; Carrère et al., 2010). High temperature above 170 °C would lead to a reaction called Maillard reaction in which forms carbohydrate and amino acid to melanoidins which are hard or impossible to be degraded (Dwyer et al., 2008). The treatment time is less important unless the temperature is lower than 100 °C. Generally, 30 min is employed (Carrère et al., 2010). Alkaline treatment was reported to be effective to enhance sludge biodegradability, and NaOH provided the highest efficacy among KOH, $Mg(OH)_2$, and $Ca(OH)_2$ (Kim et al., 2003). The combination of thermal and alkaline treatment could lower the required temperature from 170 °C to around 120 to 130 °C to achieve a similar result (Tanaka et al., 1997; Kim et al., 2003; Park et al., 2005).

In this study, secondary sludge with pH 12 adjusted with NaOH was treated at 121 °C for 30 min, and used for lipid production in *Trichosporon oleaginosus*. Secondary sludge with pH of 2 and 6.44 (natural pH of the sludge) was also treated in similar way to compare with alkaline treatment. Characteristics of the sludge used are presented in Table 6.1. The SS, DOC, TDN, DAN, TDP, metals and pH of the different SS concentration sludge before and after treatment were measured and results are shown in Tables 6.2 and 6.3.

Pre-treatment is aimed to obtain a high solubilisation on sludge, and thus could be easily used by microorganisms. SS was decreased after acidic (pH 2)/alkaline (pH 12) thermal or thermal (pH nature) treatment. It is due to the hydrolysis of the non-soluble complex compounds to soluble compounds. This can be observed in the difference of dissolved matters concentration including DOC, TDN, DAN, and TDP before and after treatment (Table 6.2). The pre-treatment resulted in the disintegration and extraction of intracellular (within the microbial cell) and

extracellular (within the polymeric network) protein, carbons, and phosphorous from the sludge matrix. Protein under aggressive acid or alkaline condition would be further decomposed to ammonia nitrogen, and hence led to the increase on concentration of ammonia nitrogen and total dissolved nitrogen. However, the decrease of ammonia nitrogen concentration occurred in alkaline treatment (Table 6.2). It is predicated that ammonium (NH_4^+) was converted into ammonia gas (NH_3) as pH of the solution is higher than 9.25 (Sharma et al., 1998). NH_3 finally escaped from the solution, and resulted in the ammonia nitrogen concentration reduction. PO_4^{3-} slightly increased by around 10 mg/L after acidic-thermal treatment, and decreases were observed on PO_4^{3-} concentration after thermal or alkaline-thermal treatment. Normally, PO_4^{3-} is associated with carbon (organic compounds) or metals (precipitates) in the sludge. After treatment, organic compounds were broken down, and some of PO_4^{3-} would be released; however, when the pH is greater than 7, PO_4^{3-} would be precipitated by the metals. Thermal or alkaline-thermal treatment increased sludge pH which led to the formation of PO_4^{3-} precipitates and resulted in PO_4^{3-} concentration reduction (Figure 6.1).

Very slight effect was observed on metals concentrations after pre-treatment; and no clear trends (increase or decrease) for each metal before and after pre-treatment were observed; however, the metals concentrations are higher with pH 2 treatment than with nature pH and pH 12 (Table 6.3). With low pH 2, pH was slightly increased to 2.6 from 2 after treatment, while it is still in acidic condition. With pH natural, pH was increased from 6.44 to above 7 after treatment except with SS concentration of 30 g/L (pH 6.84). With pH 12, pH was decreased from 12 to around 10 to 8 after treatment. It indicates that it is in alkaline condition for sludge treated at pH nature and 12, which results in the precipitation of the metals, and thus a lower concentration of metals were obtained in sludge treated at pH nature and 12.

Comparing acidic and alkaline pre-treatment, alkaline treatment provided higher soluble matters DOC, TDN, DAN, and TDP. It has been reported that alkaline pre-treatment on sludge could greatly increase biogas production in sludge digestion (Park et al., 2005; Dwyer et al., 2008; Carvajal et al., 2013). It suggested that alkaline treatment would be more suitable for increasing sludge biodegradability.

6.5.2 Struvite formation

Numerous studies have been conducted on improving lipid accumulation in microorganism with increasing on C/N ratio (Huang et al., 2011; Ruan et al., 2012; Gao et al., 2013). As stated above, the chemical-thermal treatment has increased DOC as well as DAN and TDP. In order to increase C/N ratio, the removal of nitrogen is necessary. Struvite consists of equal molar concentrations magnesium, ammonium and phosphorus ($MgNH_4PO_4 \cdot 6H_2O$). It forms according to the general reaction (Equation 6.1):

Equation 6. 1 $Mg^{2+} + NH_4^- + PO_4^{3-} + 6H_2O \rightarrow MgNH_4PO_4 \cdot 6H_2O$

The formation of struvite would reduce ammonia nitrogen concentration and consequently reduce total nitrogen concentration, and hence increase C/N ratio. In addition, struvite has shown excellent quality as fertilizer due to its low solubility which provides long effect in the field. According to the result of ammonia concentration in the treated sludge, the $MgSO_4 \cdot 7H_2O$ was added with a 1: 1.2 molar ratio of NH_4^+ to Mg^{2+}. The theoretical struvite formation was calculated based on the reduction of ammonia nitrogen. According to Equation 6.1, every 18 g of ammonia will produce 137 g of struvite (Considering drying at 105 ° has removed $6H_2O$ in $MgNH_4PO_4 \cdot 6H_2O$); therefore, if ammonia reduction is A, the struvite formation amount will be 137A/18. The C/N ratio of sludge before and after struvite formation is given in Table 6.4.

For enhancing struvite formation to achieve complete ammonia nitrogen removal, excess Mg^{2+} was added and pH of the solution was adjusted to 9, which was reported to be the optimal pH of the reaction (Mamais et al., 1994). After struvite formation, ammonia nitrogen was still left in the experiment, it is due to the fact that the formation is affected by several parameters including PO_4^{3-} concentration, other iron (such as Ca^{2+}) presence, and $Mg(OH)_2$ formation (pH>8). After pre-treatment, PO_4^{3-} molar concentration is lower than that of NH_4^+ (Table 6.2). Study has found that the optimal struvite formation required the molar ratio of NH_4^{2+}: Mg^{2+}: PO_4^{3-} at 1:1.2:1.2, indicating that PO_4^{3-} was excess. In addition, the effect of irons such as Na^+, K^+, and Ca^{2+} could also inhibit the formation of struvite (Hassan et al., 1996; Emsley, 2000; Kim et

al., 2007). The generation of $Mg(OH)_2$, which consumes Mg^{2+} resulting in the reduction of Mg^{2+} concentration, would also be a reason of the low struvite formation.

The results showed that the practical struvite formation amount is slightly lower than the theoretical formation amount which is considered as the loss of NH_4-N during handling. Slight reduction of DOC/TDN and TC/TN ratios was observed before and after struvite formation. In struvite formation, ammonia nitrogen was precipitated and eliminated from the solution; however, its concentration is rather low comparing with the total dissolved nitrogen (10% w/w) and total nitrogen (1% w/w). Therefore, the reduction of ammonia nitrogen has very small impact on the concentration of total dissolved nitrogen and total nitrogen. The dissolved organic carbon and total carbon remained almost the same, and thus led to a similar DOC/TDN and TC/TN before and after struvite formation.

6.5.3 Lipid accumulation in *Trichosporon oleaginosus* with pre-treated sludge

The lipid content in the sludge was given in Figure 6.2. With alkaline-thermal pre-treatment, the lipid accumulation is slightly higher than with acidic-thermal and thermal pre-treatment. As stated that C/N ratio normally has great impact on lipid accumulation, but it is seen that the C/N ratio was similar with different pre-treatment (Table 6.4), which would lead to the similar lipid accumulation in the strain. Unlike as expected that struvite formation would enhance lipid accumulation it didn't impact on the lipid accumulation due to the slight change of C/N ratio with or without the formation (Table 6.4). With the same pre-treatment, lipid accumulation in the pre-treated sludge with struvite formation is less than that without struvite formation. In order to completely precipitate NH_4^+, excess Mg^{2+} was added (1:1.2 molar ratio of NH_4^+ to Mg^{2+}) (Mamais et al., 1994; Kim et al., 2007). The trace amount of minerals (several ppm) is essential in the microorganism lives; however, they would become inhibition when the concentration of Mg^{2+} is above a certain level. Based on calculation, there would be around 120 mg/L Mg^{2+} left in the solution which was further present in medium. This concentration is considered so high that Mg^{2+} inhibits the lipid accumulation in the strain. Due to that the formation amount of struvite isn't significant and the formation didn't show any improvement on lipid accumulation, it wouldn't be practical to form struvite in the pre-treated sludge and then used as medium for lipid accumulation in *Trichosporon oleaginosus*.

Comparing with acidic-thermal and thermal treatment (around 48 h), alkaline-thermal treatment (around 42 h) reduced the time to obtain the maximum lipid accumulation in *Trichosporon oleaginosus*. This is would be due to the alkaline treatment converted more big molecules to small ones which is readily up-taken by the strain than other two pre-treatment, and results in rapid lipid accumulation. Fermentation time is directly associate the production cost as it determines the utilities cost as well as labor cost. Short fermentation time reduced the production cost, and additionally decreased the contamination risk. Hence, alkaline-thermal treatment on sludge would be a promising method for lipid accumulation (Table 6.5); however, it still should be investigated if it would be cost-efficient method as it involved in the addition of NaOH and energy (for heating).

6.5.4 Lipid profile of *Trichosporon oleaginosus* with pre-treated sludge

Different pre-treated sludges were used as media for lipid production by *Trichosporon oleaginosus*, and the produced lipid was further transferred to biodiesel, fatty acid methyl esters (FAMEs). The fatty acid profile showed that the majority of the compounds are C14, C16 and C18 with some amount of C15 and C 17 (Figure 6.3) by *Trichosporon oleaginosus*.

The saturation degree (the sum of Cn:0 fraction) of the oil obtained from these sludge mediums are around 40%, which is rather higher than soybean oil (20%). The results are similar as reported (Seo et al., 2013). It indicates that the biodiesel from sludge derived oil has high oxidation stability and density, but poor cold flow property (problem in working on low temperature).

Treated with struvite formation on the sludge medium induced the C18:1 fraction increase and C18:3 fraction decrease in the case of pH nature. It consequently increased the mono-unsaturation fraction and reduced the poly-unsaturation fraction, while the saturation degree was still similar which indicates that using the treated sludge with or without further struvite formation as medium for lipid production wouldn't impact on biodiesel properties much.

6.6 Conclusions

Acidic-thermal, thermal, and alkaline-thermal treatment on sludge increased the concentration of dissolved organic carbon, dissolved nitrogen, and dissolved phosphorus which are essential in microorganism cultivation. Using alkaline-thermal pre-treated sludge as medium lowered the fermentation time from 48 (thermal or acidic-thermal pre-treaded) to around 42 h to reach the maximum lipid accumulation. It would reduce the cost counting for utilities and labor cost, and contamination risk. Using struvite formation to reduce nitrogen (ammonia nitrogen) amount and hence to increase C/N ratio was not an efficient way as ammonia nitrogen contributes to a very small weight of total nitrogen. Biodiesel produced by *Trichosporon oleaginosus* cultivated with (acidic-thermal, thermal, and alkaline-thermal) pre-treated sludge mainly contains C14, C16, and C18, which are also rich in currently commercial biodiesel produced from soybean seed, canola, and sunflower seed oils. It reveals that lipid produced from sludge is a promising biodiesel production source.

6.7 Acknowledgements

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6.8 References

Angerbauer, C., Siebenhofer, M., Mittelbach, M., Guebitz, G. M., 2008. Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production. Bioresour. Technol. 99, 3051-3056.

APHA, 2005. Standard methods for examination of waters and wastewaters. Washington, DC: American Public Health Association Publications. ISBN 0875531547.

Brar, S. K., Verma, M., Tyagi, R. D., Valero, J. R., Surampalli, R. Y., Banerji, S. K., 2004. Comparative rheology and particle size analysis of various types of Bacillus thuringiensis fermented sludges. Journal of Residue Science and Technology 1, 231-237.

Brennan, L., Owende, P., 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews 14, 557-577.

Camacho, P., Ewert, W., Kopp, J., Panter, K., Perez-Elvira, S. I., Piat, E., 2008. Combined experiences of thermal hydrolysis and anaerobic digestion latest thinking on thermal hydrolysis of secondary sludge only for optimum dewatering and digestion. Proceedings of the Water Environment Federation 2008, 1964-1978.

Cao, Y., Yao, J., Li, J., Chen, X., Jinyong, W., 2010. Breeding of high lipid producing strain of Geotrichum robustum by ion beam implantation. Electron. J. Biotechnol. 13.

Carrère, H., Dumas, C., Battimelli, A., Batstone, D. J., Delgenès, J. P., Steyer, J. P., Ferrer, I., 2010. Pretreatment methods to improve sludge anaerobic degradability: A review. J. Hazard. Mater. 183, 1-15.

Carvajal, A., Peña, M., Pérez-Elvira, S., 2013. Autohydrolysis pretreatment of secondary sludge for anaerobic digestion. Biochem. Eng. J. in press.

Cheirsilp, B., Suwannarat, W., Niyomdecha, R., 2011. Mixed culture of oleaginous yeast Rhodotorula glutinis and microalga Chlorella vulgaris for lipid production from industrial wastes and its use as biodiesel feedstock. New Biotechnol. 28, 362-368.

Dwyer, J., Starrenburg, D., Tait, S., Barr, K., Batstone, D. J., Lant, P., 2008. Decreasing activated sludge thermal hydrolysis temperature reduces product colour, without decreasing degradability. Water Res. 42, 4699-4709.

Emsley, J. (Ed) (2000). *The shocking history of phosphorus: a biography of the devil's element.* London: Macmillan Publishers Ltd.

Eskicioglu, C., Kennedy, K. J., Droste, R. L., 2008. Initial examination of microwave pretreatment on primary, secondary and mixed sludges before and after anaerobic digestion. Water Sci. Technol. 57, 311-317.

Folch, J., Lees, M., G.H., S. S., 1957. A simple method for the isolation and purification of total lipides from animal tissues. Journal of Biological Chemistry 226, 497-509.

Gao, D., Zeng, J., Zheng, Y., Yu, X., Chen, S., 2013. Microbial lipid production from xylose by *Mortierella isabellina*. Bioresour. Technol. 133, 315-321.

Gonzalez-Garcia, Y., Hernandez, R., Zhang, G., Escalante, F. M. E., Holmes, W., French, W. T., 2013. Lipids accumulation in *Rhodotorula glutinis* and *Cryptococcus curvatus* growing on distillery wastewater as culture medium. Environmental Progress & Sustainable Energy 32, 69-74.

Halim, R., Gladman, B., Danquah, M. K., Webley, P. A., 2011. Oil extraction from microalgae for biodiesel production. Bioresource Technology 102, 178-185.

Hassan, M., Blanc, P. J., Granger, L.-M., Pareilleux, A., Goma, G., 1996. Influence of nitrogen and iron limitations on lipid production by *Cryptococcus curvatus* grown in batch and fed-batch culture. Process Biochem. 31, 355-361.

Hoekman, S. K., Broch, A., Robbins, C., Ceniceros, E., Natarajan, M., 2012. Review of biodiesel composition, properties, and specifications. Renewable and Sustainable Energy Reviews 16, 143-169.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal 54, 621-639.

Huang, X., Wang, Y., Liu, W., Bao, J., 2011. Biological removal of inhibitors leads to the improved lipid production in the lipid fermentation of corn stover hydrolysate by *Trichosporon cutaneum*. Bioresour. Technol. 102, 9705-9709.

Kim, D., Ryu, H.-D., Kim, M.-S., Kim, J., Lee, S.-I., 2007. Enhancing struvite precipitation potential for ammonia nitrogen removal in municipal landfill leachate. J. Hazard. Mater. 146, 81-85.

Kim, J., Park, C., Kim, T.-H., Lee, M., Kim, S., Kim, S.-W., Lee, J., 2003. Effects of various pretreatments for enhanced anaerobic digestion with waste activated sludge. J. Biosci. Bioeng. 95, 271-275.

Lian, J., Garcia-Perez, M., Coates, R., Wu, H., Chen, S., 2012. Yeast fermentation of carboxylic acids obtained from pyrolytic aqueous phases for lipid production. Bioresource Technology 118, 177-186.

Mamais, D., Pitt, P., Yao, W. C., Loicano, J., , D., 1994. Determination of ferric chloride dose to control struvite precipitation in anaerobic digesters. Water Environ. Res. 66, 912-918.

Marker, T., Petri, J., Kalnes, T., McCall, M., Mackowiak, D., Jerosky, B., 2005. Opportunities for biorenewables in oil refineries. Report no. DE-FG36-05GO15085.

Meesters, P. A. E. P., Huijberts, G. N. M., Eggink, G., 1996. High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. Appl Biochem Biotechnol 45, 575-579.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., Xian, M., 2009. Biodiesel production from oleaginous microorganisms. Renewable Energy 34, 1–5.

Park, C., Lee, C., Kim, S., Chen, Y., Chase, H. A., 2005. Upgrading of anaerobic digestion by incorporating two different hydrolysis processes. J. Biosci. Bioeng. 100, 164-167.

Pereyra-Irujo, G. A., Izquierdo, N. G., Covi, M., Nolasco, S. M., Quiroz, F., Aguirrezábal, L. A. N., 2009. Variability in sunflower oil quality for biodiesel production: A simulation study. Biomass Bioenergy 33, 459-468.

Ruan, Z., Zanotti, M., Wang, X., Ducey, C., Liu, Y., 2012. Evaluation of lipid accumulation from lignocellulosic sugars by *Mortierella isabellina* for biodiesel production. Bioresour. Technol. 110, 198-205.

Seo, Y. h., Lee, I. g., Han, J. i., 2013. Cultivation and lipid production of yeast *Cryptococcus curvatus* using pretreated waste active sludge supernatant. Bioresource Technology 135, 304-308.

Sharma, V. K., Bloom, J. T., Joshi, V. N., 1998. Oxidation of ammonia by ferrate(vi). Journal of Environmental Science and Health, Part A 33, 635-650.

Su, Y., Mennerich, A., Urban, B., 2012. Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios. Bioresource Technology 105, 67-73.

Tanaka, S., Kobayashi, T., Kamiyama, K. I., Bildan, L. N. S., 1997. Effects of thermochemical pretreatment on the anaerobic digestion of waste activated sludge. Water Sci. Technol. 35, 209-215.

Tang, T. (2011).Intracellular lipids measurement and production with yeast *Cryptococcus curvatus* from sweet sorghum bagasse. In *Environmental Engineering*, Vol. Master, 74 Carbondale Southern Illinois University

Tommasi, T., Sassi, G., Ruggeri, B., 2008. Acid pre-treatment of sewage anaerobic sludge to increase hydrogen producing bacteria HPB: effectiveness and reproducibility. Water Sci. Technol. 58, 1623-1628.

Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., Sádaba, I., Ruiz-Vázquez, R. M., Torres-Martínez, S., Garre, V., 2009. Biodiesel production from biomass of an oleaginous fungus. Biochemical Engineering Journal 48, 22-27.

Wu, S., Hu, C., Zhao, X., Zhao, Z., 2010. Production of lipid from N-acetylglucosamine by *Cryptococcus curvatus*. Eur. J. Lipid Sci. Technol. 112, 727-733.

Xu, H., Miao, X., Wu, Q., 2006. High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. J Biotechnol 126, 499–507.

Yezza, A., Tyagi, R., Valéro, J., Surampalli, R., 2005. Wastewater sludge pre-treatment for enhancing entomotoxicity produced by *Bacillus thuringiensis var. kurstaki*. World J. Microbiol. Biotechnol. 21, 1165-1174.

Zhang, D., Chen, Y., Zhao, Y., Zhu, X., 2010. New sludge pretreatment method to improve methane production in waste activated sludge digestion. Environ. Sci. Technol. 44, 4802-4808.

Zhang, G., French, W. T., Hernandez, R. e., Hall, J., Sparks, D., Holmes, W. E., 2011. Microbial lipid production as biodiesel feedstock from N-acetylglucosamine by oleaginous microorganisms. Journal of Chemical Technology & Biotechnology 86, 642-650.

Table 6.1 Characteristics of secondary sludge from CUQ

Characteristics	Concentration
Total solids (TS)	26.14 ± 0.39 g/L
Total suspended solids (TSS)	20.44 ± 0.54 g/L
Volatile solids (VS)	19.06 ± 0.77 g/L
Total carbon (TC)	41.26 ± 1.13 g/100 g TS
Dissolved organic carbon (DOC)	1.25 ± 0.11 g/L
Total nitrogen (TN)	5.77 ± 0.32 g/100 g TS
Total dissolved nitrogen (TDN)	0.20 ± 0.03 g/L
Dissolved ammonia nitrogen (DAN)	92.90 ± 3.46 mg/L
Total phosphorous (TP)	1.33 ± 0.17 g/100 g TS
Total dissolved phosphorous (TDP)	88.20 ± 2.19 mg/L
Cd	<0.04 mg/L
Cr	< 0.1 mg/L
Cu	2.0 ± 0.1mg/L
Fe	81.5 ± 2.3 mg/L
Mg	24.2 ± 1.0 mg/L
Mn	1.5 ± 0.1 mg/L
Ni	0.2 ± 0.0 mg/L
Pb	< 0.2 mg/L
Zn	1.7 ± 0.1 mg/L
pH	6.44 ± 0.00

Table 6.2 Characteristic of different SS concentration sludge before and after pre-treatment

Item	Treatment		SS=10 g/L	SS=15 g/L	SS=20 g/L	SS=25 g/L	SS=30 g/L
	Before/After	Name					
SS (g/L)	After	aci.-ther	7.47 ± 0.59	11.50 ± 0.38	15.53 ± 0.77	19.82 ± 0.68	22.33 ± 1.33
		ther.	6.67 ± 0.09	11.91 ± 0.29	17.78 ± 0.61	20.13 ± 0.89	23.13 ± 0.94
		alk.-ther.	6.07 ± 0.14	10.04 ± 0.48	12.57 ± 0.32	16.18 ± 0.33	18.87 ± 0.71
DOC (g/L)	Before		0.83 ± 0.06	1.04 ± 0.23	1.25 ± 0.22	1.48 ± 0.12	1.57 ± 0.10
	After	aci.-ther	1.41 ± 0.06	2.16 ± 0.06	2.31 ± 0.07	2.75 ± 0.11	3.09 ± 0.09
		ther.	1.23 ± 0.03	1.89 ± 0.01	2.03 ± 0.05	2.21 ± 0.05	2.65 ± 0.04
		alk.-ther.	2.02 ± 0.12	3.39 ± 0.10	4.53 ± 0.22	5.52 ± 0.18	6.04 ± 0.24
TDN (g/L)	Before		0.07 ± 0.01	0.13 ± 0.01	0.20 ± 0.01	0.30 ± 0.03	0.45 ± 0.02
	After	aci.-ther	0.66 ± 0.04	0.76 ± 0.03	0.91 ± 0.01	1.20 ± 0.08	1.38 ± 0.04
		ther.	0.40 ± 0.03	0.56 ± 0.01	0.70 ± 0.05	1.04 ± 0.03	1.22 ± 0.08
		alk.-ther.	0.68 ± 0.02	0.74 ± 0.03	0.98 ± 0.10	1.46 ± 0.09	1.84 ± 0.02
NH_4-N (mg/L)	Before		74.37 ± 3.42	82.73 ± 2.79	93.42 ± 1.38	96.14 ± 2.26	101.17 ± 3.94
	After	aci.-ther	105.2 ± 4.29	116.23 ± 1.17	132.40 ± 2.43	137.43 ± 2.09	156.71 ± 1.38
		ther.	97.60 ± 2.02	106.29 ± 3.11	113.14 ± 1.56	154.2 ± 1.47	167.67 ± 5.23
		alk.-ther.	75.39 ± 1.89	34.22 ± 0.49	37.40 ± 2.17	34.12 ± 0.52	31.93 ± 1.88
TDP (mg/L)	Before		60.19 ± 1.64	73.14 ± 2.33	88.21 ± 2.12	92.19 ± 1.49	107.14 ± 3.06
	After	aci.-ther	82.19 ± 1.84	109.85 ± 2.07	133.20 ± 2.19	140.07 ± 2.66	149.27 ± 3.08
		ther.	70.07 ± 1.43	91.46 ± 2.08	125.22 ± 1.38	137.88 ± 5.67	142.04 ± 3.19
		alk.-ther.	63.17 ± 2.22	74.93 ± 1.24	89.44 ± 2.68	96.74 ± 2.53	110.89 ± 1.11
PO_4^{3-} (mg/L)	Before		22.24 ± 1.08	26.47 ± 0.91	31.59 ± 1.12	38.23 ± 0.77	47.19 ± 0.95
	After	aci.-ther	28.67 ± 1.19	34.67 ± 1.58	40.59 ± 1.74	51.67 ± 1.69	59.69 ± 2.07
		ther.	19.43 ± 0.57	21.42 ± 0.18	25.14 ± 3.28	30.03 ± 2.26	32.24 ± 1.77
		alk. -ther.	16.46 ± 0.47	16.23 ± 0.82	17.55 ± 0.33	19.17 ± 0.12	19.34 ± 0.46

Before=before treatment; After=after treatment; SS= suspended solids; DOC=dissolved organic carbon; TDN=total dissolved nitrogen; TDP=total dissolved phosphorus; aci.-ther=acidic-thermal; ther.=thermal; alk.-ther.=alkaline-thermal.

Table 6.3 Metals concentrations in different SS concentration sludge before and after treatment

Metals	Treatment		SS=10 g/L	SS=15 g/L	SS=20 g/L	SS=25 g/L	SS=30 g/L
	Before/After	Name					
Cd (mg/L)	Before		<0.04	<0.04	<0.04	<0.04	<0.04
	After	aci.-ther	<0.04	<0.04	<0.04	<0.04	<0.04
		ther.	<0.04	<0.04	<0.04	<0.04	<0.04
		alk.-ther.	<0.04	<0.04	<0.04	<0.04	<0.04
Cr (mg/L)	Before		<0.1	<0.1	<0.1	<0.1	<0.1
	After	aci.-ther	0.3	0.3	0.4	0.5	0.6
		ther.	<0.1	<0.1	<0.1	<0.1	<0.1
		alk.-ther.	<0.1	<0.1	<0.1	<0.1	<0.1
Cu (mg/L)	Before		2.0	2.0	2.0	3.0	3.2
	After	aci.-ther	3.0	3.3	4.1	4.5	5.5
		ther.	1.7	2.2	2.5	3.0	3.7
		alk.-ther.	1.7	1.8	2.0	3.1	3.2
Fe (mg/L)	Before		71.4 ± 1.6	78.2 ± 3.2	81.5 ± 0.8	87.6 ± 1.9	91.3 ± 2.5
	After	aci.-ther	89.3 ± 2.1	97.1 ± 0.8	118.4 ± 2.5	129.4 ± 1.7	135.3 ± 3.5
		ther.	50.6 ± 2.0	50.3 ± 3.1	54.1 ± 1.5	54.2 ± 1.5	56.7 ± 5.2
		alk.-ther.	43.2 ± 1.9	44.6 ± 1.0	49.6 ± 1.3	51.5 ± 1.1	51.6 ± 0.7
Mg (mg/L)	Before		17.6 ± 0.3	18.4 ± 1.2	25.2 ± 0.7	32.1 ± 1.4	33.4 ± 1.1
	After	aci.-ther	18.9 ± 1.2	25.3 ± 1.0	31.2 ± 0.8	35.4 ± 1.6	40.1 ± 1.9
		ther.	22.0 ± 1.1	33.1 ± 1.6	35.1 ± 1.2	35.8 ± 0.7	37.2 ± 1.0
		alk.-ther.	15.2 ± 0.8	15.7 ± 0.4	18.3 ± 0.5	21.1 ± 1.0	31.7 ± 0.9
Mn (mg/L)	Before		1.0	1.1	1.5	1.5	1.6
	After	aci.-ther	2.3	3.1	3.5	4.0	4.7
		ther.	0.5	0.5	0.5	1.1	1.3
		alk.-ther.	0.4	0.5	0.5	0.5	0.8
Ni (mg/L)	Before		0.2	0.2	0.2	0.2	0.2
	After	aci.-ther	0.16	0.2	0.2	0.2	0.2
		ther.	0.1	0.1	0.1	0.1	0.1
		alk.-ther.	0.1	0.1	0.1	0.1	0.1
Pb (mg/L)	Before		<0.2	<0.2	<0.2	<0.2	<0.2
	After	aci.-ther	<0.2	<0.2	<0.2	<0.2	<0.2
		ther.	<0.2	<0.2	<0.2	<0.2	<0.2
		alk.-ther.	<0.2	<0.2	<0.2	<0.2	<0.2
Zn (mg/L)	Before		1.3	1.4	1.7	1.9	2.8
	After	aci.-ther	3.5	5.5	5.5	5.8	6.4
		ther.	0.5	0.5	0.7	0.8	1.1
		alk.-ther.	0.5	0.5	0.5	0.5	0.9

Before=before treatment; After=after treatment; SS= suspended solids; aci.-ther=acidic-thermal; ther.=thermal; alk.-ther.=alkaline-thermal.

Table 6.4 **Sludge characteristics variation before and after struvite formation**

pH	SS (g/L)	Theoretical struvite formation (g)	Practical struvite formation (g)	DOC/TDN[a] ratio before struvite formation	DOC/TDN ratio after struvite formation	TC/TN[b] ratio before struvite formation	TC/TN ratio after struvite formation
2	10	0.32 ± 0.01	0.29 ± 0.01	2.13	2.76	13.19	13.78
	15	0.35 ± 0.01	0.34 ± 0.02	2.44	2.99	15.13	15.89
	20	0.35 ± 0.01	0.35 ± 0.01	2.77	3.16	17.75	18.15
	25	0.45 ± 0.01	0.44 ± 0.01	2.92	3.62	18.23	18.76
	30	0.61 ± 0.01	0.58 ± 0.02	2.95	4.14	18.65	18.99
6.44 (nature pH)	10	0.31 ± 0.01	0.30 ± 0.02	2.64	2.83	19.28	19.33
	15	0.34 ± 0.01	0.34 ± 0.01	2.81	3.01	20.54	20.67
	20	0.36 ± 0.02	0.34 ± 0.01	2.92	3.24	21.12	22.23
	25	0.39 ± 0.01	0.39 ± 0.02	3.04	3.33	21.86	21.91
	30	0.43 ± 0.02	0.42 ± 0.01	3.08	3.56	22.19	22.26
12	10	0.27 ± 0.01	0.27 ± 0.00	4.12	4.45	14.64	14.76
	15	0.17 ± 0.01	0.16 ± 0.01	4.38	4.55	14.89	14.95
	20	0.17 ± 0.01	0.14 ± 0.01	4.63	4.74	15.14	15.33
	25	0.15 ± 0.01	0.14 ± 0.01	4.69	4.83	15.38	15.46
	30	0.15 ± 0.01	0.15 ± 0.01	4.81	4.96	15.62	15.67

[a] *Dissolved organic carbon to total dissolved organic carbon ratio;*

[b] *Total carbon to total nitrogen ratio.*

Table 6.5 Comparison of lipid accumulation in *Trichosporon oleaginosus* with different medium

Medium	Fermentation time (h)	Biomass concentration (g/L)	Lipid content (% w/w biomass)	References
Glycerol (32 g/L)	48	10.7	46.1	(Meesters et al., 1996)
Chitin (70 g/L)	120	18.1	54.2	(Wu et al., 2010)
N-acetylglucosamine (50 g/L)	72	19.8	17.3	(Zhang et al., 2011)
Sweet sorghum hydrolysates (30 g/L)	48	10.8	73.3	(Tang, 2011)
Pyrolytic aqueous phases of wood (30 g/L)	120	6.9	32.6	(Lian et al., 2012)
Distillery wastewater (COD = 30 g/L)	144	7.0	25.7	(Gonzalez-Garcia et al., 2013)
Alkaline pre-treated activated wastewater sludge (1 g/L)	24	9.8	23.0	(Seo et al., 2013)
Acidic-thermal pre-treated sludge (SS 30 g/L)	48	19.6	37.1	This study
Thermal pre-treated sludge (SS 30 g/L)	48	20.4	35.2	This study
Alkaline-thermal pre-treated sludge (SS 30 g/L)	42	20.1	38.8	This study

Table 6.6 Fatty acid composition in biodiesel produced from different oils

Oils	C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	References
Sunflower	0.1	0	6.4	0.1	0.1	3.6	21.7	66.3	1.5	(Hu et al., 2008; Pereyra-Irujo et al., 2009)
Soybean	0.1	0	11.6	0.2	0.1	3.9	23.7	53.8	5.9	(Xu et al., 2006)
Palm	1.1	0	42.5	0.2	0.1	4.2	41.3	9.5	0.3	(Marker et al., 2005)
Canola	0	0	4.2	0.3	0.1	2.0	60.4	21.2	9.6	(Pereyra-Irujo et al., 2009)
Rapeseed	1.1	0	4.2	0.1	0	1.6	59.5	21.5	8.4	(Brennan and Owende, 2010; Hoekman et al., 2012)
T.O. in pH 2 WOSF	3.1	1.8	14.6	11.9	1.4	18.6	24.4	18.8	4.2	This study
T.O. in pH 2 WSF	2.7	1.7	15.1	9.8	1.5	18.5	25.5	13.0	5.3	This study
T.O. in pH nature WOSF	2.6	1.8	12.6	8.1	1.6	18.2	13.9	11.8	21.6	This study
T.O. in pH nature WSF	2.7	1.9	15.5	9.1	1.6	18.6	25.9	13.6	4.4	This study
T.O. in pH 12 WOSF	1.8	1.4	13.1	5.7	1.5	20.0	15.4	11.7	21.9	This study
T.O. in pH 12 WSF	2.5	1.7	13.4	7.2	1.5	16.3	14.0	12.9	23.3	This study

T.O.= Trichosporon oleaginosus; WSF=with struvite formation; WOSF=without struvite formation.

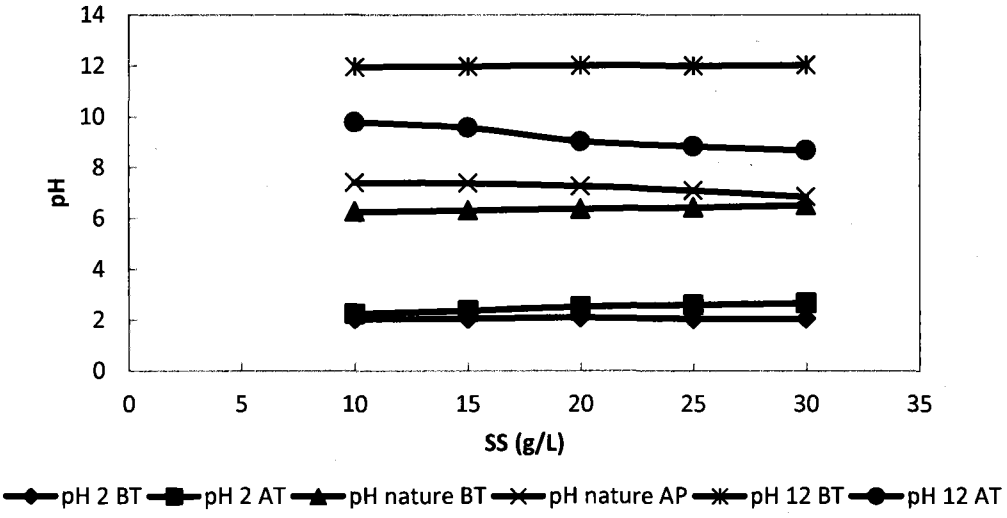
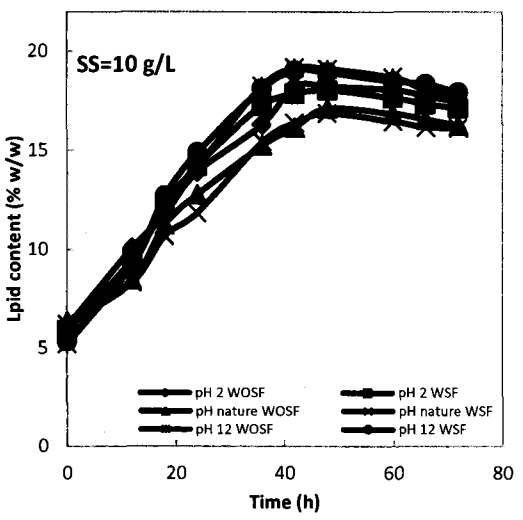
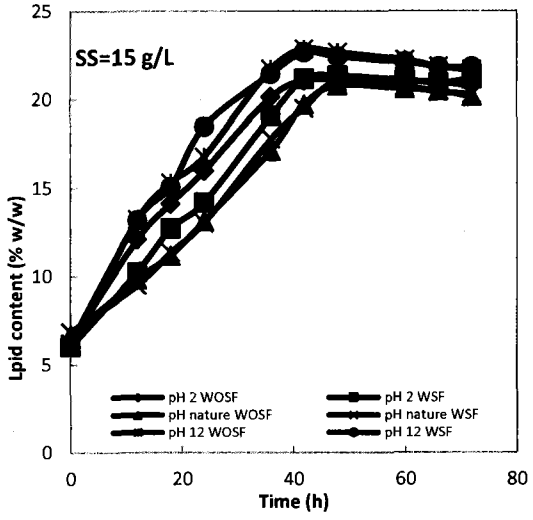
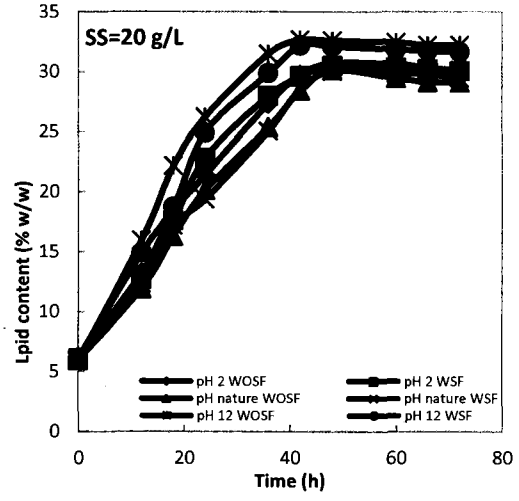
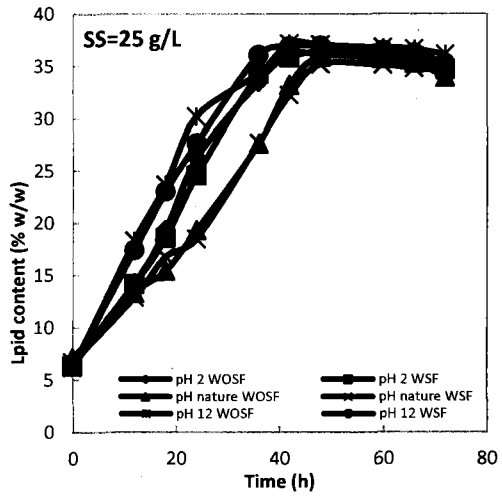


Figure 6.1 **The sludge pH variation before and after pre-treatment at different SS concentration, the standard division is less than 0.3% (BT=before treatment; AT=after treatment)**


SS=10 g/L
Lpid content (% w/w)
Time (h)
pH 2 WOSF
pH nature WOSF
pH 12 WOSF
pH 2 WSF
pH nature WSF
pH 12 WSF


SS=15 g/L
Lpid content (% w/w)
Time (h)
pH 2 WOSF
pH nature WOSF
pH 12 WOSF
pH 2 WSF
pH nature WSF
pH 12 WSF


SS=20 g/L
Lpid content (% w/w)
Time (h)
pH 2 WOSF
pH nature WOSF
pH 12 WOSF
pH 2 WSF
pH nature WSF
pH 12 WSF


SS=25 g/L
Lpid content (% w/w)
Time (h)
pH 2 WOSF
pH nature WOSF
pH 12 WOSF
pH 2 WSF
pH nature WSF
pH 12 WSF

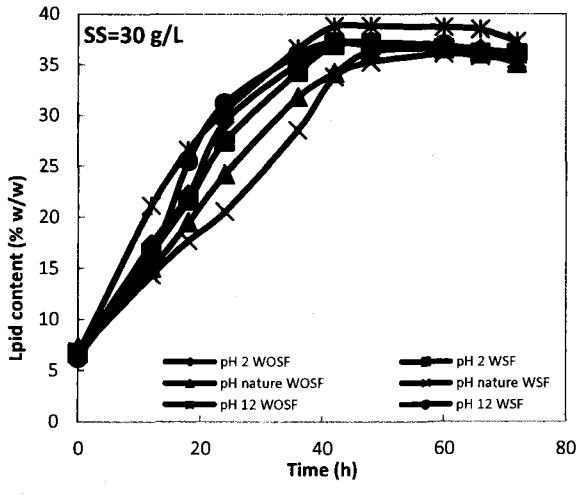

SS=30 g/L
Lpid content (% w/w)
Time (h)
pH 2 WOSF
pH nature WOSF
pH 12 WOSF
pH 2 WSF
pH nature WSF
pH 12 WSF

Figure 6.2 **Pre-treatment and struvite formation impact on lipid accumulation; the standard division is less than 5% (pH 2 WOSF represents pre-treated at pH 2 without struvite formation following; pH 2 WOSF represents pre-treated at pH 2 with struvite formation following; pH nature WOSF represents pre-treated at pH nature without struvite formation following; pH nature WSF represents pre-treated at pH nature with struvite formation following; pH 12 WOSF represents pre-treated at pH 12 without struvite formation following; pH 12 WSF represents pre-treated at pH 12 with struvite formation following)**

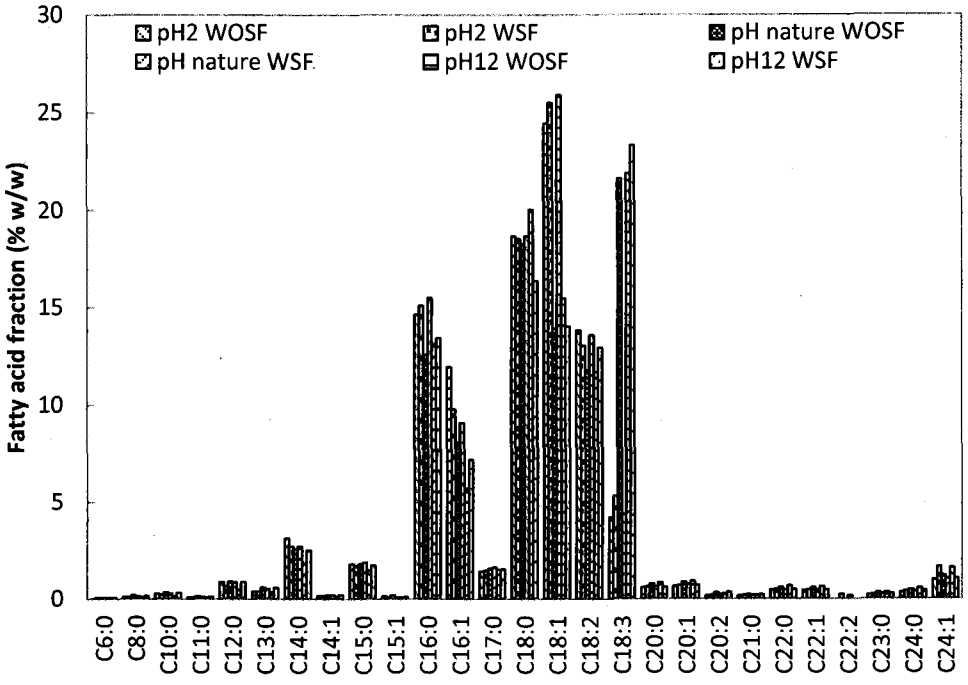


Figure 6.3 **Fatty acid composition of *Trichosporon oleaginosus* (pH 2 WOSF represents pre-treated at pH 2 without struvite formation following; pH 2 WOSF represents pre-treated at pH 2 with struvite formation following; pH nature WOSF represents pre-treated at pH nature without struvite formation following; pH nature WSF represents pre-treated at pH nature with struvite formation following; pH 12 WOSF represents pre-treated at pH 12 without struvite formation following; pH 12 WSF represents pre-treated at pH 12 with struvite formation following)**

ENERGY BALANCE AND GREENHOUSE GAS EMISSIONS OF BIODIESEL PRODUCTION FROM OIL DERIVED FROM WASTEWATER AND WASTEWATER SLUDGE

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7 ENERGY BALANCE AND GREENHOUSE GAS EMISSIONS OF BIODIESEL PRODUCTION FROM OIL DERIVED FROM WASTEWATER AND WASTEWATER SLUDGE

7.1 Résumé

Il a été reconnu que les huiles issues de microorganismes et des boues d'épuration sont des remplacements comparables de matières premières de production de biodiesel traditionnel, qui est énergétique et coûteux. Le bilan énergétique et des émissions de gaz à effet de serre (GES) sont des facteurs essentiels pour évaluer la faisabilité d'un procédé de production de biodiesel. Cette étude a évalué le bilan énergétique et les émissions de GES de la production de biodiesel à partir d'huile microbienne et des boues d'épuration. Les résultats montrent que l'équilibre énergétique et les émissions de GES de biodiesel produit à partir d'huile microbienne sont affectés de manière significative par les méthodes de culture et les sources de carbone. Pour le microorganisme phototrophe (microalgues), le système d'étang ouvert donne un gain énergétique (3.6 GJ/tonne de biodiesel produite) plus élevé que celui donné par le photo-bioréacteur. Pour les microorganismes hétérotrophes, le bilan énergétique dépend du type de la source de carbone. Trois sources de carbone incluant l'amidon, la cellulose et les eaux usées de l'industrie d'amidon (SIW), ont été utilisées dans cette étude et ont montré que l'utilisation des SIW comme source de carbone donne le bilan énergétique le plus favorable. Lorsque l'huile extraite de boues municipales est utilisée pour la production de biodiesel, le gain d'énergie par tonne de biodiesel produite est 29.7 GJ, qui est plus élevé que le gain d'énergie par tonne de biodiesel produit par des microorganismes cultivés sur SIW. L'étude des émissions de GES montre que la production de biodiesel à partir de microbes ou de l'huile extraite de la boue est un procédé de capture du dioxyde de carbone net sauf dans le cas ou l'amidon est utilisé comme matière première pour la production microbienne. Un haut de capture de 40 tonnes de dioxyde de carbone par tonne de biodiesel produit a été estimé.

Mots clés : Biodiesel; boues; bilan énergétique; huile microbienne; émissions de gaz à effet de serre

7.2 Abstract

It has been recognized that oils derived from microorganism and wastewater sludge are comparable replacements of traditional biodiesel production feedstock, which is energy intensive and costly. Energy balance and greenhouse gas (GHG) emissions are essential factors to assess the feasibility of the production. This study evaluated the energy balance and GHG emissions of biodiesel production from microbial and wastewater sludge oil. The results show that energy balance and GHG emissions of biodiesel produced from microbial oil are significantly impacted by the cultivation methods and carbon source. For phototrophic microorganism (microalgae), open pond system gives 3.6 GJ higher energy gain than photo bioreactor system in per tonne biodiesel produced. For heterotrophic microorganisms, the energy balance depends on the type of carbon source. Three carbon sources including starch, cellulose, and starch industry wastewater (SIW) used in this study showed that utilization of SIW as carbon source is most favorable energy balance. When oil extracted from municipal sludge is used for biodiesel production, the energy gain is up to 29.7 GJ per tonne biodiesel produced, which is higher than the energy gain per tonne of biodiesel produced from SIW cultivated microbes. GHG emissions study shows that biodiesel production from microbes or sludge oil is a net carbon dioxide capture process except when starch is used as raw material for microbial oil production, and the highest capture is around 40 tonnes carbon dioxide per tonne of biodiesel produced.

Keywords: Biodiesel; wastewater sludge; microbial oil, energy balance; Greenhouse gas emissions

7.3 Introduction

The fact that fossil fuel is subjected to depletion and is the major contributor of greenhouse gas emissions necessitates to develop the alternate sources of energy, which could be sustainable and environment friendly. Biodiesel, fatty acid methyl esters (FAMEs), has grabbed great attention due to the advantages such as; it is renewable, sustainable, environment friendly (burns much cleaner than petroleum diesel), compatible with current commercial diesel engines, as well as has excellent lubricity and could provide energy density similar to diesel. As a feasible energy source, biodiesel production should be a sustainable and net energy gain process (the difference between the energy output and the energy input is positive) [1]. The energy input in the production of biodiesel is mainly from feedstock oil production (feedstock cultivation, harvesting, and oil extraction), biodiesel synthesis, and biodiesel purification process. Generally, the synthesis and the purification are similar in most of the biodiesel production processes, which suggests that feedstock oil production is the major factor affecting the energy balance. Traditionally biodiesel is derived from plants or seed oils and animal fats through transesterification. However, oil and fats are not sustainable feedstock due to their strong competition with food requirements and kitchen utilization. Some researchers have reported that biodiesel produced from two major raw materials, soybean and sunflower oils, were energetically unfavorable due to the low oil yield of the crops (energy loss of 32% for soybean and 118% for sunflower) [2]. Therefore, it has forced the researchers and engineers to search for replacement of the traditional oils and lipids as raw materials, which should be abundant, sustainable, and energetically favorable (positive energy balance). Oleaginous microorganisms have shown a great advantage as lipid source due to their faster growth rate and larger lipid contents (up to 95% microorganism dry weight) compared to oilseed crops and animals [3, 4]. In addition, the possibility of increasing lipid content of microorganisms by controlling the cultivation condition (which is not possible in case of plants) and using wastewater as carbon source offers another significant advantages [5]. Sludge from municipal and industrial wastewater treatment plant is also reported as a promising raw material for biodiesel production as it has been generated in vast quantities around the world and contains a significant amount of oils and fats (up to 25% sludge dry weight) [6].

Current reports on energy balance of biodiesel production from different feedstock are summarized in Table 7.1 [7-18]. Energy ratio (energy output to input) is used to represent energy balance. When the ratio is greater than 1, the process is considered as an energy gain or energetically favorable, when the ratio is smaller than 1, the process is considered as an energy loss. From Table 7.1, it can be seen that the energy ratio largely varies with feedstock source and each country, which is understandable as the energy cost of materials (fertilizer, equipment, pesticide, and herbicide) is different, as well as climate and soil conditions in countries. Therefore, energy balance is a climate, region, and feedstock dependent.

The purpose of this study is to evaluate the energy balance and GHG emissions during biodiesel production from oil derived from wastewater and wastewater sludge, which so far has not been reported in the literature. This study also attempted to provide an insight of biodiesel production from waste (wastewater and sludge), and revealed that the process can be applied for a practical production.

7.4 Study basic

In this study, the energy and mass balance is calculated based on per tonne of biodiesel produced either from microalgae or the wastewater sludge. The calculation starts at raw collection (sludge), lipids extraction or production (microorganism) and ends until pure biodiesel is produced. The electricity, fossil fuels, steam, and methanol used in the process are considered direct energy, which means that energy contents of these items are used in the calculation, while other materials (fertilizer, acid, base, and so on) used during the production is considered indirect energy in which energy consummation during production of these materials are used in the calculation. Substitute approach was used in the calculation. The related energy terms, total energy input, energy credit, net energy input, energy balance, and energy ratio, are defined as follows:

- ***Total energy input***: the sum of sub-processes energy input;
- ***Energy credit***: the energy allocated in co-products;
- ***Net energy input***: the difference between total energy input and energy credit;

- ***Energy balance***: the difference between the energy contained in the produced biodiesel and the net energy input to produce the same amount of biodiesel.
- ***Energy ratio***: energy output (energy content of the produced biodiesel) to net energy input.

7.5 Energy balance of biodiesel production from microbial oil

Microorganisms for biodiesel production include the phototrophic microalgae, which uses sunlight as energy source and carbon dioxide as carbon source for their growth and the heterotrophic microorganisms (bacteria, fungi, yeast, and heterotrophic microalgae) which obtain their energy through metabolizing organic carbon such as glucose and starch. Microalgae are the most commonly used microorganism in biodiesel production due to the feasibility of large scale production [19]. Therefore, microalgae are used as source of oil to evaluate the energy balance and GHG emissions during biodiesel production. The production of biodiesel mainly consists of five steps: microalgae cultivation, microalgae harvesting, lipid extraction, lipid transesterification, and biodiesel purification. The cultivation methods are different depending on the microalgae types (phototrophic or heterotrophic microalgae). Generally, phototrophic microalgae can be produced in open pond or photo bioreactor, while heterotrophic microalgae are generally produced through fermentation. In order to obtain pure biodiesel, purification step is required after the transesterification. The details of each step of the production are discussed in the following sections.

7.5.1 Phototrophic microalgae

Two types of phototrophic microalgae cultivation system, open pond and photo bioreactor, are commercially used. Open pond microalgae cultivation is easier to operate, while photo bioreactors cultivation produces microalgae with higher lipid content (up to 70% microalgae dry weight) [20]. Therefore, in this study the energy balance of biodiesel production employing both the system (ponds and photo bioreactors microalgae) is investigated. In calculation, carbon dioxide served as carbon source, which is assumed to be supplied by adjacent coal-fired power plant.

7.5.1.1 Microalgae cultivated in open pond

The process of biodiesel production from microalgae obtained from open pond is schematically shown in Figure 7.1.

Microalgae cultivation. In the calculation, the microalgae cultivations are assumed to occur in open ponds with area of 400 hectare. Flue gas after two-stage cooling to around 30 ºC is continuously injected into the ponds. The first cooling stage, required 0.51 m^3 cold distilled water (per tonne of biodiesel produced) to lower the flue gas temperature so that the temperature of the cold distilled water increased to 50 ºC. Normally, the temperature of flue gases cannot go down to the required temperature (30^0C), therefore, after first stage cooling [21], the second stage cooling with tap water will be performed to achieve 30 ºC temperature. The distilled water (at 50 ºC) obtained from the first stage cooling was sent to wash biodiesel during the process (Figure 7.1). The water used for cooling flue gas in the second stage is sent to heat transesterification reactor after it is heated to 65 ºC (Figure 7.1); and the water circulates between flue gas cooling system and transesterification reactor. Fertilizers, urea (22.3 MJ /kg produced) and diammonium phosphate (DAP) (13.2 MJ/kg produced) as nutrients are also added into the open ponds [22]. The amount of nutrients added are determined based on the nitrogen and phosphorous contents of algae cell (around 5.5% N of the algae dry weight and around 1.1% P of the algae dry weight) [23]. Mixing with paddle wheel (0.1 W/m^2) is performed during the entire cultivation period [24, 25]. Once the microalgae concentration of the pond reaches about 0.5 kg/m^3 [25], which normally takes a week to a month depending on the season, the microalgae will be sent for harvesting. In this study, it is assumed that microalgae are harvested after two weeks. It is reported that the annual microalgae productivity is around 22 to 30 $g/m^2/d$ and the lipid content of the microalgae could reach 50% in open pond [22, 26]. In the calculation, the average annual microalgae productivity, 26 $g/m^2/d$, and the average lipid content of the microalgae, 25% w/w, are employed. It is assumed that water loss during evaporation is 2% of the total volume per week (two weeks for one microalgae harvesting), and salt (NaCl) loss during harvesting is 0.134 kg/kg dry algae [27].

Harvesting, drying and grinding of algal biomass. When the algae concentration of the open pond reaches at 0.5 kg/m^3, the algae solution will pass through screen (1 mm mesh) to

concentrate the biomass to 2 kg/m^3, and thereafter will be sent to centrifuge by pumping (32.6 kJ/m^3) [28], then will be dewatered by centrifugation (1 kWh/m^3) to get a cake with solid content of 15% w/w. The water obtained from the dewatering (centrifuge) step is sent back to the algae cultivation pond for reuse, and the dewatered algae is dried prior to lipid extraction step in order to prevent water effect on the extraction. For algae drying, several methods have been reported. One is solar drying process, which requires no energy input; however, it is so slow that it cannot be relied for commercial applications. Natural gas drying system has also been used, which has an energy consumption of 3.6 MJ in per kg water removed [29]. Compared to solar and natural gas drying, steam drying is more suitable method, which consumes 134 kWh to produce per tonne of dry product, hence, steam drying of the centrifuged algal biomass is adopted in these calculations [30]. Normally, the algae biomass is in bulk form after drying, in order to reduce the effect on the extraction efficiency, grinding (16 kWh/tonne product) should be performed to powder the biomass [31].

Lipid extraction. Solvent extraction is a mature and efficient method. Several types of solvents such as n-hexane, methanol, and chloroform/methanol have been used, and among all, chloroform/methanol showed the best performance for lipid extraction from microbes [6]. Therefore, a mixture of chloroform and methanol (2:1 v/v) is used with 20 mL solvents per g dry microalgae. The extraction is carried out at 25 °C for 5 h in extraction reactor under mixing (30 kWh/tonne lipid produced) and the extraction efficiency is assumed to be 96% [27]. After extraction, the solid (algae biomass) and liquid (lipid in solvents) will be separated by centrifugation. The lipid will be obtained after solvents evaporation with rotary evaporator (60 ºC). The centrifuged algae cake will also go to evaporation for residual solvents recovery. The recovered solvents will be mixed with fresh solvent and charged back to the extraction reactor. It is assumed that the solvent loss during the evaporation is 0.05% w/w [32].

Transesterification and biodiesel recovery. Biodiesel is synthesized through transesterification, in which 1 mole lipid reacts with 3 moles of primary alcohol to produce 3 moles alkyl esters (biodiesel) and 1 mole glycerol in the presence of catalyst (Equation 7.1).

Equation 7.1

$$CH_2OOR_1 - CHCOOR_2 - CH_2COOR_3 (triglyceride) + 3EtOH (methonal / ethanol) \xrightarrow{(Catalyst)} CH_3COOR_1 + CH_3COOR_2 + CH_3COOR_3 + CH_2OH - CHOH - CH_2OH (glycerol)$$

Where R_1, R_2, and R_3 denote fatty acid chains; the products, CH_3COOR_1, CH_3COOR_2, and CH_3COOR_3 represent alkyl (methyl, propyl, or ethyl) esters.

Compared to ethanol, methanol is cheaper and more efficient [33], thus methanol is often employed in the transesterification step. In large scale biodiesel production, base catalytic process is most often applied in order to have fast reaction rate (less than 1 h for base catalytic process and several hours for acid catalytic process) and low requirement of the catalyst (0.3 - 4% w/w of the oil for base versus 5 -25% w/w of the oil for acid catalytic process) [34, 35].

The lipid obtained from the extraction step will be sent into the transesterification reactor (Figure 7.1). In the reactor, methanol (methanol to lipid molar ratio of 6:1) and catalyst, NaOH (2 w/w % of the lipid), are also added. The reaction (Equation 7.1) is accomplished at 65 ºC in an hour under mixing, and the transesterification efficiency is assumed to be 99% [6, 36]. The methanol consumption and the energy used for mixing are 96 g and 0.030 kWh per kg biodiesel produced [27], respectively. Methanol recovery is normally necessary after the reaction due to its excess addition in order to enhance the reaction rate. Therefore, after the reaction is complete, the mixture is distilled (625 kW) at 1500 kg/h flow rate for methanol recovery and the recovered methanol will be recycled by mixing with fresh methanol and injected back to the reactor. Methanol recovery efficiency of 96% is assumed [33]. To separate biodiesel from glycerol and catalyst, hot water (50 ºC) is normally used. The residue (mixture of biodiesel, glycerol, and catalyst) of the distillation (methanol recovery) will be sent to washing tank in which hot water (50 ºC) (obtained from flue gas cooling in microalgae cultivation system) is present. After washing and settling, the solution becomes two layers (phase separation) with biodiesel in the top layer and the mixture of catalyst, water, and glycerol in the bottom layer. The top layer will go to distillation to remove the small amount of residual water (1 g water per liter biodiesel), and finally the pure biodiesel is obtained [37]. The bottom layer first passes

through neutralization reactor to remove NaOH, and then through distillation to dry the glycerol (1.3 MJ/kg glycerol purified) [27].

Based on the process (Figure 7.1), the mass and energy balance of per tonne of biodiesel production from open pond microalgae is presented in Table 7.2. According to the calculation, in order to produce 1 tonne of biodiesel, 4.21 tonnes of dry microalgae and 32.3 GJ of energy is required. In the process, apart from one tonne biodiesel, 3.21 tonnes algae cake (residual biomass) and 140 kg glycerol are also simultaneously produced. Normally, algae cake can be used as animal feed, fertilizer, or raw material of ethanol production due to the large contents of starch (40% w/w) and protein (20% w/w) in the open pond algae cell [22, 29], while glycerol is valuable for food and pharmaceutical industries. Therefore, the energy input used for biodiesel production should be the difference between the total energy input and the energy contained in algae cake and glycerol. Researchers have indicated that using algae cake to produce ethanol was more energy efficient than other utilization and reported an energy gain of 8.2 MJ per liter of ethanol produced from algae cake (667 L ethanol/tonne starch and the starch content of algae cake is around 35 % w/w) [22, 29, 38].Therefore, taking energy credit of ethanol production from algae cake and glycerol as a by-product, the energy consumption to produce 1 tonne of biodiesel is reduced to 18.5 GJ. The energy contents (per kg) of biodiesel and glycerol are 37.8 MJ and 16.5 MJ, respectively [8, 9]. It indicates that 19.1 GJ is gained to produce per tonne of biodiesel from microalgae cultivated in open ponds with energy output/input ratio of 2.03.

7.5.1.2 Microalgae cultivated in photo bioreactor

The process of biodiesel production from microalgae obtained from photo bioreactor is schematically shown in Figure 7.2. Apart from the cultivation step, other steps of biodiesel production (microalgae harvesting, lipid extraction, and transesterification) using photo bioreactor microalgae are similar to that of open pond system; therefore, only the cultivation in photo bioreactor is discussed in detail in this section.

Microalgae cultivation. Plate photo bioreactor, carboy photo bioreactor, and tubular photo bioreactor have been reported for cultivating microalgae; however, tubular photo bioreactor is

considered suitable for large scale cultivation [39]. It was reported that 16.3 hectares land area could be sufficient to build a total volume of 10120 m^3 tubular photo bioreactor farm in which horizontal bubble columns with 0.2 m diameter, 2.0 m length, 0.15 m^3 effective cultivation volumes, and 0.35 m space between two bubble columns, were used to cultivate microalgae. In photo bioreactor cultivation system, the reported lipid content of microalgae was up to 70% (microalgae dry weight basis) and the yearly microalgae biomass productivity in photo bioreactor was around 1500 to 2200 g algae biomass/m^3/d [3, 39].

To evaluate energy balance, it is assumed that the microalgae cultivation occurs in horizontal bubble column tubular photo bioreactors built in a farm occupying a 400 hectares land area (240000 m^3 total microalgae cultivating volume). The average values of lipid content 35% w/w of microalgae and yearly productivity 1850 g algae biomass/m^3/d of the microalgae biomass and are used in the calculation. Flue gas is (after cooling to 25 °C by a similar procedure as described in the open pond cultivation system) continuously injected into nutrient solution (urea and DAP) and then the nutrient solution is fed to the bioreactors. Similar to the open pond system, 0.51 m^3 volume of water from the first cooling stage (at 50 ºC) is sent to wash one tonne biodiesel. The water from second cooling stage (at 65 ºC) is sent to heat the transesterification reactor and the water is considered to circulate between flue gas cooling system and transesterification reactor. As mentioned in the open pond microalgae cultivation, significant mixing is required in microalgae cultivation because of the need of the transfer of carbon dioxide, nitrogen, phosphate, protons, and minerals; therefore, aeration (0.4 W/m^2) is performed to accomplish an appropriate mixing [27, 28]. When the microalgae concentration is reached up to 1.5 kg/m^3, which normally takes 7 to 15 days (average value of 11 days is assumed in these calculations) depending on the season, it is assumed that the algae solution will be sent to a collecting tank for the harvesting) [22, 28].

In photo bioreactor cultivation system, light is significantly important as it is the energy source. Either sunlight or artificial light can be used. Even though, use of artificial light is independent of the weather and seasons; however, it is rather expensive and not sustainable for a large scale cultivation system [20]. Therefore, sunlight is assumed to be the light source for the calculation.

Microalgae harvesting, lipid extraction, and transesterification are assumed to be conducted in a similar way as described in the section of microalgae cultivation in open pond.

Based on the process (Figure 7.2), the energy balance of per tonne of biodiesel produced in a microalgae photo bioreactor is presented in Table 7.3. According to the calculation, 3.01 tonnes of photo bioreactor microalgae is required to produce 1 tonne of biodiesel. A total energy input of 32 GJ is needed. After taking credit from microalgae cake, used for ethanol production and the credit from glycerol, it is revealed that 1 tonne of biodiesel produced from photo bioreactor microalgae will provide 15.6 GJ energy gain with output/input energy ratio of 1.71.

7.5.2 Heterotrophic microalgae

The average lipid content of heterotrophic microalgae is 50% w/w [40] and is used in this study to evaluate the energy balance of biodiesel production.

The process is shown in Figure 7.3. Apart from the microalgae cultivation step, other steps of biodiesel production using heterotrophic microalgae are similar to those of autotrophic microalgae. Therefore, only heterotrophic microalgae cultivation in the bioreactor is described in detail in the following section.

Microalgae cultivation. Martek Biosciences Corporation built a fermentation facility with a total volume of 1200 m^3 consisting with eight 150 m^3 tanks to produce oils [41]. To evaluate the energy balance, it is assumed that the microalgae cultivation is carried out in fermentor with an effective cultivation capacity of 1200 m^3. The algae productivity is assumed to be 50 kg/ m^3/d [22].

Unlike autotrophic microalgae which grow through photosynthesis, heterotrophic microalgae consume organic carbon as food to obtain energy for growth. Therefore, carbon source has to be provided in the cultivation medium. Normally, glucose is used as a carbon source; however, the high cost of glucose (350 US$/tonne) has motivated researchers to find an alternative to glucose. Recently, it was reported that sugar cane juice (260 US$/tonne), starch (230 US$/tonne) or Jerusalem artichoke (165 US$/tonne) could be used as carbon source [42, 43]. Comparing the price, Jerusalem artichhoke showed advantage; however, the utilization efficiency of Jerusalem artichoke as carbon source is 55%, which would increase the amount

required and hence increase the cost. Thus, starch could be considered a cheaper carbon source. Moreover, some researchers have reported that cellulose-hydrolyzate could be used as a carbon source [44]. Furthermore, wastewater use as carbon source is also proposed, and it is known that wastewater is rich in nutrients (nitrogen and phosphorus) which would eliminate the need of nutrient addition [22]. Thus, starch, cellulose, and wastewater from starch production industry, are assumed to be the carbon source to evaluate the energy balance. When starch is used as carbon source, the dissolved starch (9.1 kJ/per gram starch produced) [45] and nutrients are well mixed and fed to the fermentor. When cellulose is employed, hydrolysis of cellulose will be performed prior to feeding the fermentor, and then the hydrolyzed cellulose ($(C_6H_{10}O_5)_n$) and dissolved nutrients will be fed to the fermentor. The energy consumption in cellulose hydrolysis is 4.2 kJ for treating per gram cellulose [46]. When wastewater from starch production industry (SIW), which has a starch content of 2 kg/m^3, is the carbon source, sterilization (20.9 kJ/1000 m^3) [47] will be performed, and then fed to the fermentor. The typical composition of heterotrophic algae is $CH_{1.8}N_{0.17}O_{0.56}$; therefore, the addition of carbon source and fertilizers (urea and DAP) are calculated based on the C and N fraction in $CH_{1.8}N_{0.17}O_{0.56}$. The fermentation occurs at room temperature (25 ºC) and mixing is performed throughout the cultivation. It was reported that around 0.5 kWh energy was needed per cubic meter volume to meet the mixing requirement [48]. It is assumed the mixing efficiency has been improved by 30% during the last 30 years; hence energy consumption is assumed to be 0.35 kWh for per cube meter volume in the calculation. When the microalgae concentration in the fermentor reaches 50 kg/m^3, which normally takes 7 d [22], the algae harvesting will be conducted by pumping (32.6 kJ/m^3) [28, 44].

Based on the process (Figure 7.3), the energy balance of per tonne of biodiesel produced employing heterotrophic microalgae and fed with starch, cellulose, or wastewater as carbon source is calculated and presented (Table 7.4). According to the calculation, in order to produce one tonne of biodiesel, 2.1 tonnes of heterotrophic microalgae is required. The energy consumption depends on the type of carbon source used to cultivate microalgae. The total energy input is 44.8 GJ, 31.4 GJ, and 16.1 GJ when corn starch, cellulose, or starch industry wastewater (SIW) is used as carbon source, respectively. After taking credit from algae cake

used for ethanol production, and from glycerol as byproduct, the net energy gain to produce one tonne of biodiesel using heterotrophic microalgae with starch, cellulose, and SIW as carbon source is 1.5 GJ, 11.8 GJ, and 27.2 GJ, with output/energy ratio of 0.96, 1.46 and 3.60, respectively. Thus, use of SIW as carbon source would highly increase the energy gain. It indicates that wastewater could be used for biodiesel production.

From the foregoing evaluation of the energy balance for biodiesel production employing microalgae, it can be concluded that the cultivation system (open ponds, photo bioreactor, and fermentation using heterotrophic algae), as well as the carbon source have a great impact on the energy balance. Therefore, special consideration should be given to the selection of a cultivation system in biodiesel production using microalgae.

7.6 Biodiesel production using sludge

Various types of wastewater sludge have been reported to contain important oil concentration (15-25% w/w). The primary sludge exhibited higher oil content compared to other types of sludge (secondary, digested or mixed sludge) [6, 36]. In this study, an average oil content of 20% of dry weight of primary sludge is employed in the calculations. Biodiesel production from sludge can be divided into two types, one is called two-step process in which oil is first extracted from the sludge followed by transesterification to synthesize biodiesel (Equation 7.1), and the other is one-step process where sludge is directly used as feedstock to form biodiesel without extraction step. The two types of production processes are discussed separately in this section. At present, biodiesel production from sludge is tested on bench scale, thus technological information on overall continuous industrial processes is not completely available. Therefore, in order to evaluate the energy balance, the overall processes of sludge biodiesel production is designed (Figures 7.4 and 7.5) and is described below.

7.6.1 Two-step process for biodiesel production from wastewater sludge

The process is schematically shown in Figure 7.4.

Sludge transportation. Generally, wastewater treatment plants are built in suburban area due to the concern of smell, while biodiesel production sites are in the city. Therefore, prior to using the sludge to produce biodiesel, the dewatered sludge (20% solid content) from wastewater treatment plants has to be transported to the biodiesel production site. It is assumed that they are 20 km away, and the sludge transport is accomplished by diesel vehicles which consume 3.5 kJ to transport 1 kg load for 1 km [2].

Sludge drying. Sludge drying is performed similar to microalgae drying. The dewatered sludge (20% solids content) transported from wastewater treatment plant will be placed into steam drying system and dried until the solids content of the sludge is reached 95% in order to minimize free water impact on the oil extraction efficiency [6, 36].

Oil extraction. The dried sludge is powdered with grinding machine performed similar to microalgae grinding. A mixture of solvents, 60% hexane +20% methanol + 20% acetone (v/v), exhibited a good performance of oil extraction from sludge [6]. Therefore, the powdered sludge was mixed (150 rpm) with the solvent mixture of hexane, methanol and acetone (3:1:1 v/v/v) for 1 h at 50 ºC in a traction reactor (2381 W/m^3) [49]. The solvent mixture used is 10 mL for per gram of dry sludge, and the extraction efficiency is assumed to be 96% [36]. After extraction, the sludge and solvent solution will be centrifuged (0.5 kWh/m^3), and the lipid in solvent (supernatant) will be obtained after evaporation at 60 ºC, and it is assumed that the solvent loss during recovery is 0.05% [6, 27].

Transesterification. Transesterification (Equation 7.1) is the key step of biodiesel production. Unlike microalgae biodiesel production, where base is often used as a catalyst, in the case of sludge, acid catalyst is used because of the fact that sludge oil contains a large portion of free fatty acids which would lead to soap formation if base is used as a catalyst.

In transesterification reactor, the oil obtained from the extraction step is mixed with methanol and sulphuric acid (1% v/v sulphuric acid in the methanol) in methanol to lipid molar ratio of 6:1. According to Equation 7.1, 1 molar of lipid requires 3 molar of methanol to form FAMEs in

transesterification. The excess addition of methanol in the process is to enhance the conversion. The reaction will proceed at 50 ºC under mixing (0.030 kWh per kg biodiesel) [27] and the transesterification efficiency is 99% [6, 36]. The methanol and energy consumption for mixing is 96 g and 0.03 kWh per kg biodiesel produced [27]. After the reaction, the mixture will go to distillation (625 kW) at 1 500 kg/h flow rate for methanol recovery (96% recovery efficiency) and the recovered methanol will be then mixed with fresh methanol and injected back to the reactor [33]. Biodiesel purification will be conducted similar to biodiesel production from microalgae.

The residual sludge after extraction will be sent to evaporator for solvent recovery. There are mainly two ways to deal with the residual sludge; one is directly transported to landfill and the other is sent to agriculture land to be used as fertilizer. It is assumed that landfill and agriculture land are 20 km away from the biodiesel production site. The waste is transported by diesel vehicles which consumes 3.5 kJ to transport 1 kg of load for 1 km [2].

The energy balance of two-step biodiesel production (Figure 7.4) from sludge is calculated and presented in Table 7.5. According to the calculation, 5.26 tonnes of dry sludge can generate one tonne of biodiesel with a total energy input of 12.4 GJ. Because of use of sludge for the biodiesel production, sludge is not transported to landfill, but instead to biodiesel production site, therefore, the energy (fuel utilization) that should have been consumed to dispose the 26.3 tonnes of sludge with 20% solids content (5.26 tonnes dry sludge) during transportation is saved. In addition, when the residual sludge (phosphorus as P_2O_5 content is 1.6% w/w) is used as fertilizer, there is credit for replacing commercial fertilizer production (8.31 GJ/tonne) [50]. Therefore, taking credit for 26.3 tonnes of sludge disposal (fertilizer replacement) and glycerol, the net energy input will be 8.28 GJ with residual sludge to landfill and 7.58 GJ with residual sludge used as fertilizer. The energy gain per tonne of biodiesel produced will be 29.36 and 30.09 GJ with residual sludge to landfill and as fertilizer, respectively, in two-step biodiesel production process.

7.6.2 One-step biodiesel production from wastewater sludge

One-step biodiesel production is a process in which dry sludge is directly used for transesterification step without oil extraction (Figure 7.5). In one-step biodiesel production from sludge, transportation and sludge drying are similar as in two-step biodiesel production process. Transesterification process is described as below.

Transesterification. The sludge from steam drying step after grinding is directly placed into transesterification reactor and reacts with methanol at 50 ºC for 4 h, in which one gram sludge will be mixed with 5 mL of 1% H_2SO_4 in methanol [6]. After the reaction, methanol will be recovered through distillation (similar to two-step process), and the rest of the solution will be mixed with 5% solution of NaCl (25 mL/g dry sludge), and hexane (5 mL/g dry sludge) will be added to extract biodiesel followed by centrifugation. The hexane extraction will be performed three times. The supernatants will be then washed with $NaHCO_3$ (5 mL of 2% w/v solution of $NaHCO_3$ for 30 mL supernatant). The top layer (hexane and biodiesel) is collected and subjected to evaporation to recover hexane (in a similar way as solvent recovery in two-step process) and the solvents recovered by evaporation will be collected and reused in biodiesel extraction step. Glycerol purification will be performed similar to two-step process. According to published reports, the biodiesel yield is around 100 kg/tonne dry sludge [6, 36]. The residual sludge after solvents recovery will be transported to landfill or agriculture land.

The energy balance of one-step biodiesel production process (Figure 7.5) from sludge is computed and presented in Table 7.6. According to the calculation, 10 tonnes of dry sludge can produce one tonne of biodiesel with a total energy input of 17.3 GJ. The credit taken is similar as used in two-step process. The energy gain per tonne of biodiesel produced will be 26.2 and 27.5 GJ with residual sludge to landfill or used as fertilizer, respectively.

Comparing with biodiesel production from microalgae, wastewater sludge as raw material for biodiesel production provides substantial higher energy gain (Figure 7.6). It shows that wastewater sludge is a promising raw material for biodiesel production. The one-step process of biodiesel production from sludge is simpler than the two-step process due to the elimination of extraction step; however, its biodiesel yield is only half of two-step process due to low

efficiency (only 50% of lipid in the sludge can be converted into biodiesel in one-step process). It is obvious that the two-step process is more feasible in terms of energy balance compared to the one-step process.

7.7 Greenhouse gas emissions

This study accounts for CO_2, CH_4, and N_2O emissions originated from specific sources of energy and materials consumed, the use of fuels, electricity, and chemicals [22]. Intergovernmental Panel on Climate Change (IPCC) global warming potentials are applied to CH_4 (21 CO_2-eq) and N_2O (310 CO_2-eq) emissions to calculate the CO_2 equivalent (CO_2-eq) emissions of the biodiesel production processes.

In the production process, carbon dioxide emissions are from the utilization of fertilizers, fuels, and electricity. The local (Québec, Canada) power is assumed as hydro power. Positive/negative value of the calculation represents that the process produces/reduces (capture) GHG emissions. The study accounts for avoidance of CO_2 emissions due to credits including:

- The fuel saving, in sludge disposal due to reduction of sludge amount during biodiesel production;
- Emissions of carbon dioxide from the equal amount of fossil diesel replaced by produced biodiesel;
- Emissions of carbon dioxide from sludge land filling replaced by biodiesel production;
- GHG emitted from the production process of the replaced industrial glycerin;
- Residual sludge used as fertilizer replacing commercial fertilizer production.

The emission coefficients of the fertilizer, electricity, and fuels are presented in Table 7.7 [21, 51-55]. GHG emissions of biodiesel production from microalgae are shown in Table 7.8, and the emissions from photo bioreactor and fermentor were calculated in a similar way as open ponds. GHG emission of two-step biodiesel production from sludge is shown in Table 7.9, and the emission from one-step production was calculated in a similar way as two-step production.

According to the calculation, biodiesel production from phototrophic microalgae and sludge has negative GHG emissions, which suggests that they are carbon dioxide capture processes (Figure 7.7). GHG emissions from heterotrophic microalgae biodiesel production are affected by carbon source. When cellulose and wastewater are used as carbon source, the production processes are carbon dioxide capture processes, while it is a GHG production process when starch is the carbon source. Biodiesel production from sludge with residual sludge used as fertilizer has higher reduction of GHG emissions than sending residual sludge to landfill due to the credit taken for the replacement of the commercial fertilizer production and the emission reduction from sludge land application instead of residual sludge to landfill. The highest reduction of carbon dioxide emission is from biodiesel production with one-step process using residual sludge as fertilizer. It is mainly because of the residual sludge use as fertilizer in agriculture field and refraining the sludge disposal in landfill.

7.8 Conclusions

Biodiesel production from lipid produced by phototrophic and heterotrophic microalgae showed greater advantages on energy savings and GHG emissions reduction compared to that from traditional feedstock such as seed oils (Table 7.1). Different microalgae cultivation systems (open pond, photo bioreactor, and fermentor) lead to a significant difference in energy input. For phototrophic microalgae, open pond system provides higher energy gain due to operational ease than photo bioreactor system; energy ratio is 2.03 for open pond and 1.71 for photo bioreactor. However, both cultivation systems heavily depend on the climate, therefore, the biodiesel production from phototrophic microalgae are not suitable in the cold regions. Heterotrophic microalgae are widely applicable as there is no demand on light. Even though heterotrophic microalgae biodiesel production shows energy loss (energy ratio 0.96 <1) when starch is used as the carbon source, while using cellulose and wastewater as carbon source the process increases the energy ratio to 1.46 and 3.60, respectively. In addition, there are further difficulties to commercially use fermentor for microalgae production due to the huge capital cost. Wastewater sludge gives high energy gain in biodiesel production, and the process also

minimizes the problem of sludge disposal, which is energy consuming and resource wasting. Therefore, in future the wastewater sludge could be a compatible feedstock for biodiesel production as it provides energy gains and environmentally friendly solution.

The evaluation of GHG emissions of biodiesel production from microalgae indicates that phototrophic microalgae or heterotrophic microalgae fed with cellulose and wastewater is a carbon dioxide capture or recovery method. Use of wastewater sludge for biodiesel production leads to great reduction in GHG emissions, as well as the residual sludge used as fertilizer has a great impact on GHG emissions.

Based on the estimation on energy balance and GHG emissions of biodiesel production, wastewater sludge as raw material has the great advantage. However, the biodiesel production from wastewater sludge is still in research stage, therefore, further large scale studies are required to realize the benefits of this new biotechnology.

7.9 Acknowledgements

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7.10 References

[1]. Thamsiriroj T, Murphy JD. The impact of the life cycle analysis methodology on whether biodiesel produced from residues can meet the EU sustainability criteria for biofuel facilities constructed after 2017. Renewable Energy 2011;36(1): 50-63.

[2]. Pimentel D, Patzek TW. Ethanol production using corn, switchgrass, and wood; biodiesel production using soybean and sunflower. Nat Resour Res 2005;14(1): 65–76.

[3]. Chisti Y. Biodiesel from microalgae. Biotechnol Adv 2007;25(1): 294–306.

[4]. Gouda M, Omar S, Aouad L. Single cell oil production by *Gordonia* sp. DG using agro-industrial wastes. World J Microbiol Biotechnol 2008;24(9): 1703–1711.

[5]. Widjaja A, Chien C-C, Ju Y-H. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J Taiwan Inst Chem Eng 2009;40(1): 13–20.

[6]. Dufreche S, Hernandez R, French T, Sparks D, Zappi M, Alley E. Extraction of lipids from municipal wastewater plant microorganisms for production of biodiesel. J Amer Oil Chem Soc 2007;84(2): 181–187.

[7]. Hill J, Nelson E, Tilman D, Polasky S, Tiffany D. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. PNAS 2006;103(7): 11206–11210.

[8]. Fore SR, Porter P, Lazarus W. Net energy balance of small-scale on-farm biodiesel production from canola and soybean. Biomass Bioenergy 2011;35(5): 2234–2244.

[9]. Smith EG, Janzen HH, Newlands NK. Energy balances of biodiesel production from soybean and canola in Canada. Can J Plant Sci 2007;87(4): 793–801.

[10]. Sheehan J, Camobreco V, Duffield J, Graboski M, Shapouri H. Life cycle inventory of biodiesel and petroleum diesel for use in an urban bus. 1998;Report by U.S. Department of Agriculture and U.S. Department of Energy, 1998.

[11]. Kamahara H, Hasanudin U, Widiyanto A, Tachibana R, Atsuta Y, Goto N, Daimon H, Fujie K. Improvement potential for net energy balance of biodiesel derived from palm oil: A case study from Indonesian practice. Biomass Bioenergy 2010;34(12): 1818–1824.

[12]. Yáñez Angarita EE, Silva Lora EE, da Costa RE, Torres EA. The energy balance in the Palm Oil-Derived Methyl Ester (PME) life cycle for the cases in Brazil and Colombia. Renewable Energy 2009;34(12): 2905–2913.

[13]. de Souza SP, Pacca S, de Ávila MT, Borges JLB. Greenhouse gas emissions and energy balance of palm oil biofuel. Renewable Energy 2010;35(11): 2552–2561.

[14]. Chen H, Chen G. Energy cost of rapeseed-based biodiesel as alternative energy in China. Renewable Energy 2011;36(5): 1374–1378.

[15]. Janulis P. Reduction of energy consumption in biodiesel fuel life cycle. Renewable Energy 2004;29(6): 861–871.

[16]. Thamsiriroj T, Murphy JD. Is it better to import palm oil from Thailand to produce biodiesel in Ireland than to produce biodiesel from indigenous Irish rape seed? Applied Energy 2009;86(5): 595-604.

[17]. Thamsiriroj T, Murphy JD. Can Rape Seed Biodiesel Meet the European Union Sustainability Criteria for Biofuels? Energy Fuels 2010;24(3): 1720-1730.

[18]. Lardon L, Helias A, Sialve B, Steyer J-P, Bernard O. Life-cycle assessment of biodiesel production from microalgae. Environ Sci Technol 2009;43(17): 6475–6481.

[19]. Meng X, Yang J, Xu X, Zhang L, Nie Q, Xian M. Biodiesel production from oleaginous microorganisms. Renewable Energy 2009;34(1): 1–5.

[20]. Douskova I, Doucha J, Livansky K, Machat J, Novak P, Umysova D, Zachleder V, Vitova M. Simultaneous flue gas bioremediation and reduction of microalgal biomass production costs. Appl Microbiol Biotechnol 2009;82(1): 179–185.

[21]. Doucha J, Straka F, Lívanský K. Utilization of flue gas for cultivation of microalgae *Chlorella* in an outdoor open thin-layer photobioreactor. J Appl Phycol 2005;17(5): 403–412.

[22]. Alabi AO, Tampier M, Bibeau E. Microalgae technologies and processes for biofuelsbioenergy production in British Columbia. Report submitted to the British Columbia Innovaion Council, 2009.

[23]. Borowitzka M. Algal biotechnology products and processes — matching science and economics. J Appl Phycol 1992;4(3): 267–279.

[24]. Wagener K. Mass cultures of marine algae for energy farming in coastal deserts. Int J Biometeorol 1983;27(3): 227–233.

[25]. Seambiotic Ltd. Algae pilot plant ashkelon, Israel 2006;Available at http://www.seambiotic.com/uploads/Seambiotic%20Ltd.%20-%20Algae%20Pilot%20Plant%20white%20paper.pdf (accessed 17/09/2011).

[26]. Pedroni PM, Lamenti G, Prosperi G, Ritorto L, Scolla G, Capuano F, Valdiserri M. Enitecnologie R&D project on microalgae biofixation of CO_2: outdoor comparative tests of biomass productivity using flue gas CO_2 from a NGCC power plant. 2004;Available at http://uregina.ca/ghgt7/PDF/papers/nonpeer/075.pdf. (accessed 004/008/2011).

[27]. Batan L, Quinn J, Willson B, Bradley T. Net energy and greenhouse gas emission evaluation of biodiesel derived from microalgae. Environ Sci Technol 2010;44(20): 7975–7980.

[28]. Jorquera O, Kiperstok A, Sales EA, Embiruçu M, Ghirardi ML. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresour Technol 2010;101(4): 1406–1413.

[29]. Sander K, Murthy G. Life cycle analysis of algae biodiesel. Int J LCA 2010;15(7): 704–714.

[30]. High Efficient Drying Equipment. Avaialbe at http://www.alibaba.com/product-gs/407620763/High_Efficient_Drying_Equipment.html (accessed 407620710/407620705/407622011).

[31]. Ball Grinding Machine. Available at http://www.alibaba.com/product-gs/387012885/Hot_Sale_Ball_Grinding_Machine_of.html (accessed 387012808/387012804/387012011).

[32]. Pokoo-Aikins G, Heath A, Mentzer RA, Sam Mannan M, Rogers WJ, El-Halwagi MM. A multi-criteria approach to screening alternatives for converting sewage sludge to biodiesel. J Loss Prev Process Ind 2010;23(3): 412–420.

[33]. Dhar B, Kirtania K. Excess methanol recovery in biodiesel production process using a distillation column: A simulation study. Chemical Engineering Research Bulletin 2009;13(2): 55–60.

[34]. Prueksakorn K, Gheewala SH. Energy and greenhouse gas implications of biodiesel production from *Jatropha curcas L.*, In *The 2nd Joint International Conference on "Sustainable Energy and Environment (SEE 2006)"*, Bangkok, Thailand, 21-23 November 2006.

[35]. Kywe T, Oo M. Production of biodiesel from Jatropha Oil (Jatropha curcas) in pilot plant. WASET 2009;14(1): 78–86.

[36]. Willson RM, Wiesman Z, Brenner A. Analyzing alternative bio-waste feedstocks for potential biodiesel production using time domain (TD)-NMR. Waste Manage 2010;30(10): 1881–1888.

[37]. Gonzalo A, Garcìla M, Luis Salnchez J, Arauzo J, Penìfa J. Water cleaning of biodiesel. effect of catalyst concentration, water amount, and washing temperature on biodiesel obtained from rapeseed oil and used oil. Ind Eng Chem Res 2010;49(9): 4436–4443.

[38]. Lorenz D, Morris D. How much energy does It take to make a gallon of ethanol? 1995;Available at http://www.carbohydrateeconomy.org/library/admin/uploadedfiles/How_Much_Energy_Does_it_Take_to_Make_a_Gallon_.html (accessed 21/07/2011).

[39]. Sanchez Miron A, Contreras Gomez A, Garcia Camacho F, Molina Grima E, Chisti Y. Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. J Biotechnol 1999;70(1-3): 249–270.

[40]. Miao X, Wu Q. Biodiesel production from heterotrophic microalgal oil. Bioresour Technol 2006;97(6): 841–846.

[41]. BENT. Market completes purchase of fermentation plant; Winchester, Kentucky facility to manufacture essential fatty acid products. Business Wire Columbia 1995.

[42]. Cheng Y, Lu Y, Gao C, Wu Q. Alga-based biodiesel production and optimization using sugar cane as the feedstock. Energy Fuels 2009;23(8): 4166–4173.

[43]. Cheng Y, Zhou W, Gao C, Lan K, Gao Y, Wu Q. Biodiesel production from Jerusalem artichoke (Helianthus Tuberosus L.) tuber by heterotrophic microalgae *Chlorella protothecoides*. J Chem Technol Biotechnol 2009;84(5): 777–781.

[44]. Behrens P. Photobioreactors and fermentors: the light and dark sides of growing algae. editors. Algal Culturing Techniques, New York: Elsevier Academic Press; 2005, p. 189–204

[45]. Hilton JW, Atkinson JL, J SS. Evaluation of the net energy value of glucose (cerelose) and maize starch in diets for rainbow trout (Salmo gairdnerz). Br J Nutr 1987;58(2): 453–461.

[46]. Kim J, Lee Y, Torget R. Cellulose hydrolysis under extremely low sulfuric acid and high-temperature conditions. Appl Biochem Biotechnol 2001;91-93(1): 331–340.

[47]. Gadgil A. 1995;Avaiable at http://www.lbl.gov/Education/ELSI/Frames/Sustain21-f.html (accessed 23/06/2011).

[48]. Osen'kina V, Tokarev I, Padalko V, Shemetov S. Comparative tests of 100-m^3 capacity fermenters. Chem Pet Eng 1982;18(9): 417–419.

[49]. Berk Z. Chapter 7 - Mixing. editors. Food Process Engineering and Technology, San Diego: Academic Press; 2009, p. 175-194

[50]. Energy Information Handbook. Appendix C: Units, Equivalents and Energy Constants of the Energy Information Handbook. University of Florida Cooperative Extension Service 1990.

[51]. Kim H, Kim S, Dale BE. Biofuels, land use change, and greenhouse gas emissions: Some unexplored variables. Environ Sci Technol 2009;43(3): 961–967.

[52]. Sustainable Aerobic Landfill Technologies. The sustainable landfill from SALT Inc. 2009;Available at http://www.saltinc.ca/files/SALT%20Advantages.pdf. (accessed 14/07/2011).

[53]. Sander K, Murthy G. Life cycle analysis of algae biodiesel. The International Journal of Life Cycle Assessment 2010;15(7): 704-714.

[54]. Coronado CR, de Carvalho Jr JA, Silveira JL. Biodiesel CO_2 emissions: A comparison with the main fuels in the Brazilian market. Fuel Processing Technology 2009;90(2): 204-211.

[55]. Daniel P. 1999;Annual Report of Canada's Climate Change Voluntary Challenge and Registry Inc. (VCR), 1999.

Table 7.1 **Energy balance of biodiesel produced from different feedstock oils**

Feedstock oil	Energy ratio	Country	Reference
Soybean oil	0.61	America	[27]
Soybean oil	0.75	America	[2]
Soybean oil	2.0	America	[7]
Soybean oil	2.05	America	[8]
Soybean oil	2.41	Canada	[9]
Soybean oil	1.94	Argentina	[38]
Sunflower oil	3.2	America	[10]
Sunflower oil	0.46	America	[2]
Palm oil	3.5	Indonesia	[11]
Palm oil	4.7	Brazil	[12]
Palm oil	2.33	Brazil	[13]
Palm oil	4.9	Colombia	[12]
Palm oil	1.64	Ireland	[16]
Rapeseed oil	0.91	China	[14]
Rapeseed oil	3.0	Europe	[15]
Rapeseed oil	1.19	Ireland	[16]
Rapeseed oil	0.97	Ireland	[17]
Canola oil	1.78	America	[8]
Canola oil	2.08	Canada	[9]
Microalgae	1.07	America	[27]
Microalgae	0.97-1.24	Europe	[18]

Table 7.2 Energy and mass balance of per tonne biodiesel produced from open pond microalgae

Inputs	Quantity required (ton)	Energy (kWh)	Energy required (GJ)	Energy contained in chemical used/produced (GJ)
Microalgae cultivation	4.21			
Water	336.7			0.02
Salt	0.56			0.34
Urea	0.41			9.07
DAP	0.20			2.60
Mixing+pumping		426.67	1.54	
Harvesting				
Centrifuge+drying+grinding		2735.69	9.84	
Lipid extraction				
Chloroform	0.08			0.61
Methanol	0.02			0.44
Mixing+centrifuge+evaporation		194.98	0.70	
Transesterification+methanol recovery				
NaOH	0.02			0.37
Methanol	0.1			2.0
Mixing+evaporation		1209.02	4.35	
Biodiesel purification				
Water	0.51			0.02
HCl	0.018			0.08
Mixing+distillation		91.88	0.33	
Total energy input (a)			32.31	
Energy credit				
Algae cake	3.20			11.44
Glycerol	0.14			2.34
Total credit (b)			13.77	
Net energy input (a-b)=c			18.53	
Biodiesel yield (d)	1			37.8
Net energy gain (d-c)=e1			19.14	
Energy balance for per tonne biodiesel produced (f1)			19.14	
Energy output to input ratio d/c=h1	2.03			

Table 7.3 **Energy and mass balance of per tonne biodiesel produced from photo bioreactor microalgae** [a]

Inputs	Quantity required (ton)	Energy (kWh)	Energy required (GJ)	Energy contained in chemical used/produced (GJ)
Microalgae cultivation	3.01			
Salt	0.40			0.25
Urea	0.29			6.48
DAP	0.14			1.86
Mixing+pumping		1672.71	6.02	
Harvesting			8.83	
Lipid extraction			0.62	0.75
Transesterification+methanol recovery			4.35	2.37
Biodiesel purification			0.33	0.10
Total energy input (a)			31.96	
Energy credit				
Algae cake	2.0			7.53
Glycerol	0.14			2.34
Total credit (b)			9.87	
Net energy input (a-b)=c			22.09	
Biodiesel yield (d)	1			37.8
Net energy gain (d-c)=e2			15.58	
Energy balance for per tonne biodiesel produced (f2)			15.58	
Energy output to input ratio d/c=h2	1.71			

[a] *The energy input calculation in harvesting, lipid extraction, transesterification and methanol recovery, and biodiesel purification, are similar as Table 7.2.*

Table 7.4 **Energy and mass balance of per tonne biodiesel produced from fermentor microalgae with starch as carbon source [a,b]**

Inputs	Quantity required (ton)	Energy (kWh)	Energy required (GJ)	Energy contained in chemical used/produced (GJ)
Microalgae cultivation	2.10			
Salt	0.28			0.17
Starch[1]	2.15			22.80
Cellulose[2]	2.26			9.46
SIW[3]	1200			26.28
Urea	0.20			4.54
DAP	0.10			1.30
Mixing+pumping		632.65	2.28	
Other process are calculated as Table 7.2				
Total energy input (a)				
Starch[1]			44.77	
Cellulose[2]			31.42	
SIW[3]			16.12	
Total credit (b)				
Starch[1]			5.58	
Cellulose[2]			5.58	
SIW[3]			5.64	
Net energy input (a-b)=c				
Starch[1]			39.19	
Cellulose[2]			25.84	
SIW[3]			10.47	
Biodiesel yield (d)	1			37.8
Energy balance for per tonne biodiesel produced (f3)				
Starch[1]			-1.52	
Cellulose[2]			11.83	
SIW[3]			27.19	
Energy output to input ratio				
d/c=h3				
Starch[1]	0.96			
Cellulose[2]	1.46			
SIW[3]	3.60			

[a] *The energy input calculation in harvesting, lipid extraction, transesterification and methanol recovery, and biodiesel purification, are similar as Table 7.2.*

[b] *In each calculation, only one of the [1], [2], and [3] will take place.*

Table 7.5 Energy and mass balance of two-step biodiesel (per tonne) production from sludge [a]

Inputs	Quantity required (kg)	Energy (kWh)	Energy required (GJ)	Energy contained in chemical used/produced (GJ)
Transportation	26300		1.83	
Sludge drying		789.1542	2.84	
Lipid extraction				
Hexane	10.32			0.46
Methanol	4.04			0.08
Acetone	4.05			0.12
Mixing+centrifuge+evaporation		573.8636	2.06	
Transesterification				
H_2SO_4	16.19			0.12
Methanol	99.76			2.00
Mixing+evaporation		552.30	1.99	
Biodiesel and glycerin purification				
Water for biodiesel washing	505			0.07
NaOH for neutralization	13.15			0.24
Mixing+distillation		91.88	0.33	
Residue sludge handling				
Transportation to landfill/agriculture land	4.25		0.30	
Total energy input (a)			12.44	
Total credit (b)				
Landfill[4]			4.16	
Fertilizer[5]			4.86	
Net energy input (a-b)=c				
Landfill[4]			8.28	
Fertilizer[5]			7.58	
Biodiesel yield (d)	1000			37.8
Energy balance for per tonne biodiesel produced (f4)				
Landfill[4]			29.39	
Fertilizer[5]			30.09	
Energy output and input ratio d/c= h4				
Landfill[4]	4.55			
Fertilizer[5]	4.97			

[a] *In each calculation, only one of the [4], and [5] will take place.*

Table 7.6 Energy and mass balance of one-step biodiesel (per tonne) production from sludge

Inputs	Quantity required (kg)	Energy (kWh)	Energy required (GJ)	Energy contained in chemical used/produced (GJ)
Transportation	50000		3.47	
Sludge drying		1500	5.40	
Transesterification				
H_2SO_4	61.54			0.46
Methanol	189.62			2.00
NaCl	125			0.08
$NaHCO_3$	100			0.10
Hexane	163.46			0.73
Mixing+evaporation		883.37	3.18	
Biodiesel and glycerin purification				
Water for biodiesel washing	480			0.07
NaOH for neutralization	50			0.92
Mixing+distillation		73.68	0.27	
Residue sludge handling				
Transportation to landfill/agriculture land	9000		0.63	
Total energy input (a)			17.27	
Total credit (b)				
Landfill[6]			5.78	
Fertilizer[7]			7.11	
Net energy input (a-b)=c				
Landfill[6]			11.49	
Fertilizer[7]			10.16	
Biodiesel yield (d)	1000			37.8
Energy balance for per tonne biodiesel produced (f5)				
Landfill[4]			26.18	
Fertilizer[5]			27.51	
Energy output and input ratio d/c= h5				
Landfill[6]	3.28			
Fertilizer[7]	3.71			

[a] *In each calculation, only one of the [6], and [7] will take place.*

Table 7.7 Emission coefficients of materials, electricity, and fuels

Substances	Emission coefficients (kg CO_2-equivalent)	References
Urea (/tonne)	732	[20]
DAP (/tonne)	894	[20]
Diesel vehicle (/km/tonne)	0.11	[16]
Electricity (/kWh)	0.0014	[10]
Starch production (tonne)	230	[51]
Corn starch for ethanol (/tonne)	10.97	[53]
Cellulose (/tonne)	10.97	[53]
Glycerin (/tonne)	1.66	[55]
Wastewater (/kg COD)	0.9	[52]
Sludge lipid landfill (/tonne)	25560	[54]
Sludge land application	1140	[54]
Sludge landfill(/tonne)	29400	[54]
Biodiesel (/tonne)	2830	[54]
Biodiesel displace diesel (/tonne)	3750	[21]

Table 7.8 GHG emissions of biodiesel production from open pond microalgae

Items	Quantity	Emission coefficient (kg CO_2-equivalent)	Emission (kg CO_2/ton biodiesel)
Carbon dioxide consumed	7368.3 kg	1 per kg	-7368.3
Fertilizer			
Urea	0.41 kg	732 per tonne	300.12
DAP	0.20 kg	894 per tonne	178.80
Electricity	4231.57 kWh	0.0014 per kWh	5.92
Diesel[a]	1.52 tonnes 20km	0.11 per km per tonne	3.33
Biodiesel	1 tonne	2830 per tonne	2830
Credit			
Algae cake starch instead corn starch	1.68 tonnes	10.97 per tonne	18.47
Residual algae cake for fertilizer	0.13 tonnes	893 per tonne	116.09
Glycerin	0.14 tonnes	1.66 per tonne	0.23
Displaces	1 tonne biodiesel	3220 per tonne biodiesel	3220
Total GHG			-7404.92

[a] *Diesel using for sludge transportation from biodiesel production site to landfill or agriculture land.*

Table 7.9 GHG emissions of two-step biodiesel production from sludge

Items	Quantity	Emission coefficient (kg CO_2-equivalent)	Emission (kg CO_2/ton biodiesel)
Electricity	2007 kWh	0.0014 per kWh	2.81
Diesel[a]	26.3 tonne, 20 km	0.11 per kg per tonne	57.87
Diesel[b]	4.25 tonne, 20 km	0.11 per kg per tonne	9.35
Biodiesel	1 tonne	2830 per tonne	2830
Credit			
Diesel[c]	26.3 tonne, 20 km	0.11 per kg per tonne	57.87
Sludge lipid landfill	1 tonne	25560 per tonne	25560
Fertilizer	4.16 tonne	894 per tonne	75.25
Sludge land application instead of landfill	4.25 tonne	1140 per tonne	4846
Glycerin	0.14 tonne	1.66 per tonne	0.23
Displaces	1 tonne diesel	3220 per tonne biodiesel	3220.05
Total GHG (residual to landfill)			-26196.7
Total GHG (residual using as fertilizer			-31118

[a] *Diesel using for sludge transportation from wastewater treatment plant to biodiesel production site.*

[b] *Diesel using for sludge transportation from biodiesel production site to landfill or agriculture land.*

[c] *Diesel using for sludge transportation from wastewater treatment plant to landfill.*

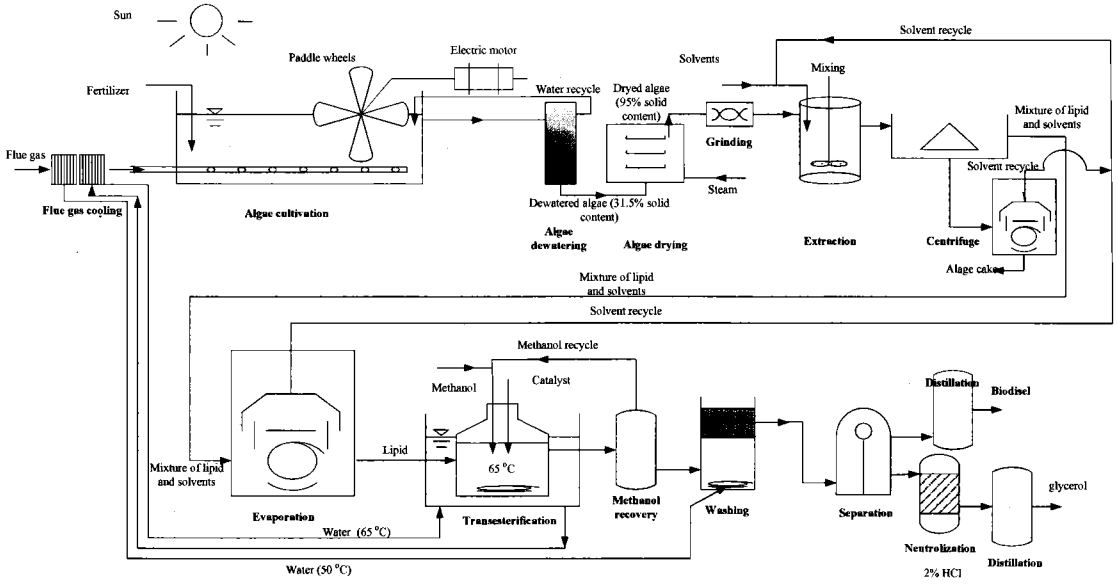

Figure 7.1 **Biodiesel derived from microalgae cultivated in open ponds**

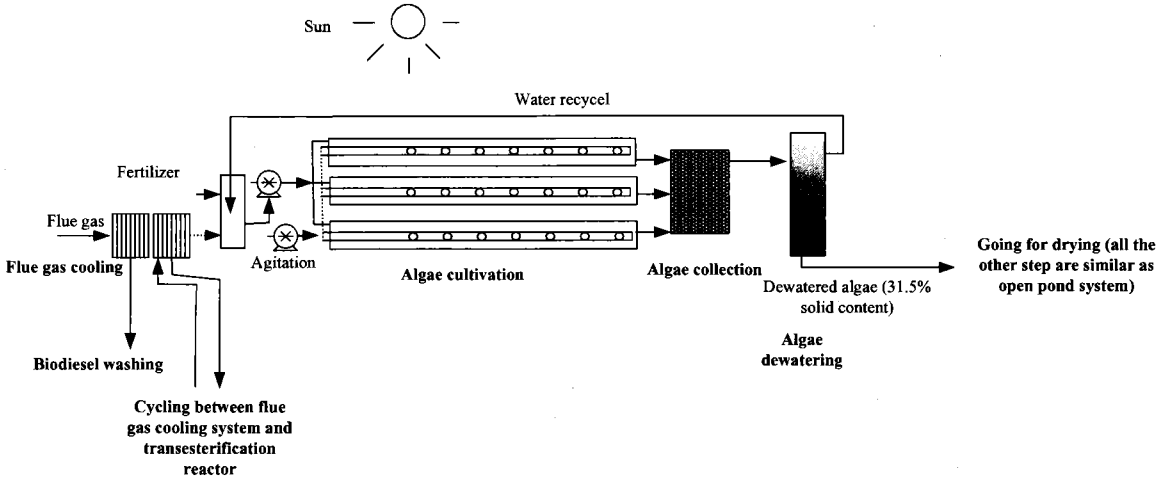

Figure 7.2 **Biodiesel derived from microalgae cultivated in photo bioreactors (only cultivation step is shown here as the other steps are similar as open pond Figure 7.1).**

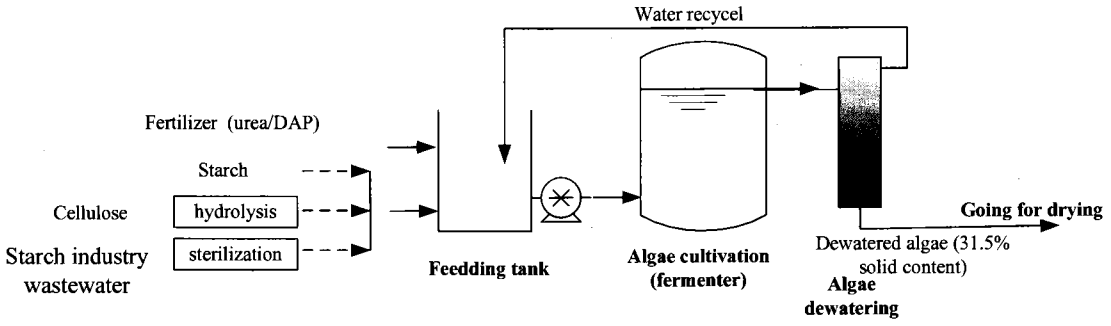

Figure 7.3 **Biodiesel productions from heterotrophic microalgae (only cultivation step is shown here as the other steps are similar as open pond Figure 7.1)**

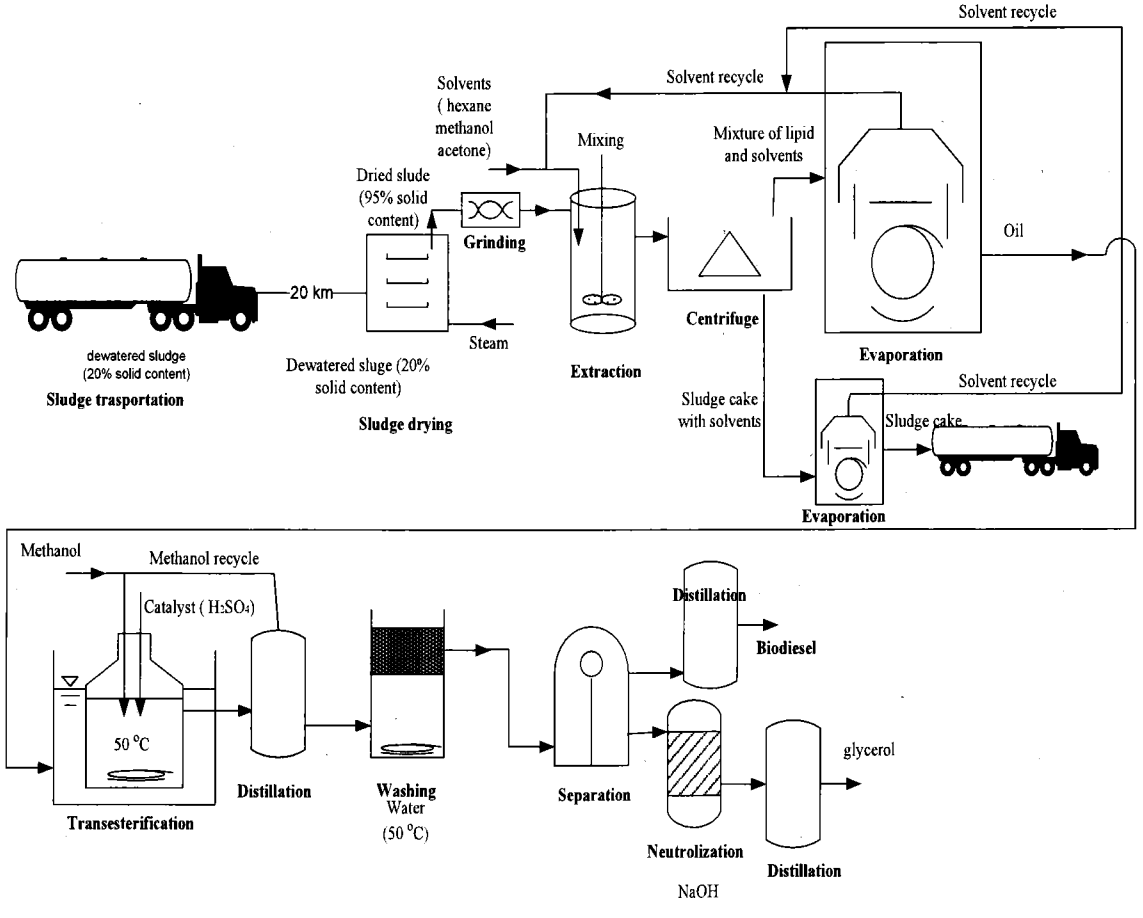

Figure 7.4 **Two-step biodiesel production from wastewater sludge**

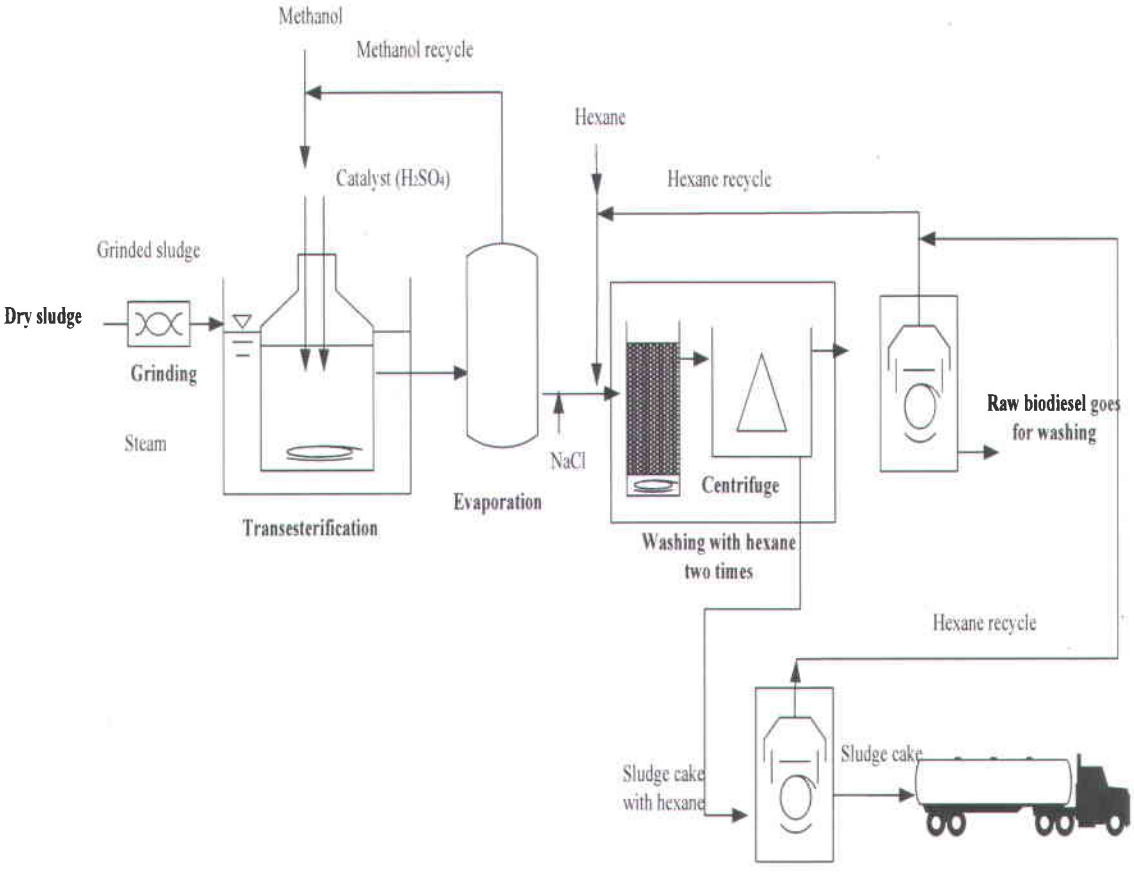

Figure 7.5 **One-step biodiesel production from wastewater sludge (other steps including sludge transportation, drying, and biodiesel recovery steps are similar as two-step production Figure 7.4)**

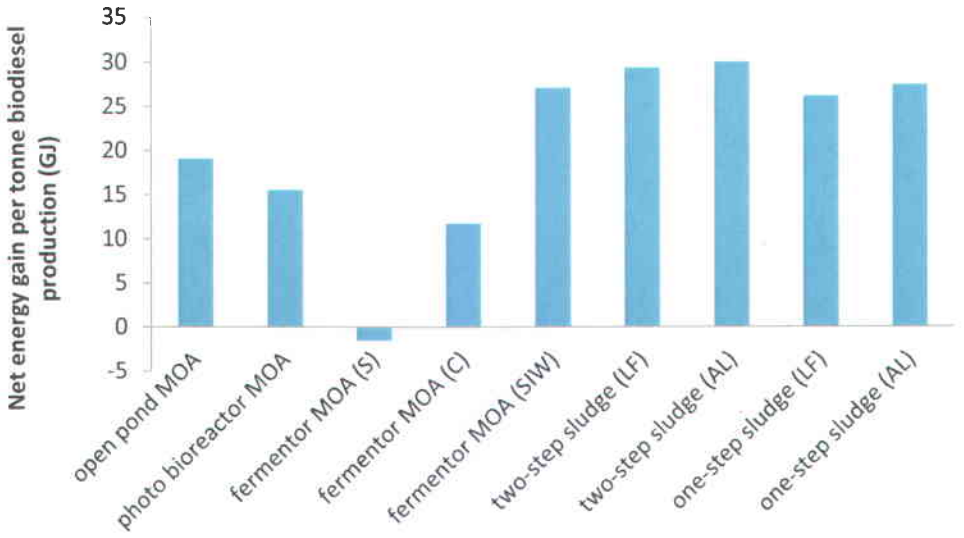

Figure 7.6 **Comparison of energy balance of biodiesel production from microalgae and sludge (MOA – microalgae; S – starch; C – cellulose; SIW – starch industry wastewater; LF –landfill; AL – agriculture land)**

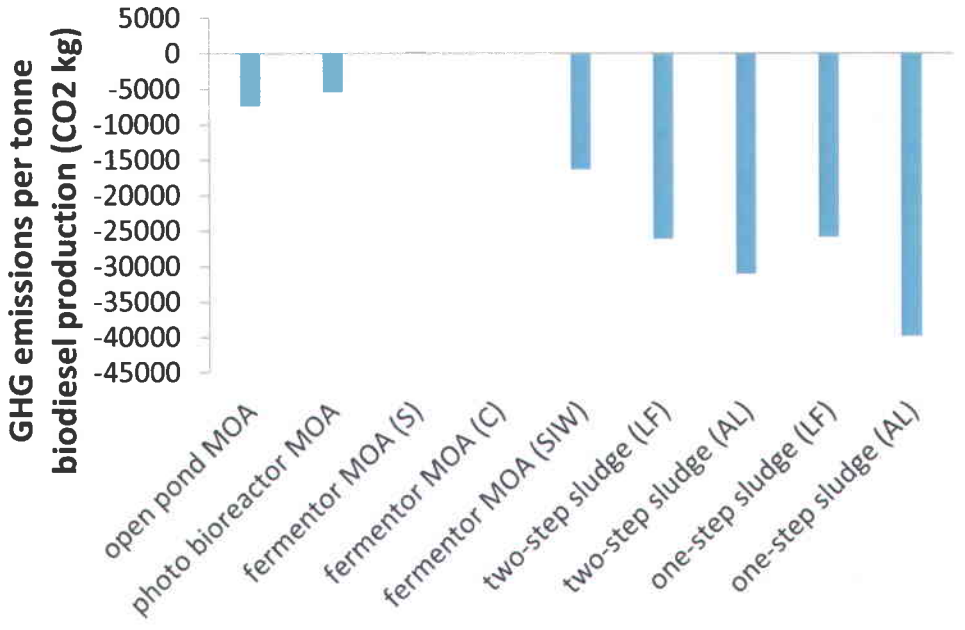

Figure 7.7 **Comparison of GHG emissions of biodiesel production from microalgae and sludge (MOA – microalgae; S – starch; C – cellulose; SIW – starch industry wastewater; LF –landfill; AL – agriculture land**

COST ESTIMATION OF BIODIESEL PRODUCTION FROM WASTEWATER SLUDGE

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ENERGY ECONOMICS (UNDER REVIEW)

8 COST ESTIMATION OF BIODIESEL PRODUCTION FROM WASTEWATER SLUDGE

8.1 Résumé

Deux procédés de production de biodiesel à partir de boues d'épuration ont été conçus et leur coût a été estimé à l'aide du logiciel Superpro Designer. L'un des procédés consiste à utiliser les boues contenant les lipides comme matière première pour la production de biodiesel. L'estimation du coût a montré que le coût unitaire de production est de 0.43 US$/kg biodiesel produit à partir de boues d'épuration. Les études de l'impact des méthodes d'élimination des boues résiduelles (mise en décharge, valorisation comme engrais et substrat pour la culture de microorganismes oléagineux) ont montré que les boues résiduelles utilisées comme engrais pourraient réduire le coût de production unitaire. Une autre option consiste à utiliser les boues comme milieu de fermentation de microorganismes oléagineux. Les lipides accumulés dans ces microorganismes sont ensuite convertis en biodiesel. Le calcul a montré que le coût unitaire de production était de 0.51 US$/kg biodiesel avec des boues résiduelles envoyées à l'enfouissement. Le coût unitaire de production est significativement affecté par la teneur en lipides des microorganismes. Ainsi, le coût de production unitaire plus faible est obtenu avec la teneur en lipides plus élevée. Le coût actuel de production de biodiesel commercial est d'environ 1.0 US$/kg biodiesel. Ceci indique que la production de biodiesel à partir des boues est un procédé prometteur pour une éventuelle utilisation.

Mots clés : Biodiesel; boues; bilan énergétique; huile microbienne

8.2 Abstract

Two processes of biodiesel production from wastewater sludge were designed and the cost was estimated with SuperPro Designer. One is to utilize the lipid contained in raw sludge for biodiesel production. The estimation showed that the unit production cost was 0.43 US$/kg biodiesel produced with residual sludge landfilling. Studies on the impact of disposal methods of residual sludge (landfilling, fertilizer, and medium for oleaginous microorganism cultivation) showed that residual sludge used as fertilizer could reduce the unit production cost. Another process is that sludge was used as fermentation medium of oleaginous microorganism, and the accumulated lipid in the microorganism was converted to biodiesel. The calculation showed that the unit production cost was 0.51 US$/kg biodiesel produced with residual sludge landfilling. When residual sludge used as fertilizer instead of landfilling, the unit production cost was reduced 8 cents. It was also observed that the unit production cost was significantly affected by lipid content of microorganism. The higher of the lipid content resulted in the lower unit production cost. The current commercial biodiesel production cost is around 1.0 US$/kg biodiesel. It indicates that biodiesel production from sludge is a promising process.

Keywords: Biodiesel; wastewater sludge; microbial oil, cost

8.3 Introduction

Biodiesel, one of the best alternatives of petro-fuel, has attracted considerable attention due to the energy crisis. Current method of production is to convert edible oils to biodiesel through transesterification. However, the high price of edible oil requires a search for cheap replacement.

Wastewater sludge is widely produced in large quantity. It was reported that wastewater sludge contained 5 to 20% lipid w/w dry sludge which is comparable to plant seeds (Turovskiy and Mathai 2006). When sludge is used as lipid source, the cost of biodiesel production would be highly reduced as sludge is cost free. In addition, wastewater sludge has been found as a suitable medium to cultivate microorganism due to the fact that the sludge is rich in nutrients such as carbon, nitrogen, and phosphorus (Angerbauer et al. 2008). Oleaginous microorganism such as microalgae has been investigated for lipid production (Gao et al. 2010). Therefore, use of sludge as nutrient medium to cultivate oleaginous microorganism for lipid production would decrease biodiesel production cost. Wastewater sludge is free.

Computer simulations to model and predict the costs of production have been used with success for many industrial processes (Ramirez et al. 2008; Vázquez and Rodríguez 2011; Qureshi et al. 2013). They provide the ability to estimate the effect of raw materials, utilities, the product productivity, and the technologies for product recovery on the product production cost. Beginning with a basic scenario and designing the model to simulate those conditions effectively allows the user to estimate results of alternative processes with confidence. Superpro designer, a simulation program that is able to estimate both process and economic parameters, has been widely used for bioprocess cost estimation (Petrides 2003; Kwiatkowski et al. 2006).

So far, economic evaluation of lipid production using wastewater sludge followed by extraction of lipids and their conversion to biodiesel has not been published. Economic analysis of the entire process for biodiesel production provides the approximate cost of biodiesel produced on a commercial scale. The goal of the project was to design a process to produce biodiesel using

wastewater sludge as a direct lipid source and cultivation medium to grow oleaginous microorganisms for economic evaluation.

8.4 Basic information

SuperPro Designer facilitates modeling, evaluation and optimization of integrated processes in a wide range of industries (Pharmaceutical, Biotech, Specialty Chemical, Food, Consumer Goods, Mineral Processing, Microelectronics, Water Purification, Wastewater Treatment, Air Pollution Control, etc.). In the study, SuperPro Designer was employed to estimate the cost of biodiesel production.

Wastewater sludge was found to have a lipid content of 11% w/w dry matter in our lab study. In addition, it was observed that oleaginous microorganism could accumulate lipid in sludge medium. In order to investigate the cost feasibility of biodiesel production from these sludge derived lipid. The cost of two basic scenarios namely biodiesel production by transesterification of raw sludge lipid and microbial lipid were estimated. The estimation information was summarized in Table 8.1.

8.5 Results

8.5.1 Biodiesel from raw sludge lipid

8.5.1.1 Description of the process

In wastewater treatment plant (WWTP), dewatering is normally performed before the sludge is transferred to landfills or for other usages. The dewatered sludge generally has a solid concentration of 3% w/v. Study reported that water content had great impact on lipid extraction (Dufreche et al. 2007; Willson et al. 2010); therefore, the first step of biodiesel production from raw sludge lipid is sludge drying which is to minimize the water effect on lipid extraction (Figure 8.1). In the study, rotary dryer with steam as heating agent was selected as its high efficiency.

After drying, sludge is normally in bulk form. In order to provide a better contact between sludge and lipid extraction solvent (Dufreche et al. 2007; Mondala et al. 2009), grinding was used to reduce the particle size of the sludge from bulk to fine powders. The powdered sludge was then mixed with organic solvents to extract lipid from sludge in extractors. In the extraction, mixture of hexane, acetone, and methanol was used in a ratio of 2:1:1 due to their high lipid recovery efficiency (96%) (Mondala et al. 2009).

After extraction, centrifugation was employed to separate the liquid part (lipid in solvents) from the solid (residual sludge). Then the liquid phase was sent to solvent evaporator to recover the solvents and the residue (lipid) was collected in a storage tank. It was assumed that the solvent loss during the process was 0.05% w/w (Batan et al. 2010). The recovered solvents were then reused in lipid extraction process after mixed with fresh solvents.

The lipid in the storage tank was then transferred to transesterification reactor to synthesis biodiesel with methanol in the presence of sulfide acid (catalyst). In the reaction, 3 molars methanol reacts with 1 molar lipid to form 3 molar biodiesel and 1 molar glycerol. In order to enhance the reaction shifting to the biodiesel production side, excess methanol is generally used. Sulfide acid was selected as catalyst due to the high free fatty acid content in the raw sludge lipid (> 5%). In the study, methanol to lipid molar ratio used was 6:1 with sulfide acid addition of 5 v/v methanol. The reaction preformed at 50 °C for 12 h to achieve a transesterfication efficiency of 99% (Mondala et al. 2009).

After reaction, the mixture (biodiesel, excessed methanol, sulfuric acid, by-product glycerol) was first subjected to evaporator to recover the extra methanol which would be then mixed with fresh methanol to synthesis biodiesel in transesterification reactor. The remaining mixture was then washed with warm water (50 °C), and allowed for phase separation. The top layer (raw biodiesel) was then distilled to remove the moisture, and finally biodiesel was obtained. The bottom part mainly containing glycerol, sulfide acid and water, was neutralized in neutralization reactor by sodium hydroxide. The heavy part (sodium sulfide) was settled and removed, while the light part (water and glycerol) was distilled to remove water. Glycerol with less than 1% of water was then obtained.

Based on 260 tonnes dry sludge utilization per day, the biodiesel production is 9380 tonnes per year along with 1313 tonnes by-product glycerol. The detailed mass balance is given in Table 8.2.

8.5.1.2 Economic evaluation

Capital investment is the sum of direct and indirect fixed capitals. For design purpose, the various items of direct fixed capital (DFC) and indirect fixed capital are estimated based on the total equipment purchase cost (PC) using several multipliers. Table 8.3 provides ranges and average values for the multipliers and a skeleton for the calculations of capital investment from equipment cost. Therefore, to calculate the capital investment, the equipment cost has to be first calculated.

Equipment cost: the equipment purchase cost can be estimated from vendor quotations, published data, company data compiled from previous projects, and by using process simulators and other computer aids. Generally, cost data for one or two discrete equipment sizes is available, but the cost for a different size piece of equipment has to be estimated. In such cases, the scaling law can be used as suggested in Equation 8.1:

Equation 8. 1 $Cost2=Cost1\ (size2/size1)^{I}$

Where the index I value normally falls between 0.5 and 1.0 with an average value for vessels of around 0.6. Generally 0.6 is applied when I value is unknown (Zhuang et al. 2007). In this study, the equipment cost is from vendor quotations. The total equipment cost was 1 428 000 $.

Capital cost: Based on the equipment cost, capital cost from piping, instruments, insulation, electrical facilities, etc (Table 8.3) was estimated to be 7 356 000 $.

Operation cost: the operating cost to run a plant is the sum of all expenses associated with raw materials, labor, utilities, equipment, and lab/QA/QC. Dividing the annual operating cost by the annual production rate yields the unit production cost (in $/kg).

Raw materials accounts for the cost of all chemicals utilized for biodiesel production. The price of a raw material can vary widely depending on its required purity. Various raw materials can be found in the Chemical Marketing Reporter. More recently, a number of websites have come

online where buyers can find pricing information. In this study, raw materials include wastewater sludge (lipid source), mixture of hexane: acetone: methanol (solvents), methanol (reactant of transesterification), and H_2SO_4 (catalyst), and NaOH (to neutralize H_2SO_4). Sludge is a waste thus it is considered as cost free. In the process, solvents after extraction were recovered and reused. However, 0.05% w/w solvent loss was assume; therefore, it counts for a part of raw material cost. Other chemical cost is calculated based on the amount used (Table 8.2).

Labor is estimated based on the total number of operators and the operation time. In a single product facility, the number of operators in each shift must be based on maximum demand during that shift. In the study, labor cost is calculated by the program.

Lab/QC/QA refers to the cost of off-line analysis, quality control (QC), and quality assurance (QA) costs. This cost is usually 10-20% of the operating labor cost. In this study, the average value 15% is taken to calculated lab/QC/QA cost.

Utilities include heating (steam) and cooling (cooling or chilled water) utilities as well as electricity. The amounts are calculated as part of the material and energy balances. In terms of unit cost, electricity costs is 0.10 $/kWh. Heating steam, cooling water, chilled water are 2.0, 0.1, 0.4 $/1000 kg, respectively.

Equipment-dependent is from the depreciation of the fixed capital investment, maintenance of equipment, insurance, and local (property) taxes. For preliminary cost estimates, the entire fixed capital investment is usually depreciated linearly over a 10-year period. The annual equipment maintenance cost is normally estimated as a 10 per cent of the equipment's purchase cost (Petrides 2003). Insurance value for bioprocessing facilities is generally in the range of 0.5-1% of DFC. In this study 1% DFC is taken for insurance cost. The local tax is usually 2-5% of DFC and 2% is taken in this study. The factory expense represents overhead cost incurred by the operation of non-process-oriented facilities and organizations including accounting, payroll, fire protection, security, cafeteria, etc. A value of 5-10% of DFC is appropriate for these costs and 5% is taken in this study.

By sum of the raw material, labor, utilities, lab/QA/QC, and equipment-dependent cost, the total of annul operation cost was 5034000. The detailed cost of the process (raw materials, equipment, labor, lab/QC/QA, and utilization) is shown in Table 8.4. The unit biodiesel production cost (annul operation cost by annul production rate) was then estimated to be 0.53 $/kg (0.47 $/L).

Glycerol was produced as by-product along with biodiesel in the process. Glycerol has great value in pharmaceutical industries, thus it is considered as credits (0.3 cent/kg biodiesel) (Yang et al. 2012). Additionally, due to the biodiesel production, sludge volume is reduced from per 260 tonnes to 231.4 tonnes. It suggests that sludge disposal volume is reduced and hence the disposal fee is saved when residual sludge is considered to send to landfill. Therefore, the avoidance of the reduced volume sludge can be considered as credit (0.97 cent/kg biodiesel) (Wheeler et al. 2008). After taken credits, the net unit biodiesel production is 0.43 $/kg (0.38 $/L).

8.5.2 Biodiesel from lipid extracted from microorganism cultivated with sludge

8.5.2.1 Description of the process

As mentioned, wastewater sludge generally has a solid content of 3% w/w or 30 g/L. In the study, the sludge with 30 g/L was used as medium for lipid accumulation in oleaginous microorganism after being sterilized at 121 °C for 15 min. The fermentation occurred at 28 °C with 0.5 vvm aeration 200 rpm agitation. According to lab study, it was assumed that the fermentation broth had a 30 g/L dry matter concentration with lipid content of 40% w/w dry matters after 48 h fermentation.

After fermentation, the sludge-biomass was harvested with centrifugation. To further remove water, drying was employed. Thereafter, lipid extraction with chloroform and methanol in 2:1 volume ratio (1 kg of biomass in 20 L of solvent mixture) was performed (Cheng et al. 2011) followed by centrifugation to separate the liquid (lipid in solvent) from the solid (residual sludge-biomass). Then the liquid part was subjected to evaporation to recover solvents from lipid. It was assumed that the solvent loss during the process was 0.05% w/w (Batan et al. 2010).

The recovered solvents would be then reused for lipid extraction after mixed with fresh solvents. The lipid was stored for biodiesel synthesis.

Biodiesel was synthesized in transesterification reactor by reacting with methanol. Unlike biodiesel synthesis from raw sludge lipid with H_2SO_4 as catalyst, sodium hydroxide was used in biodiesel synthesis from microbial lipid due to its acceptable free fatty acid content (<2%). In the study, methanol to lipid molar ratio 6:1 with 2% NaOH w/w lipid was utilized. After reaction, the steps are similar as descripted in biodiesel production from raw sludge lipid. The schematic process is shown in Figure 8.2. Based on 260 tonnes dry sludge utilization, the biodiesel production rate is 31756 tonnes per year along with 4446 tonnes by-product glycerol.

8.5.2.2 Economic evaluation

The cost estimation is done in the similar method as biodiesel production from raw sludge lipid. The detailed cost of the process (raw materials, equipment, labor, Lab/QC/QA, and utilization) is shown in Table 8.5.

Based on the results, the unit production cost of biodiesel is 0.63 $/kg (0.55 $/L). Credits were taken from by-product glycerol production and the avoidance of sludge landfilling. After subtracting the credits, the net unite biodiesel production is 0.51 $/kg (0.46 $/L).

The detailed calculations from the program are shown in the Annex. The study showed that the major cost of biodiesel production from raw sludge lipid and microbial lipid was from raw material and utilities. This is due to the organic solvent loss and large energy consumption in the extraction and solvent recovery. It indicates that new lipid extraction technology is required.

8.6 Sensitivity analysis

The sensitivity of the key process parameters including management of residual sludge (to landfill; used as fertilizer or microorganism cultivation) or residual sludge-biomass (to landfill; used as fertilizer) and lipid content of sludge-biomass (40%, 50%, and 60% w/w), was studied. The result is shown in Table 8.6. It is assumed that residual sludge has a fertilizer value of 77 $/tonne (EPB 296 2004).

When raw sludge lipid was used for biodiesel production, the residual sludge handling approach had great impact on the cost. The cost was 0.43, 0.36, 0.67 $/kg with residual sludge to landfill, used as fertilizer, and microorganism cultivation medium, respectively (Table 8.6). Using residual sludge for microorganism cultivation medium increased the biodiesel production rate from 9380 to 21780 tonnes per year compared with residual sludge to landfill or using as fertilizer, but the unit production cost increased as well due to the extra equipment required.

Comparing the results (Table 8.6), when sludge directly is used as lipid source (0.53 $/kg) the cost was lower than that using sludge to cultivate oleaginous microorganism to produce lipid (0.63 $/kg), even though the lipid content in sludge is only 11% w/w while lipid content in microorganism is 40% w/w. The high cost of biodiesel production from oleaginous microorganism cultivated with sludge is due to the sludge sterilization and fermentation. As lipid content increases from 40% to 50% w/w, the cost reduced 14 cents. With further increasing lipid content 10% w/w, the cost decrease was only 8 cents. It suggested that lipid content impact on unit biodiesel production cost becomes small when lipid content was higher than 50% w/w sludge-biomass.

8.7 Conclusions

Cost estimation showed that using sludge directly as lipid source is more feasible than using sludge as nutrients media to cultivate oleaginous microorganisms which can be converted to biodiesel used as lipid source. The sensitivity studies showed that the handling methods of residual sludge and lipid content of microorganism had great impact on the unit production cost. Comparing with the current commercial biodiesel production cost (around 1.0 US$/kg biodiesel), biodiesel produced from sludge derived oil is more cost feasible. Using sludge for biodiesel production also reduced sludge amount which provides a way of its management. It suggested that the process would be promising in biodiesel production as well as in sludge management.

8.8 Acknowledgements

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8.9 References

Angerbauer, C., Siebenhofer, M., Mittelbach, M., Guebitz, G. M., 2008. Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production. Bioresour. Technol. 99, 3051-3056.

Gao, C., Zhai, Y., Ding, Y., Wu, Q., 2010. Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Appl. Energy 87, 756-761.

Kwiatkowski, J. R., McAloon, A. J., Taylor, F., Johnston, D. B., 2006. Modeling the process and costs of fuel ethanol production by the corn dry-grind process. Industrial Crops and Products 23, 288-296.

Petrides, D., 2003. Bioprocess design and economics. Oxford University Press, 2003.

Qureshi, N., Saha, B. C., Cotta, M. A., Singh, V., 2013. An economic evaluation of biological conversion of wheat straw to butanol: A biofuel. Energy Conversion and Management 65, 456-462.

Ramirez, E. C., Johnston, D. B., McAloon, A. J., Yee, W., Singh, V., 2008. Engineering process and cost model for a conventional corn wet milling facility. Industrial Crops and Products 27, 91-97.

Turovskiy, I. S., Mathai, P. K., 2006. Wastewater sludge processing: Chapter 2. Sludge quantities and characteristics. Wiley-Interscience; 1 edition (Jun 2 2006), 30-59.

Vázquez, M. V., Rodríguez, M. E. G., 2011. Computer simulation of microbial transglutaminase production from potato wastes. Procedia Food Science 1, 736-741.

Table 8.1 Basic information of the study

Items	Description
Capacity	260 tonne dry sludge per day
Plant location	Near wastewater sludge treatment
Construction period	30 months
Project life time	15 years
Production level in the 15 years	100%
Income tax	30%
Cost unit	$ refers to US$

Table 8.2 Mass balance of biodiesel production from raw sludge lipid

Process	Component	Input (tonne/d)	Output (tonne/d)
Sludge drying	Sludge (3% w/v)	8 666.67	0
	Water	0	8 406.67
	Dry sludge	0	260
	Total	8 666.67	8 666.67
Grinding	Bulk dry sludge	260	0
	Powdered dry sludge	0	260
	Total	260	260
Extraction	Powdered dry sludge	260	0
	Hexane	954.2	0
	Acetone	494	0
	Methanol	494	0
	Mixture 1 (solvent phase)	0	1 967.16
	Solid phase	0	235.04
	Total	2 202.2	2202.2
Evaporation	Mixture 1	1 967.16	0
	Lipid	0	24.96
	Recovered solvents	0	1 932.49
	Loss of solvents	0	9.71
	Total	1 967.16	1 967.16
Transesterification	Lipids	24.96	0
	Methanol	5.29	0
	H2SO4	0.41	0
	Mixture 2	0	30.66
	Total	30.66	30.66
Methanol recovery	Mixture 2	30.66	0
	Methanol recovered	0	2.64
	Mixture 3	0	28.02
	Total	30.66	30.66
Water washing	Mixture 3	28.02	0
	Water (50 °C)	0.03	0
	Diluted mixture 3	0	28.05
	Total	28.05	28.05
Phase separation	Diluted mixture 3	28.05	0
	Mixture 4 (raw biodiesel)	0	24.72
	Mixture 5 (crude glycerol)	0	3.33
	Total	28.05	28.05
Biodiesel drying	Mixture 4	24.72	0
	Biodiesel	0	24.71
	Water	0	0.01
	Total	24.72	24.72

Glycerol purification	Mixture 6	3.33	0
	NaOH	0.35	0
	Glycerol	0	3.21
	Salt	0	0.44
	Water	0	0.03
	Total	3.33	3.33

Table 8.3 Calculation information of capital investment

Items	Values
Year of analysis	2011
Depreciation	15 years
Salvage	5%
Total plant direct cost (TPDC)	
Equipment Purchase Cost (PC)	From references
Installation	0.40 x PC
Process Piping	0.35 x PC
Instrumentation	0.40 x PC
Insulation	0.03 x PC
Electrical Facilities	0.1 x PC
Unlisted equipment purchase cost (UEPC)	0.20 x PC
Unlisted equipment installation	0.35 x UEPCPC
TOTAL PLANT INDIRECT COST (TPIC)	
Engineering	0.25 x TPDC
TOTAL PLANT COST (TPC)	TPDC+TPIC
Contractor's fee	0.05 x TPC
Contingency	0.10 x TPC
Direct fixed capital (DFC)	TPC+ Contractor's fee+ Contingency
Startup and validation cost	5% DFC
Maintenance	1% DFC
Insurance	1% DFC
Local taxes	2% DFC
Factory expense	5% DFC

Table 8.4 The detailed cost report of biodiesel production from raw sludge lipid

Item	Name	Cost ($/yr)
Raw materials	Reactant (Methanol); Lost solvent (Hexane, Acetone, methanol); Catalyst (Sulfuric acid); Neutralizer (Sodium hydroxide); Lipid source (sludge: zero cost)	1 494 000
Equipment	Dryer; conveyor; grinder; extractor; evaporator, storage tank; transesterification reactor; mixer; centrifuge; distillation columns	894 000
Labor	53283 hours per yr	1 648 000
Lab/QC/QA	Laboratory/quality control/quality assurance	134 000
Utilities	Electricity; steam; cooling water; chilled water	864 000
Total		5 034 000
Unit biodiesel cost	9380 tonnes/yr	0.53 $/kg (0.47 $/L)
Revenue (glycerol)	1313 tonnes/yr	28 890
Credit from avoidance of sludge disposal	28.6 tonnes per 260 tonnes; 110 $/per tonne sludge landfilling	972 840
Net unit biodiesel cost	(total cost –revenue-credit)/production rate	0.43 $/kg (0.38 $/L)

Table 8.5 **The detailed cost report of biodiesel production from lipid extracted from microorganism cultivated with sludge**

Item	Name	Cost ($/yr)
Raw materials	Reactant (Methanol); Lost solvent (chloroform, methanol); Catalyst (sodium hydroxide); Neutralizer (HCl); Nutrient medium (sludge: zero cost)	6 026 000
Equipment	Dryer; conveyor; grinder; extractor; evaporator, storage tank; transesterification reactor; mixer; centrifuge; distillation columns	5 782 000
Labor	53283 hours per yr	1 256 000
Lab/QC/QA	Laboratory/quality control/quality assurance	188 000
Utilities	Electricity; steam; cooling water; chilled water	6 693 000
Total		19 946 000
Unit biodiesel cost	31755.817 tonnes/yr	0.63 $/kg (0.55 $/L)
Revenue (glycerol)	4445.814 tonnes/yr	97 808
Credit from avoidance of sludge disposal	104 tonnes per 260 tonnes; 110 $/per tonne sludge landfilling 156 tonnes per 260 tonnes; 77 $/per tonne residual biomass land application (using for stabilization and applying to the land)	4 461 600
Net biodiesel cost	(total cost –revenue-credit)/production rate	0.51 $/kg (0.46 $/L)

Table 8.1 **Summary of the cost estimates**

Case	I (raw sludge as lipid source)			II (microorganism as lipid source)					
	RRL	RRF	RRM	M40		M50		M60	
				RL	RF	RL	RF	RL	RF
Total capital investment (million $)	39.34	39.34	212.83	197.01	197.01	197.01	197.01	197.01	197.01
operating cost (million $/year)	257.19	257.19	1 727.85	1 619.88	1619.88	1619.88	1619.88	1619.88	1619.88
Production rate (tonne/year)	9 380	9 380	21 780	31 756	31 756	40 734	40 734	48 721	48 721
Payback time (years)	11.98	11.98	19.06	11.50	11.50	8.52	8.52	6.77	6.77
Unit production cost	0.53 $/kg (0.47 $/L)	0.53 $/kg (0.47 $/L)	0.90 $/kg (0.80 $/L)	0.63 $/kg (0.55 $/L)	0.63 $/kg (0.55 $/L)	0.49 $/kg (0.43 $/L)	0.49 $/kg (0.43 $/L)	0.41 $/kg (0.36 $/L)	0.41 $/kg (0.36 $/L)
Credits	0.10 $/kg (0.09 $/L)	0.17 $/kg (0.15 $/L)	0.23 $/kg (0.21 $/L)	0.12 $/kg (0.10 $/L)	0.19 $/kg (0.17 $/L)	0.12 $/kg (0.10 $/L)	0.17 $/kg (0.15 $/L)	0.12 $/kg (0.10 $/L)	0.15 $/kg (0.14 $/L)
Net unit production cost	0.43 $/kg (0.38 $/L)	0.36 $/kg (0.32 $/L)	0.67 $/kg (0.59 $/L)	0.51 $/kg (0.45 $/L)	0.43 $/kg (0.38 $/L)	0.37 $/kg (0.33 $/L)	0.32 $/kg (0.28 $/L)	0.29 $/kg (0.26 $/L)	0.26 $/kg (0.22 $/L)

RR1= raw sludge used as lipid source for biodiesel production with residual sludge to landfill; RR2= raw sludge used as lipid source for biodiesel production with residual sludge using as fertilizer; RR3= raw sludge used as lipid source for biodiesel production with residual sludge for oleaginous microorganism cultivation; M40, 50, 60= raw sludge used as oleaginous microorganism cultivation medium to accumulate 40%, 50%, and 60% (w/w) lipid in the microorganism; RL= residual sludge-biomass to landfill; RF= residual sludge-biomass using as fertilizer.

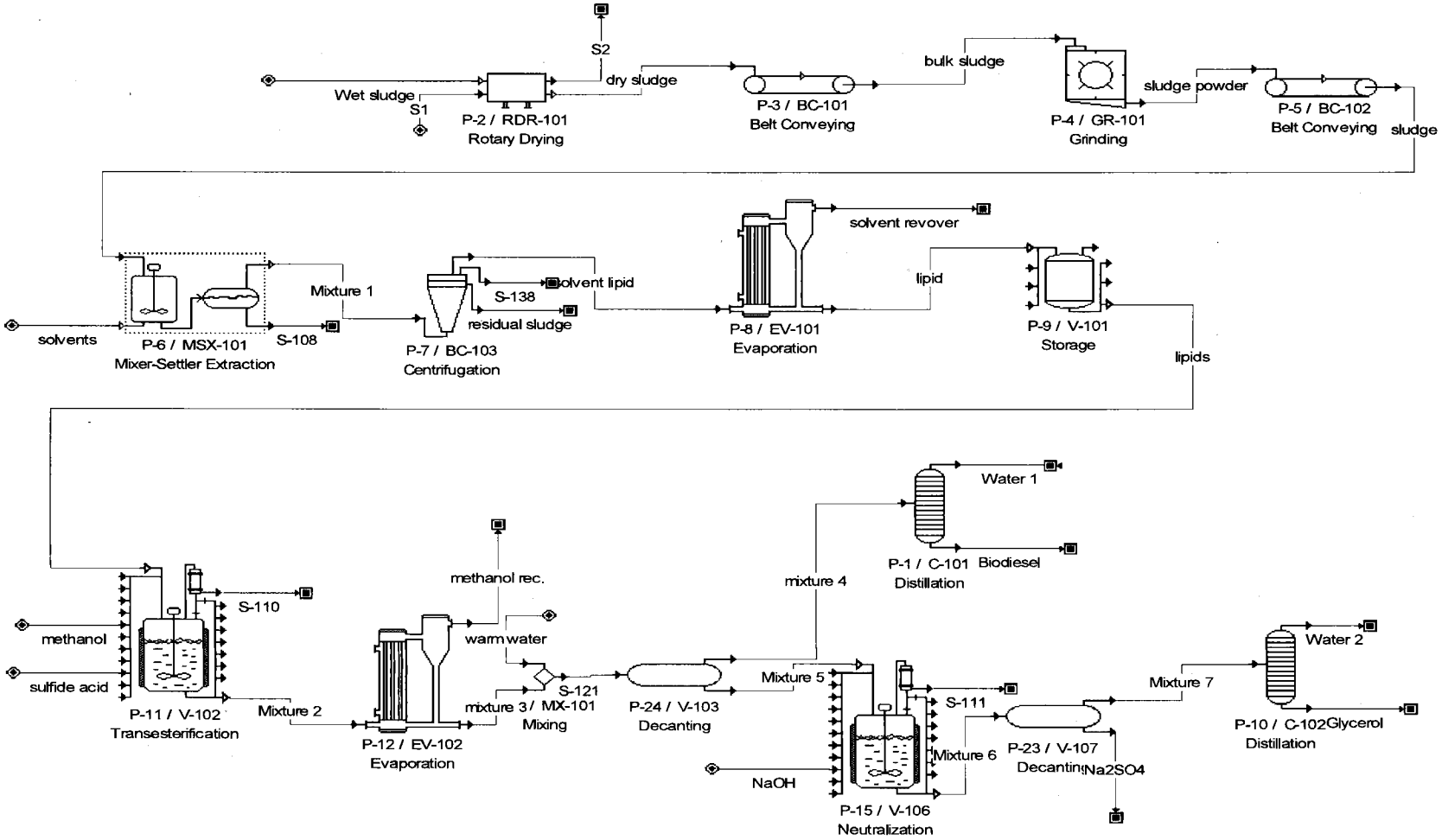

Figure 8.1 **Biodiesel production from sludge lipid**

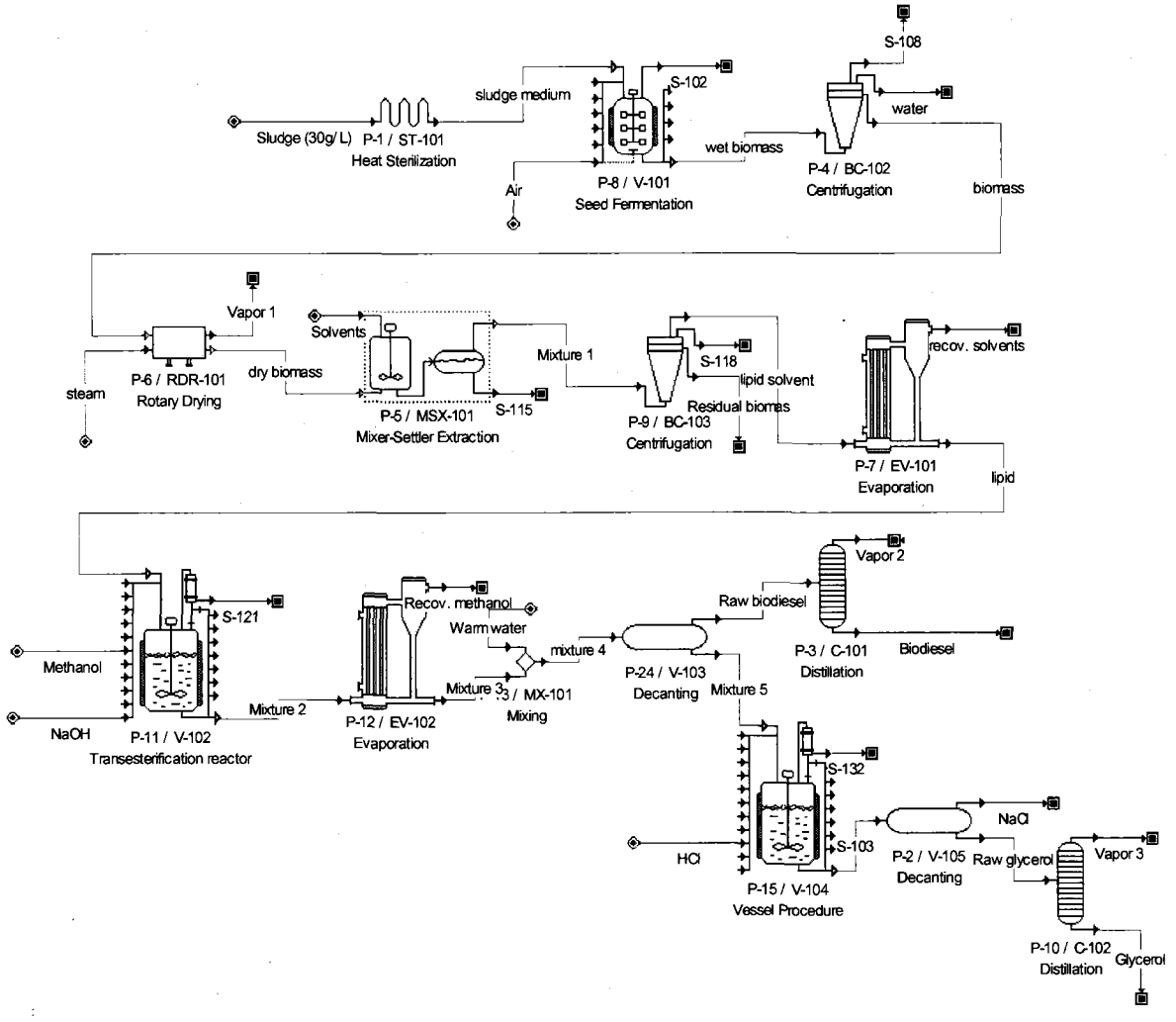

Figure 8.2 **Biodiesel production from lipid accumulated in microorganism cultivated with sludge**

CRUDE GLYCEROL APPLICATION ON THE PRODUCTION OF VALUE ADDED PRODUCT BIODIESEL

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ENVIRONMENTAL SCIENCE & TECHNOLOGY (UNDER REVIEW)

9 CRUDE GLYCEROL APPLICATION ON THE PRODUCTION OF VALUE ADDED PRODUCT BIODIESEL

9.1 Résumé

Une levure oléagineuse *Trichosporon oleaginosus* a été trouvée capable d'accumuler les lipides dans le glycérol brut et purifié. En utilisant le glycérol brut provenant de l'industrie de production de biodiesel, nous avons comparé l'accumulation des lipides dans la souche cultivée sur le glycérol brut, pur et purifié, et nous avons observé que le glycérol purifié offre un rendement similaire à celui du glycérol pur. Une inhibition a été observée en utilisant le glycérol brut. Le glycérol purifié est ensuite utilisé pour déterminer la concentration du glycérol optimale pour obtenir le meilleur rendement en lipides. Le rendement le plus élevé en lipides (0.19 g / g de glycérol) a été obtenu en utilisant une concentration de glycérol purifié de 50 g / L, dans lequel la concentration de la biomasse et la teneur en lipides étaient 11.08 g / L et 47% en poids / poids, respectivement. Le profil des acides gras a révélé que les principaux composés du biodiesel converti à partir de lipides produits à partir de *Trichosporon oleaginosus* cultivée sur le glycérol brut et purifié sont C16: 0 et C18: 1.

Mots clés : Glycérol brut ; glycérol purifié; accumulation de lipides; biodiesel

9.2 Abstract

Lipid accumulation was carried out with an oleaginous yeast *Trichosporon oleaginosus* in crude, purified, and pure glycerol. Crude glycerol was collected from biodiesel production industry. Purified glycerol was obtained from crude glycerol by lowering the pH with addition of H_3PO_4 to convert soap to free fatty acids. The optimal acid addition amount was determined and used to produce the purified glycerol. The results showed that purified glycerol provided similar performance as pure glycerol in lipid accumulation. Inhibition was shown in the usage of crude glycerol. Purified glycerol was later used to determine the optimal glycerol concentration for lipid yield. The highest lipid yield 0.19 g/g glycerol was obtained at 50 g/L purified glycerol in which the biomass concentration and lipid content were 10.75 g/L and 47% w/w, respectively. Fatty acid profiles revealed that C16 and C18 were the major compounds of the biodiesel from the lipid produced by *Trichosporon oleaginosus* cultivated with crude and purified glycerol.

Keywords: Crude glycerol; purified glycerol; lipid accumulation; biodiesel

9.3 Introduction

The dramatic increase in demand of biodiesel resulted in its increased production from various types of oils. Biodiesel production through transesterification of oils and fats generates glycerol as a by-product. About 0.10 to 0.14 kg of glycerol is generated per kilogram of biodiesel produced. It is normally called crude glycerol and is a mixture of glycerol, free fatty acids, soaps, catalyst, salts, methanol etc. The composition of crude glycerol varies from one biodiesel production plant to another and is mainly determined by the feedstock oil composition and quality, the oil and methanol molar ratio used in transesterification, type of catalyst used, and the detailed procedure such as with or without methanol recovery. Generally, the major fraction of the crude glycerol is glycerol (20 to 96% w/w), and other impurities such as water, methanol, and soap (in alkaline catalytic process).[1, 2]

Crude glycerol is a complex material, and the proper utilization to attain its maximum value is desirable for its appropriate handling. Purification of crude glycerol was the most applied method before biodiesel boom.[3] However, due to a substantial decrease in the price of purified glycerol (1.54 US$/kg before 2000 and 0.66 US$/kg after 2007),[3] the purification is getting less attractive. Therefore, direct use or partial purification of crude glycerol is becoming promising. Use of crude glycerol for biogas production through anaerobic digestion has been reported.[4, 5] Bioconversion of glycerol to lipids for biodiesel production is another interesting way of utilization of original or partially purified crude glycerol. Oleaginous microorganisms such as *Schizochytrium limacinum*, *Yarrowia lipolytica*, *Rhodotorula glutinis*, and *Cryptococcus curvatus* are able to utilize glycerol as carbon source to produce lipids.[6-9]

In this study, the composition of crude glycerol from a biodiesel production plant was determined. The treatment of crude glycerol to remove the large amount soap was performed, and the resulting glycerol was subjected to lipids production. Free fatty acids derived from soap of crude glycerol were tested to produce biodiesel with acid catalyst.

9.4 Materials and methods

9.4.1 Materials

Crude glycerol was kindly provided by a biodiesel production plant, in Quebec, Canada. Oleaginous microorganism *Trichosporon oleaginosus* (ATCC20509) was employed in this study.

9.4.2 Crude glycerol characterization

Density and pH: The density of crude glycerol was determined at room temperature. To determine the pH, 1.0 g of crude glycerol was dissolved in 50 mL of deionized (DI) water. The pH of the solution was measured by a digital pH meter at room temperature.[1]

Glycerol content: The glycerol content was determined according to the method reported.[10] 3.5-diacetyl-1.4-dihydrolutidine, a yellow complex, was formed in a two-step reaction. Glycerol reacted with sodium periodate and the formed formaldehyde, thereafter, reacted with acetyl acetone to form the complex of 3.5-diacetyl-1.4-dihydrolutidine. The complex was measured by UV-Vis Spectrophotometer at 410 nm. The glycerol content was calculated according to standard curve (=0.05645×conc.-0.07437; R^2=0.99534).

Soap content: The soap content was estimated as reported.[9] The pH of 50 g crude glycerol was adjusted to1.0 with 85% H_3PO_4. After well mixing, the solution was centrifuged at 5000 rpm (1677 *g*) for 20 min. The top red dark layer was free fatty acids (FFAs). The soap content was calculated according to FFA amount =304×FFA amount/282; where 304 is average soap molar mass and 282 is average FFA molar mass.

Biodiesel content: 5 mL of 5% NaCl was added to 2 g of crude glycerol. 5 mL hexane was used to extract biodiesel from the mixture, and the extraction was performed two times.[11] The hexane layer (top layer) of the two extractions was collected together into a pre-weighed glass tube (W_1). After evaporation of hexane with nitrogen gas, the tube was weighed (W_2). Biodiesel content was calculated as (W_2-W_1)/2×100%.

Ash content: 10 g of crude glycerol was subjected at 750 °C for 3 h.[12] After the sample was cooled down to room temperature, the residual (W_3) was measured and then the ash content was calculated (W_3/10×100%).

NaOH was the catalyst used in transesterification process in the biodiesel production site. 10 g of crude glycerol was adjusted to pH 7 with 1 M HCl and the consumed acid 1 M HCl volume (V) was recorded and used to calculate the NaOH content (=40×1×V/10; where 40 is NaOH molar mass, 1 is HCl molar concentration, V is the volume of 1 M HCl consumed to bring the pH to 7; and 10 is crude glycerol amount) in crude glycerol.

Methanol content: The methanol content was determined with Heidolph Laborota 4011 digital evaporator at 60 °C. 100 mL (107.3 g) of crude glycerol was subjected to the evaporation for 15 min. The evaporated methanol (W_4) was collected and the content was calculated as $W_4/107.3\times100\%$.

Water content: 10 g of crude glycerol was subjected to 105 °C till the weight was constant (W_5). The water and methanol content was calculated as $[(10-W_5)/10\times100\%]$. After subtracting methanol content, water content was obtained.

9.4.3 Soap conversion to free fatty acid (FFAs)

Different volume (1, 2, 3, 4, 5, 6, 7, 8, and 9 mL) of 85% phosphoric acid was added to 40 mL of crude glycerol, respectively, to determine the optimal acid addition for conversion of soap to FFAs. After well mixing, the pH was measured. Then the mixtures were allowed to separate into three layers with the top layer as FFAs, the middle layer precipitate (salt), and the bottom layer (glycerol). The separated glycerol was used for lipids production.

9.4.4 Lipids production with glycerol

Crude and purified glycerol (obtained by removing soap) was investigated for lipid production by oleaginous microorganism *Trichosporon oleaginosus*. *Trichosporon oleaginosus* was first grown in pre-culture (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) for 24 h, then inoculated to glycerol medium (10% v/v). The glycerol medium contains (per liter): 2.7 KH_2PO_4, 0.95 Na_2HPO_4, 0.404 NH_4Cl, 0.2 $MgSO_4\cdot7H_2O$, 0.1 yeast extract, 0.1 EDTA, 0.04 $CaCl_2\cdot2H_2O$, 0.0055 $FeSO_4\cdot7H_2O$, 0.0052 citric acid$\cdot H_2O$, 0.001 $ZnSO_4\cdot7H_2O$, and 0.00076 $MnSO_4\cdot H_2O$,[8, 13] and 25 g crude, purified, or pure (Certified ACS, Fisher Scientific) glycerol. Purified glycerol was used to study glycerol concentration (25, 50, 75, and 100 g/L) effect on lipid accumulation on *Trichosporon oleaginosus*. The pH of all medium was adjusted to 6.5 and then sterilized at

121°C for 15 min prior to inoculation. The fermentation was performed with shake flasks under aerobic conditions in a shaking incubator at 28 ºC and 170 rpm.

9.4.5 Residual glycerol analysis

The residual glycerol after fermentation was analyzed with the same method as described previously for crude glycerol characterization.

9.4.6 Lipid extraction from yeast biomass

The standard chloroform and methanol extraction procedure with minor modification was employed to determine the lipid content in the biomass.[14, 15] Biomass was harvested from the fermented broth by centrifugation at 5000 rpm for 15 min, washed 2 times with distilled water, and then dried by lyophilisation. 200 mg dry biomass (lyophilised) was mixed with 4 ml solvent mixture of chloroform and methanol (2:1 v/v), and then subjected to 60 °C for 4 h. The mixture was then centrifuged at 5000 rpm for 15 min and the supernatant solvent phase was withdrawn and transferred into a pre-weighed glass vial (W_6). The extraction procedure was repeated two times. Afterwards, the vial containing the total volume of the supernatant collected from each extraction was subjected to evaporation in 60 °C oven and then weighed (W_7). The lipid amount was calculated by the difference of W_6 and W_7. The lipid content in the biomass was calculated as (W_7-W_6)/200 mg ×100%. The obtained lipid was then converted to biodiesel through transesterification.

9.4.7 Free fatty acids content in lipids extracted from biomass

The titration method was used to determine FFA content in the lipids.[16] Samples collected at 48 h fermentation were used to determine FFAs content in lipids. The extraction method of lipid is the same as described above (Lipid Extraction section). The extracted lipids obtained in vials was dissolved in 5 mL hexane and transferred to a 100 mL conical flask. Hexane was then evaporated at 60 °C. 10 mL of mixture of chloroform: methanol 2:1 v/v was added to the lipids in conical flask and then two drops of phenolphthalein were added. 10 mL of mixture of chloroform: methanol 2:1 v/v with two drops phenolphthalein was added to a dry conical flask used as blank. 0.01 N KOH filled in 25 mL burette was then added to the conical flask drop by drop with gentle shaking the flask in a swirling manner. The titration was ended when a pink

colour was observed and persisted at least for 5 seconds. Thereafter, the volume of KOH used was recorded to calculate the FFA content using Equation 9.1.

Equation 9.1 **FFA content as oleic acid (%) = $28.2 \times N \times (V-B)/W_{lipid} \times 100\%$**

Where V = the volume (mL) of titration solution; B = the volume (mL) of the blank; N = the normality of the titration solution (KOH); W_{lipid} = the weight of the oil sample (grams).

9.4.8 Esterification of free fatty acids and transesterification of lipids

The FFAs obtained from soap (as described above) were converted to fatty acids methyl esters (FAMEs, biodiesel) by reacting with methanol in the absence or presence of acid. 5 mL of acidic (sulfuric acid 2% v/v in methanol) methanol was added to 0.2 g of the FFAs. The mixture was then subjected to 50 °C for 24 h. After reaction (24h), 5% NaCl solution was added (100 mL per gram lipids), and then FAMEs was extracted by washing two times with hexane (100 mL per gram lipid), and the hexane was recovered by phase separation (upper layer). The FAMEs in hexane was washed with 2% sodium bicarbonate (20 mL per gram lipid), and the top layer was then dried in oven at 60 °C.[17]

The lipids obtained by solvent extraction from *Trichosporon oleaginosus* in vials was first dissolved in hexane (5 mL), then mixed with methanol. Lipid to methanol molar ratio was 1:6 (0.3 mL methanol for per gram lipid). Sodium hydroxide (0.5 %w/w oil) was used as catalyst. The mixture was then subjected to 55 °C in oil bath for 2 h. 1.3-dichlorobenzene was used as internal standard with a concentration of 50 ppm. The procedure of FAMEs recovery was similar as that of FAMEs converted from FFAs (see above).

The FAMEs in hexane were analyzed using a Gas Chromatography Linked to Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500). The dimensions of the column used were 30 m × 0.25 mm, with a phase thickness of 0.2 μm. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1.3-dichlorobenzene was also used as internal standard with a concentration of 50 ppm.

All the experiments were performed in triplicate and average results were reported with standard deviation less than 5%.

9.5 Results and discussion

9.5.1 Crude glycerol composition

The composition of crude glycerol was determined and the results were given in Table 9.1. It was observed that the crude glycerol had low glycerol content and high soap content. It would be due to the high content of FFAs in the feedstock of alkaline catalytic biodiesel production. Soap could be an inhibitor of cell growth as it can attach on cells and interfere to the nutrient transportation from fermentation medium to cell bodies. Therefore, soap removal was performed

9.5.2 Free fatty acids recovery from soap

The high soap content in crude glycerol (Table 9.1) is due to presence of high concentration of FFAs in the feedstock oil. In alkaline condition (pH >7), FFAs react with base (NaOH or KOH) to form soap (the equilibrium of the reaction of Equation 9.2 is shifted to right). On the contrary, FFAs will be released due to the dissociation of soap (the equilibrium of the reaction of Equation 9.2 is shifted to left) at low pH (FFAs recovery process by lowering pH of the crude glycerol).

Equation 9.2 $R_X\text{-COOH} + \text{NaOH/KOH} \leftrightarrow R_X\text{-COONa/K}$

This study investigated the optimal amount of acid required for FFAs recovery (Table 9.2). The addition of acid in the crude glycerol resulted in pH reduction. After pH was less than 7 and the reaction mixture was allowed to stand, three layers were observed (the top layer as FFAs, the middle layer as salt precipitates, and the bottom layer as glycerol). This observation was different from some other reports, which obtained the middle layer as glycerol and bottom layer as salt precipitates.[18, 19] It would be due to the difference of the composition of the crude glycerol, which would lead to the variation in density of the precipitates. There is 8.11 g of FFA

in 40 mL crude glycerol (pH 1). The phase separation of samples 2 to 10 with addition of 85% H_3PO_4 started after 15 min settling, and completed in around 72 h. With addition of 1 mL of 85% H_3PO_4 (sample 1), layer separation was not observed until 3 h. The FFAs amount obtained from samples 2 to 10 was almost the same (Table 9.2), and there was only 1.32 g FFAs obtained in sample 1. Compared to other samples, sample 2 (2 mL of 85% H_3PO_4 in 40 mL of crude glycerol) gave comparable FFAs recovery efficiency (99.2% w/w) and purest glycerol (54.96% w/w) with shorter time of settling (36 h). Therefore, 2 mL acid addition to 40 mL crude glycerol is considered the optimal acid requirement for FFAs recovery when gravity settling is used for phase separation.

9.5.3 Conversion of free fatty acids to biodiesel

Due to the fact that FFA consumes alkaline catalyst (and gets converted into soap), acid catalyst can be used in the esterification process. In the process of FFA recovery from soap, acid H_3PO_4 was added to lower the pH. It suggested that the acid was present in the system and therefore, FAMEs could be formed by addition of methanol alone. Thus, this study investigated the FAMEs formation with and without addition of acid H_2SO_4 and observed that the FAMEs yield (g FAMEs/g FFAs) was 90.8% and 32.1% respectively.

According to calculations, the H^+ present in FFA is 0.49 mmol per 0.2 g FFA. 5 mL methanol was added to react with 0.2 g FFA; therefore, the H^+ concentration in the system is 0.10 mol/L. With addition of acid, H^+ concentration in the system is increased to 0.75 mol/L. Esterification is a reversible equilibrium reaction and H^+ concentration determines the equilibrium and hence the rate of reaction and conversion. The low conversion of FFAs in the reaction without acid addition would be due to the low concentration of H^+. In order to understand if the reaction time was sufficient, the time was prolonged from 24 h to 48 and 72 h and the biodiesel yield (g FAMEs/ g FFAs) was increased from 32.1% to 38.7% and 39.1%, respectively. It is thus suggested that the reaction reached its equilibrium in about 48 h in its existing condition (5 mL methanol per 0.2 g FFAs; catalyst amount 0.1 mol H^+/L; reaction temperature 50 °C).

The obtained biodiesel contains mainly C18 and C16 with little amount of C14. In the reaction without acid (as catalyst) addition, FAMEs consists of 58.3% C18, 37.1% C16, and 2.9% C14. In

case with acid (as catalyst) addition, FAMEs contains 62.5% C18, 27.9% C16, and 0.7% C14. FAMEs produced from FFA are similar as biodiesel produced from palm oil (55-68% C18 and 32-45% C16).[20] It suggested that the FAMEs generated are suitable to use as biodiesel.

9.5.4 Effect of glycerol type on the biomass production

Three different types of glycerol: pure, crude and purified (treated crude glycerol, Table 9.1) at the same concentration (25 g/L), produced 10.90, 10.32, and 7.26 g/L of biomass, respectively, at 72 h (Figure 9.1). The results showed a similar trend of yeast growth, lipids accumulation and glycerol consumption irrespective of type of glycerol used. The purified glycerol was more suitable for the biomass production compared to crude glycerol as also reported in the literature.[21] It would be due to the similarity in composition of purified glycerol and pure glycerol. A lag period was observed in biomass growth (Figure 9.1) which would be due to the fact that the inoculum was not grown in the same medium as the growth medium, and thus an adaptation period was required by the strain in the new medium. In order to avoid the lag period, the same medium should be used to produce the inoculum and biomass.

In crude glycerol, soap content was around 21% w/w. Both the soap and the cell surface are polar, and thus the soap could easily attach to the cells surface. The cell growth is negatively impacted when the cells are surrounded by soap layer, which can cause the inhibition of the nutrients transfer. The purified glycerol was obtained from crude glycerol after soap removal. This would be the reason of higher biomass density observed in the purified glycerol than in crude glycerol (Table 9.3). Contrary to pure glycerol, methanol is also present in the purified glycerol. However, it wouldn't be the cause of a slightly low biomass concentration observed in purified glycerol (10.32 g/L) comparing to pure glycerol (10.9 g/L) cultivation. The boiling point of methanol is 65 °C and sterilization of the medium by autoclaving at 121 °C for 15 min could eliminate methanol from the medium.[22] On the other hand, it has been reported that metals may inhibit biomass growth and led to a lower biomass density while cultivated in the purified glycerol than pure glycerol.[23] Therefore, the difference on biomass density in pure and purified glycerol could be due to the presence of impurities such as metals (derived from feedstock oil or the chemicals added during the biodiesel production process) in purified glycerol. FFAs, soap,

and metals are reported to be the reason of low biomass density with the crude glycerol medium comparing to that of pure glycerol. [21-22]

The highest maximum specific growth rate (μ_{max}) was observed with pure glycerol (0.035 h^{-}) as raw material followed by 25 g/L purified glycerol (0.034 h^{-}) and crude glycerol (0.025 h^{-}) (Table 9.3). A slightly lower μ_{max} value in purified glycerol than pure glycerol (Table 9.3) might be due to the impurities (metals etc.) presence in the purified glycerol. A significant difference of μ_{max} was observed in the medium with crude glycerol as raw material, which was due to a large amount of soap (impacting on transportation of nutrients) present in the crude glycerol (Table 9.3).

9.5.5 Effect of glycerol concentration on the biomass production

Purified glycerol provided high biomass concentration at the end of the fermentation process compared with the crude glycerol; therefore, purified glycerol was further used to investigate glycerol concentration effect on the biomass and lipid production by *Trichosporon oleaginosus.* , The biomass concentration increased with fermentation time (Figure 9.1). Increase in glycerol concentration from 25 to 50 g/L, the maximum biomass concentration slightly increased while further increase in glycerol concentration decreased the biomass concentration significantly (Table 9.3). A decrease in biomass concentration at high glycerol concentration was due to substrate inhibition. Similar results have been reported by other researchers.[8] In their study, *Cryptococcus curvatus* (the previous name of *Trichosporon oleaginosus*) was grown in pure glycerol with concentration from 8 to 256 g/L. The growth was restricted when the concentration of glycerol was higher than 64 g/L. An inhibitory impact of high glycerol concentration (60, 80, and 100 g/L) on growth of *Schizochytrium limacinum* (oleaginous microalgae) was also observed whereas at low glycerol concentration (25 and 35 g/L) enhanced growth .[9] However, different results were reported as well; oleaginous yeast *Yarrowia lipolytica* was not influenced by glycerol concentration in the range from 20 to 164 g/L.[7] For microalgae cultivation, the maximum biomass density (around 14 g/L) was obtained at a wide glycerol concentration range (from 35 to 85 g/L).[21] Glycerol concentration effect on biomass production occurs not only with respect to different types of microorganisms (microalgae or yeast) used to cultivate, but also in the same types microorganisms. It indicates that each microorganism has

their own feature in utilization of glycerol. The maximum biomass density (around 10.75 g/L) was obtained at glycerol concentration of 50 g/L after 72 h fermentation, which was almost the same as 25 g/L pure glycerol (10.90 g/L) or 10.3 g/L biomass at 25 g/L of purified glycerol at 72 h. It indicated that the biomass production of *Trichosporon oleaginosus* should be conducted at 25 g/L purified glycerol concentration. Increase in glycerol concentration did not appreciably increase the biomass concentration.

9.5.6 Lipid accumulation in oleaginous microorganism with glycerol

The time course of biomass concentration, lipid accumulation, and glycerol consumption with different type of glycerol is shown in Figure 9.1. The glycerol was completely consumed with 25 g/L concentration at around 72 h when pure and purified glycerol was used. After glycerol exhaustion (72 h), biomass concentration and lipid content started to decrease, which is due to the lipid consumption to maintain the cell activities. In addition, purified glycerol (44% w/w biomass) provided a little lower maximum lipid content as pure glycerol (49% w/w biomass). In case of crude glycerol (25 g/L), glycerol was still left at the end of the fermentation (120 h). It is due to the inhibition of soap that attaches on cell surface (as said before) and reduced the nutrient transportation rate. The biomass ($Y_X/_G$) and lipid ($Y_L/_G$) yields for *Trichosporon oleaginosus* while grown in different types of glycerol at 25 g/L concentration displayed the following trend, pure glycerol > purified glycerol > crude glycerol (Table 9.3). Similar trend is also reported by other researchers.[7, 9]

Moreover, it was observed that the biomass and lipid didn't increase significantly even though glycerol still remained in the medium for glycerol concentration 50, 75, and 100 (Figure 9.1). This may be due to the fact that inhibitors (such as toxic protein or ethanol) may be produced along with cell growth.[24] In order to eliminate the inhibition problem, fed-batch process approach can be adopted instead of the batch process; the concentrations of inhibitors are diluted during feeding process of a fed-batch culture. Some researchers have reported very high biomass concentration (more than 100 g/L) in fed batch fermentation.[8]

Biomass yield decreased with increase of purified glycerol concentration, and the highest value was 0.42 g/g glycerol consumed for 25 g/L and the lowest is 0.28 g/g glycerol consumed for

100 g/L glycerol concentration. Many reports have stated that environmental stress such as substrate concentration, temperature, and pH, enhance lipid accumulation.[25-27] It would be why the high lipid content $Y_{L/X}$ (g/g) was observed at high glycerol concentration (75 and 100 g/L glycerol) than in 50 g/L glycerol (Table 9.3). The greatest lipid yield (0.19 g/g glycerol) occurred at 50 g/L purified glycerol concentration. For 25 and 75 g/L glycerol concentration, the lipid yield (0.18 g/g glycerol) was slightly different from 50 g/L glycerol concentration (0.19 g/g), but it was substantially decreased to 0.13 g/g at glycerol concentration 100 g/L. It suggested that the best utilization of the purified glycerol by *Trichosporon oleaginosus* was at 50 g/L glycerol concentration. The results were different from reported by other researchers, in which 90 to 100 g/L glycerol gave the highest lipid yield.[28, 29] The difference could be due to the different strains employed and the glycerol composition.[29, 30] The lipid yield of different strains cultivated in glycerol medium by different researchers is summarized in Table 9.4. It clearly displays that the lipid yield obtained in this study is comparable with other studies. Purified glycerol can be utilized as a carbon source for lipid production from *Trichosporon oleaginosus*.

9.5.7 Fatty acid profile of biomass extracted lipid

The FFAs content were 0.44%, 1.19%, 0.46%, 0.44%, 0.43%, and 0.44% w/w on lipids derived from *Trichosporon oleaginosus* cultivated in 25 g/L pure glycerol, 25 g/L crude glycerol, and 25, 50, 75 and 75 g/L purified glycerol, respectively, for 72 h samples. The FFA content obtained in this study (less than 1.2% w/w lipids) was significantly different from those (9% w/w lipids) observed by other researchers.[31] This would be due to the different treatment of the samples. In this study, the wet biomass was dried by lyophilisation, which preserved the nature of the lipids, and then lipids were extracted with solvent. The extracted fresh lipid was directly used to determine FFA content without storage, which prevented the risk of triacylglycerol (TAG) decomposition to FFAs. In the previous study,[31] the fermentation broth was homogenized (the risk of degradation) followed by solvent extraction of lipid from wet yeast cells with no indication if fresh lipid was used for FFA content determination. A study has reported that storage of microbial lipid above freezing for 24 h could increase the FFA content from less than 0.1 to 20% w/w lipid and decreasing the TAG content from 72 to 51% w/w lipid.[32] It clearly indicated that TAG was degraded to FFA during storage.

Comparing the lipid obtained using different types of glycerol, relatively high FFA content was found when crude glycerol (1.19% w/w total lipid) was used as raw material as above descripted. This would be due to presence of FFA in crude glycerol. As discussed above, FFA existed in the crude glycerol medium due to soap dissociation at pH 6.5 (Table 9.2), therefore, FFA could attach onto cell surface. During cell harvesting, washing was performed twice with distilled water, yet FFA was not soluble in water and would remain stick to the cell surface. Thereafter, FFA was extracted along with the lipid accumulated in the cells during organic solvent extraction. It would finally result in a high FFA content in the lipid derived from the biomass grown on crude glycerol. The FFA content of lipid extracted from biomass grown on pure and purified glycerol was almost the same, and the purified glycerol concentration (25 to 100 g/L) didn't impact the FFA content (0.46%, 0.44%, 0.43%, and 0.44% w/w total lipid for 25, 50, 75 and 75 g/L purified glycerol, respectively). For all extracted lipids irrespective of glycerol type used to grow the biomass, the FFA content was lower than 2% w/w lipid, hence alkaline NaOH could be used as catalyst in the transesterification process.

The fatty acid profile of the lipid extracted from biomass is shown in Table 9.5. The majority of fatty acids are C16:0 and C18:1, which is similar to Jatropha seed oil (currently used in commercial biodiesel production practice). It suggests that the lipid from *Trichosporon oleaginosus* cultivated with glycerol is suitable in usage as biodiesel production feedstock. The saturation rate (the sum of Cn:0) of the lipid is around 30 to 40% w/w total lipid. The high rate improves oxidation stability of the biodiesel produced from the lipid.

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Notes

The authors declare no competing financial interest.

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9.7 References

(1) Hu, S.; Luo, X.; Wan, C.; Li, Y. Characterization of crude glycerol from biodiesel plants. J. Agric. Food. Chem. 2012, 60 (23), 5915-5921.

(2) Hansen, C. F.; Hernandez, A.; Mullan, B. P.; Moore, K.; Trezona-Murray, M.; King, R. H.; Pluske, J. R. A chemical analysis of samples of crude glycerol from the production of biodiesel in australia, and the effects of feeding crude glycerol to growing-finishing pigs on performance, plasma metabolites and meat quality at slaughter. Anim. Prod. Sci. 2009, 49 (2), 154-161.

(3) Yang, F.; Hanna, M.; Sun, R. Value-added uses for crude glycerol--a byproduct of biodiesel production. Biotechnol. Biofuels 2012, 5 (1), 1-10.

(4) Robra, S.; Serpa da Cruz, R.; de Oliveira, A. M.; Neto, J. A. A.; Santos, J. V. Generation of biogas using crude glycerin from biodiesel production as a supplement to cattle slurry. Biomass Bioenergy 2010, 34 (9), 1330-1335.

(5) Kolesarova, N.; Hutnan, M.; Bodik, I.; Spalkova, V. Utilization of biodiesel by-products for biogas production. J. Biomed. Biotechnol. 2011, 2011.

(6) Easterling, E. R.; French, W. T.; Hernandez, R.; Licha, M. The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of rhodotorula glutinis. Bioresour. Technol. 2009, 100 (1), 356-361.

(7) Papanikolaou, S.; Aggelis, G. Lipid production by yarrowia lipolytica growing on industrial glycerol in a single-stage continuous culture. Bioresour. Technol. 2002, 82 (1), 43-49.

(8) Meesters, P. A. E. P.; Huijberts, G. N. M.; Eggink, G. High-cell-density cultivation of the lipid accumulating yeast cryptococcus curvatus using glycerol as a carbon source. Appl. Microbiol. Biotechnol. 1996, 45 (5), 575-579.

(9) Liang, Y.; Sarkany, N.; Cui, Y.; Blackburn, J. W. Batch stage study of lipid production from crude glycerol derived from yellow grease or animal fats through microalgal fermentation. Bioresour. Technol. 2010, 101 (17), 6745-6750.

(10) Bondioli, P.; Della Bella, L. An alternative spectrophotometric method for the determination of free glycerol in biodiesel. Eur. J. Lipid Sci. Technol. 2005, 107 (3), 153-157.

(11) Dufreche, S.; Hernandez, R.; French, T.; Sparks, D.; Zappi, M.; Alley, E. Extraction of lipids from municipal wastewater plant microorganisms for production of biodiesel. J. Amer. Oil Chem. Soc. 2007, 84 (2), 181–7.

(12) Manosak, R.; Limpattayanate, S.; Hunsom, M. Sequential-refining of crude glycerol derived from waste used-oil methyl ester plant via a combined process of chemical and adsorption. Fuel Process. Technol. 2011, 92 (1), 92-99.

(13) Zheng, Y.; Chi, Z.; Ahring, B. K.; Chen, S. Oleaginous yeast cryptococcus curvatus for biofuel production: Ammonia's effect. Biomass Bioenergy 2012, 37 (0), 114-121.

(14) Folch, J.; Lees, M.; G.H., S. S. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 1957, 226 (1), 497-509.

(15) Vicente, G.; Bautista, L. F.; Rodríguez, R.; Gutiérrez, F. J.; Sádaba, I.; Ruiz-Vázquez, R. M.; Torres-Martínez, S.; Garre, V. Biodiesel production from biomass of an oleaginous fungus. Biochem. Eng. J. 2009, 48 (1), 22-27.

(16) Woyewoda, P. J. K., A.D. ; Regier, L. W.; Ackman, R. G. An improved titrametric method for determination of free fatty acid in fish oils. Environment Canada Fisheries and Marine Service Technology Branch Halifax- Nova Scotia 1976, New Series Circular No. 61, November 1976.

(17) Halim, R.; Gladman, B.; Danquah, M. K.; Webley, P. A. Oil extraction from microalgae for biodiesel production. Bioresour. Technol. 2011, 102 (1), 178-185.

(18) Swearingen, T. Separating glycerine/ffas. 2006. Avaiable at <http://journeytoforever.org/biodiesel_glycsep.html> (accessed Dec. 2012)

(19) Kelly, T. Crude glycerine and hot compost. 2006. Avaiable at <http://www.mail-archive.com/sustainablelorgbiofuel@lists.sustainablelists.org/msg63336.html> (accessed Dect. 2012)

(20) Ekpa, O. D.; Fubara, E. P.; Morah, F. N. I. Variation in fatty acid composition of palm oils from two varieties of the oil palm (eelaeis guineensis). J. Sci. Food Agric. 1994, 64 (4), 483-486.

(21) Liang, Y.; Cui, Y.; Trushenski, J.; Blackburn, J. W. Converting crude glycerol derived from yellow grease to lipids through yeast fermentation. Bioresour. Technol. 2010, 101 (19), 7581-7586.

(22) Pyle, D. J.; Garcia, R. A.; Wen, Z. Producing docosahexaenoic acid (dha)-rich algae from biodiesel-derived crude glycerol: Effects of impurities on dha production and algal biomass composition. J. Agric. Food. Chem. 2008, 56 (11), 3933-3939.

(23) Capone, D. G.; Reese, D. D.; Kiene, R. P. Effects of metals on methanogenesis, sulfate reduction, carbon dioxide evolution, and microbial biomass in anoxic salt marsh sediments. Applied and Environmental Microbiology 1983, 45 (5), 1586-1591.

(24) Nevoigt, E. Progress in metabolic engineering of saccharomyces cerevisiae. Microbiology and Molecular Biology Reviews 2008, 72 (3), 379-412.

(25) Dyal, S. D.; Bouzidi, L.; Narine, S. S. Maximizing the production of γ-linolenic acid in mortierella ramanniana var. Ramanniana as a function of ph, temperature and carbon source, nitrogen source, metal ions and oil supplementation. Food Res. Int. 2005, 38 (7), 815-829.

(26) Ruangsomboon, S. Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, botryococcus braunii kmitl 2. Biomass Bioenergy 2012, 109 (0), 261-265.

(27) Tai, M.; Stephanopoulos, G. Engineering the push and pull of lipid biosynthesis in oleaginous yeast yarrowia lipolytica for biofuel production. Metab. Eng. 2013, 15 (0), 1-9.

(28) Kitcha, S.; Cheirsilp, B. Screening of oleaginous yeasts and optimization for lipid production using crude glycerol as a carbon source. Energy Procedia 2011, 9 (0), 274-282.

(29) Yen, H.-W.; Yang, Y.-C.; Yu, Y.-H. Using crude glycerol and thin stillage for the production of microbial lipids through the cultivation of rhodotorula glutinis. J. Biosci. Bioeng. 2012, 114 (4), 453-456.

(30) Saenge, C.; Cheirsilp, B.; Suksaroge, T. T.; Bourtoom, T. Potential use of oleaginous red yeast rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. Process Biochem. 2011, 46 (1), 210-218.

(31) Thiru, M.; Sankh, S.; Rangaswamy, V. Process for biodiesel production from cryptococcus curvatus. Bioresour. Technol. 2011, 102 (22), 10436-10440.

(32) Chen, L.; Liu, T.; Zhang, W.; Chen, X.; Wang, J. Biodiesel production from algae oil high in free fatty acids by two-step catalytic conversion. Bioresour. Technol. 2012, 111 (0), 208-214.

(33) André, A.; Chatzifragkou, A.; Diamantopoulou, P.; Sarris, D.; Philippoussis, A.; Galiotou-Panayotou, M.; Komaitis, M.; Papanikolaou, S. Biotechnological conversions of bio-diesel-derived crude glycerol by yarrowia lipolytica strains. Eng. Life Sci. 2009, 9 (6), 468-478.

(34) Chatzifragkou, A.; Makri, A.; Belka, A.; Bellou, S.; Mavrou, M.; Mastoridou, M.; Mystrioti, P.; Onjaro, G.; Aggelis, G.; Papanikolaou, S. Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species. Energy 2011, 36 (2), 1097-1108.

(35) O'Grady, J.; Morgan, J. Heterotrophic growth and lipid production of chlorella protothecoides on glycerol. Bioprocess. Biosyst. Eng. 2011, 34 (1), 121-125.

(36) Xu, J.; Zhao, X.; Wang, W.; Du, W.; Liu, D. Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast rhodosporidium toruloides and the individual effect of some impurities on lipid production. Biochem. Eng. J. 2012, 65 (0), 30-36.

Table 9.1 Composition of crude and purified glycerol

Items	Crude glycerol	Purified glycerol
Glycerol content (% w/w)	31.8 ± 0.3	55.0 ± 0.2
Soap content (% w/w)	21.1 ± 0.3	ND
Catalyst content (NaOH) (% w/w)	2.8 ± 0.2	ND
Biodiesel content (% w/w)	1.2 ± 0.0	1.5 ± 0.0
Ash (% w/w)	2.3 ± 0.1	4.2 ± 0.2
Methanol (% w/w)	15.3 ± 0.3	18.5 ± 0.6
Water (% w/w)	24.4 ± 0.2	20.8 ± 0.8
pH	8.93 ± 0.04	3.93 ± 0.25
Density (g/mL)	1.073 ± 0.06	1.101 ± 0.03

ND=not detected.

Table 9.2 **Free fatty acid recovery from crude glycerol**

Sample	Crude glycerol (mL)	Acid (H_3PO_4) addition amount (mL)	pH	Free fatty acid (g)	Free fatty acid recovery efficiency (%)	Glycerol content (% w/w)
1	40	1	6.60	1.32	5.5	43.11 ± 0.41
2	40	2	3.93	8.04	99.2	54.96 ± 0.24
3	40	3	3.55	8.04	99.2	53.66 ± 0.38
4	40	4	3.22	8.05	99.3	52.49 ± 0.16
5	40	5	3.16	8.06	99.4	51.34 ± 0.25
6	40	6	3.14	8.08	99.7	50.25 ± 0.22
7	40	7	2.97	8.09	99.8	49.26 ± 0.19
8	40	8	2.76	8.09	99.8	48.33 ± 0.36
9	40	9	2.74	8.09	99.8	47.12 ± 0.22
10	40	10	2.70	8.09	99.8	46.18 ± 0.20

Table 9.3 **Growth and lipids production parameters for *Trichosporon oleaginosus* grown in different types of glycerol**

Glycerol type	Gly_0 (g/L)	C/N ratio	Time (h)	Gly_t (g/L)	X (g/L)	$P_{X/V-T}$ (g/L-d)	L (g/L)	$P_{L/V-T}$ (g/L-d)	μ (/h)	$Y_{L/X}$ (g/g)	$Y_{X/G}$ (g/g)	$Y_{L/G}$ (g/g)
Pure glycerol	25	90	72	0.17 ± 0.04	10.90 ± 0.07	3.63	5.36 ± 0.03	1.79	0.036	0.49 ± 0.05	0.44 ± 0.01	0.22 ± 0.00
Crude glycerol	25	90	72	5.66 ± 0.91	7.58 ± 0.44	2.53	2.92 ± 0.01	0.97	0.025	0.39 ± 0.01	0.39 ± 0.03	0.15 ± 0.01
Purified glycerol	25	90	72	0.33 ± 0.02	10.32 ± 0.05	3.44	4.57 ± 0.05	1.52	0.034	0.44 ± 0.01	0.42 ± 0.02	0.18 ± 0.00
	50	180	72	22.25 ± 0.05	10.75 ± 0.02	3.69	5.24 ± 0.02	1.74	0.035	0.47 ± 0.02	0.40 ± 0.02	0.19 ± 0.00
	75	270	72	49.31 ± 0.99	9.61 ± 0.5	3.20	4.63 ± 0.00	1.54	0.021	0.48 ± 0.01	0.37 ± 0.02	0.18 ± 0.02
	100	360	72	76.59 ± 0.05	6.48 ± 0.03	2.16	3.10 ± 0.03	1.03	0.019	0.48 ± 0.01	0.28 ± 0.01	0.13 ± 0.00

Representation of biomass (X, g/l), lipid (L, g/l), and initial/final glycerol (Gly0/Glyt, g/l) concentrations at 72 h fermentation at maximum concentration of lipid was achieved. YL/X, g/g -yield of lipids with respect to dry biomass, PL/V-T -lipid productivity, YX/G, g/g -biomass yield with respect to glycerol consumed, PX/V-T -biomass productivity, YL/G, g/g -lipid produced per glycerol consumed, μ -specific growth rate, and consumed glycerol values are presented for all trials. Culture conditions: growth on 500-ml flasks at 170 rpm and T=28 °C with initial pH 6.5 ± 0.1.

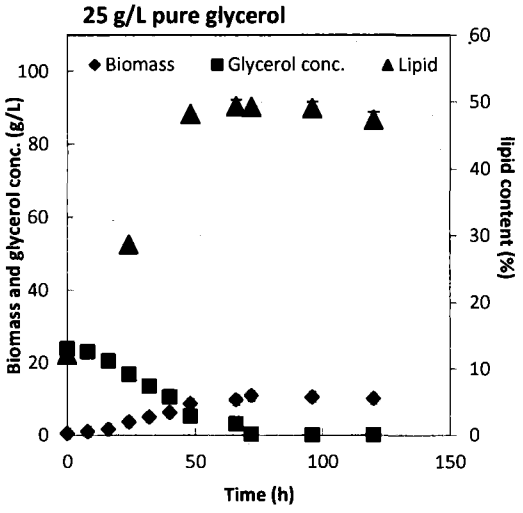
Table 9.4 Glycerol concentration effect on lipid accumulation

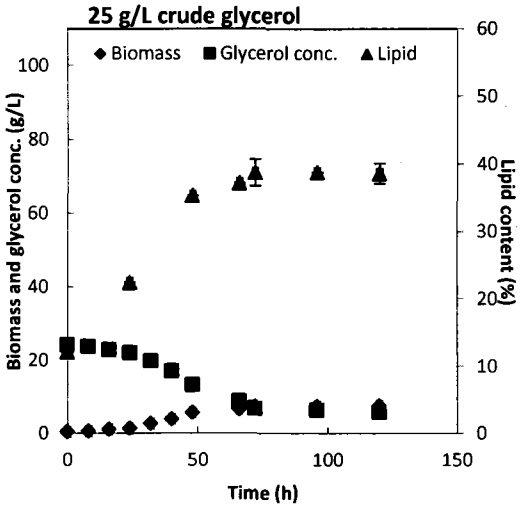
Strains	Glycerol type	Optimal glycerol conc. (g/L)	Lipid yield (g/g glycerol)	References
Aspergillus niger LFMB1	Crude glycerol	60	0.20	[33]
Aspergillus niger NRRL 364	Crude glycerol	60	0.21	[33]
Schizochytrium limacinum	Purified glycerol	35	0.26	[9]
Kodamaea ohmeri	Crude glycerol	100	0.20	[28]
Trichosporonoides Spathulata	Crude glycerol	100	0.18	[28]
Rhodotorula sp. LFMB 22	Crude glycerol	30	0.10	[34]
Chlorella protothecoides	Crude glycerol	30	0.33	[35]
Rhodotorula glutinis	Crude glycerol	100	0.10	[30]
Rhodosporidium toruloides	Crude glycerol	50	0.15	[36]
Rhodotorula glutinis	Crude glycerol	90	0.13	[29]
Trichosporon oleaginosus	Purified glycerol	50	0.19	This study

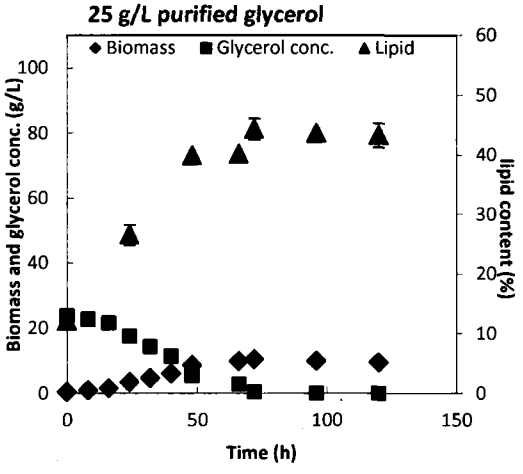
Table 9.5 **Fatty acid profile of lipid from *Trichosporon oleaginosus***

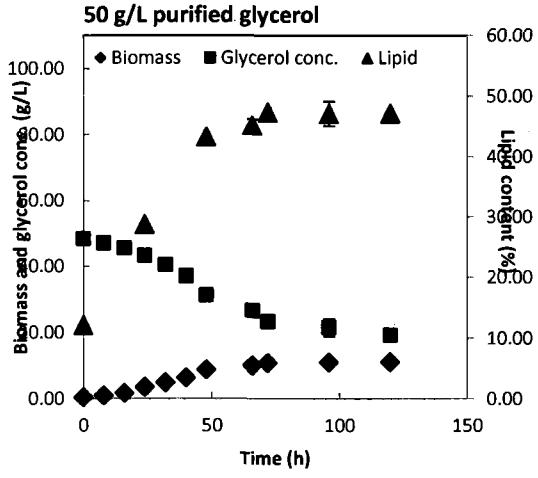
Fatty acid	Relative amount of total fatty acids (% w/w)						
	Lipid[1]	Lipid[2]	Lipid[3]	Lipid[4]	Lipid[5]	Lipid[6]	Jatropha seed oil
C14:0	0.1	2.2	0.1	0.3	0.1	0.1	-
C15:0	0.9	0.2	0.6	0.5	0.8	0.7	-
C16:0	19.3	22.6	20.1	21.0	20.7	20.2	12-20
C16:1	1.0	0.6	0.8	0.5	0.9	0.7	-
C18:0	13.4	19.5	15.2	14.9	15.0	14.2	5.0-10
C18:1	50.6	46.7	49.7	50.2	50.6	50.6	37-63
C18:2	7.5	5.8	8.6	7.9	7.2	7.8	10-19
C20:0	1.1	0.5	0.8	0.6	0.6	0.8	-

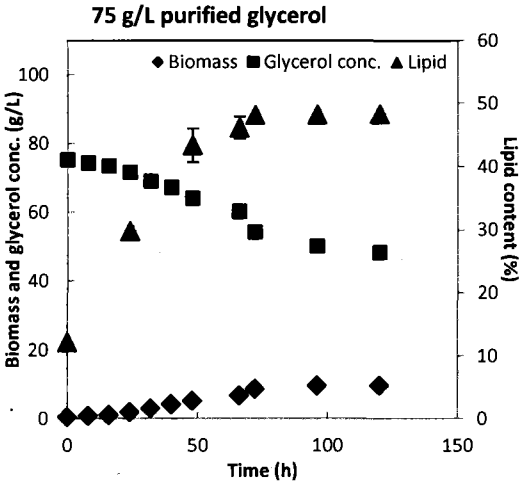
1 Lipid from Pure glycerol (25 g/L); 2 Lipid from crude glycerol (25 g/L); 3 Lipid from purified glycerol (25 g/L); 4 Lipid from purified glycerol (50 g/L); 5 Lipid from purified glycerol (75 g/L); 6 Lipid from purified glycerol (100 g/L);


25 g/L pure glycerol
Biomass
Glycerol conc.
Lipid
Biomass and glycerol conc. (g/L)
lipid content (%)
Time (h)


25 g/L crude glycerol
Biomass
Glycerol conc.
Lipid
Biomass and glycerol conc. (g/L)
Lipid content (%)
Time (h)


25 g/L purified glycerol
Biomass
Glycerol conc.
Lipid
Biomass and glycerol conc. (g/L)
lipid content (%)
Time (h)


50 g/L purified glycerol
Biomass
Glycerol conc.
Lipid
Biomass and glycerol conc. (g/L)
Lipid content (%)
Time (h)


75 g/L purified glycerol
Biomass
Glycerol conc.
Lipid
Biomass and glycerol conc. (g/L)
Lipid content (%)
Time (h)

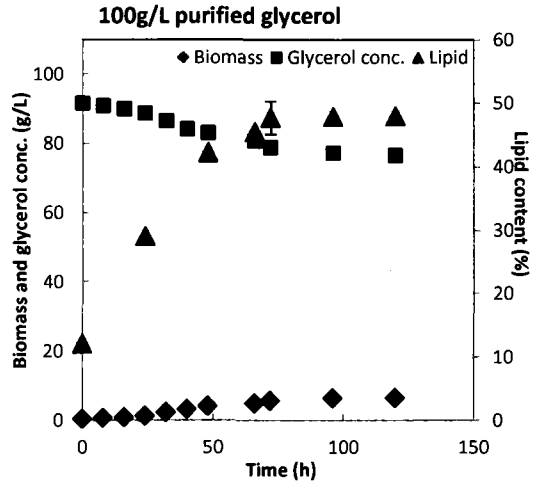

100g/L purified glycerol
Biomass
Glycerol conc.
Lipid
Biomass and glycerol conc. (g/L)
Lipid content (%)
Time (h)

Figure 9.1 **Biomass and glycerol concentration changing with time for different glycerol concentration; Data are means of three replicates with error bars indicating standard deviations**

ENERGY BALANCE OF BIOFUEL PRODUCTION FROM CRUDE GLYCEROL

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RENEWABLE ENERGY (SUBMITTED)

10 ENERGY BALANCE OF BIOFUEL PRODUCTION FROM CRUDE GLYCEROL

10.1 Résumé

Le glycérol brut, un sous-produit de la production de biodiesel, a attiré l'attention comme source de carbone. Le glycérol brut a été largement étudié dans la production de biocarburants. Le but de cette étude est d'évaluer le bilan énergétique de la production de biodiesel, d'hydrogène, de biogaz et d'éthanol à partir de 3.48 millions de kg de glycérol brut (80%). Le ratio d'énergie était de 1.32; 0.22; 0.27 et 0.52 pour la production de biodiesel, d'hydrogène, du biogaz, et d'éthanol, respectivement. Il a été constaté que l'utilisation de glycérol brut pour produire du biodiesel est un processus assurant un gain d'énergie (bilan énergétique positif). Le bilan énergétique est de 8430.56 GJ par million de kg de lipides produits (par 1 million L de biodiesel produit). En outre, il a été observé que la quantité de glycérol brut a été significativement réduite par son utilisation comme matière première dans la production du biodiesel (de 3.48 millions de L à 0.11 de millions de L). Le procédé proposé dans cette étude serait une solution pour atténuer la pression de la gestion du glycérol brut.

Mots clés : Bilan énergétique; glycérol brut ; biofuel; biodiesel

10.2 Abstract

Crude glycerol as a by-product of biodiesel production gained significant attention as carbon source. Crude glycerol has been already reported for different biofuel production. The purpose of this study is to evaluate the energy balance of the production of different biofuels such as biodiesel, hydrogen, biogas, and ethanol from 3.48 million L of 80% w/v crude glycerol. The energy ratio (energy output divided by net energy input) was 1.32, 0.22, 0.27, and 0.52 for the production of biodiesel, hydrogen, biogas, and ethanol, respectively. It was found that using crude glycerol to produce biodiesel was an energy gain (positive energy balance and energy ratio is greater than 1) process. The energy balance is 8430.56 GJ in per 1 million kg lipid produced (per 0.93 million kg biodiesel produced). In addition, the crude glycerol amount was significantly reduced in use for biodiesel production from 3.48 million L (as carbon source for lipid production) to 0.11 million L (produced in lipid transesterification). It suggested that it would be a solution for mitigating the crude glycerol management pressure.

Keywords: Energy balance; crude glycerol; biofuel; biodiesel

10.3 Introduction

Global biodiesel production amount has been sharply increased from 500 to 7500 million gallons within 10 years from 2004 to 2013, and it is predicted that it would continuously grow in the coming years [1]. Consequently, glycerol the by-product of biodiesel production through transesterification is simultaneously generated (about 0.10 to 0.14 kg glycerol per kg of biodiesel produced). The glycerol is often called as crude glycerol and is a mixture whose composition varies from one biodiesel production plant to another and is mainly determined by the feedstock oil composition and quality, the oil and methanol molar ratio, catalyst, and the detail procedure. Generally, the major fraction of the crude glycerol is glycerol (20 to 96% w/w) with some impurities such as water, methanol, soap (in alkaline catalytic process), and catalyst [2, 3].

Proper handling and use of crude glycerol grabs growing attention due to the large amount of availability. Purification was the most applied method on crude glycerol before the boom of biodiesel production and the purified glycerol was utilized in cosmetics industry [4]. However, the energy intense process becomes unfavorable on cost revenue due to the decrease of the price of purified glycerol (1.54 US$/kg before 2000 and 0.66 US$/kg after 2007). Crude glycerol as a carbon source has been greatly employed for lipid production with oleaginous microorganisms such as *Schizochytrium limacinum*, *Yarrowia lipolytica*, *Rhodotorula glutinis*, and *Cryptococcus curvatus* [5-8]. The produced lipid can be further converted to biodiesel and the crude glycerol produced along with biodiesel will be further utilized as carbon source for oleaginous microorganisms, which formed a crude glycerol utilization and production cycle. In addition, crude glycerol has been investigated for microbial hydrogen production by fermentation [9, 10]. Several microorganisms such as *Rhodopseudomonas palustris* and *Thermotoga neapolitana* are found to provide high hydrogen yield up to 0.17 g H_2/g glycerol [11-13]. Crude glycerol has also been studied for biogas production by anaerobic fermentation along with animal waste and/or sludge [14, 15]. It was observed that biogas yield in the case of crude glycerol based digestion was 825 mL/g volatile suspended solids [16], but at the same fermentation time only 269 mL/g VSS was achieved without addition of crude glycerol. Ethanol production from crude glycerol as substrate has also been reported [17, 18]. Production of

biofuels using crude glycerol would mitigate the pressure of its handling as well as the shortage of fossil fuels.

Therefore, the aim of this study was to evaluate energy balance for various biofuel productions using crude glycerol as raw material. Based on the energy balance comparison, the study proposed a feasible way of crude glycerol management through the production of biodiesel, hydrogen, biogas, and ethanol.

10.4 Methods

10.4.1 Crude glycerol based biofuel production processes

10.4.1.1 Crude glycerol based biofuel production process

A schematic diagram of biodiesel production from crude glycerol was developed based on the study of literature and is shown in Figure 10.1. Crude glycerol is fed to a fermenter to cultivate the oleaginous microorganisms. At the end of fermentation, the microbial biomass containing lipids is treated in a bead mill to break the cells and thus allow the lipid to be free from the cells. Due to lower density of lipid (0.9 g/mL) compared to water (1.0 g/mL) and cell debris (1.1 g/mL), lipid will float on the top of the phase separation tank. The separated lipid in the top layer will be collected for biodiesel synthesis through transesterification.

10.4.1.2 Crude glycerol based hydrogen production process

Dark, photo, sequential dark and photo, and combined dark and photo fermentation were used for hydrogen production from crude glycerol [12, 13, 19]. Researchers have reviewed and compared the types of fermentation processes for hydrogen production and revealed that the dark fermentation is the most suitable process for industrial scale production as it is easy to operate and provides competitive hydrogen yield [20]. A schematic diagram of hydrogen production from crude glycerol is shown in Figure 10.2. Crude glycerol is fed to an anaerobic fermenter to cultivate the hydrogen production bacteria. The products hydrogen as well as the co-product CO_2 (Equation 10.1) are collected and dispersed in a closed tank containing NaOH to

remove CO_2. The pure hydrogen gas is finally obtained. The fermentation broth (the residual liquid) is centrifuged to separate biomass solids and dried for further use.

Equation 10.1 $C_3H_8O_3 + H_2O \rightarrow 2H_2 + CO_2 + CH_3COOH$

10.4.1.3 Crude glycerol based biogas production process

Crude glycerol can be used as substrate or co-substrate of organic solid waste for biogas production through anaerobic digestion. A recent review on biogas production from crude glycerol has reported that using crude glycerol as substrate could provide higher biogas yield than using as co-substrate [21]. However, nutrient (such as nitrogen) addition is required and the biogas production wasn't stable for long term processing when crude glycerol was used as sole substrate. It was found that crude glycerol as co-substrate of wastewater sludge and animal waste digestion produced stable and comparable biogas yield and operation. In addition, as co-substrate, crude glycerol could increase methane content in the biogas [14]. Wastewater sludge is widely and abundantly produced all over the world. It is a zero cost nutrient source, which can replace the supply of expensive chemical nutrients. Therefore, in this study, crude glycerol addition to wastewater sludge was used to investigate the energy balance for biogas production. The schematic process is shown in Figure 10.3. Crude glycerol is fed to the sludge digester to produce biogas, which is collected and used as bioenergy source.

10.4.1.4 Crude glycerol based ethanol production process

Fuel ethanol is currently produced in large scale using fermentation of corn crops. The crude glycerol as carbon source has been used for ethanol production by fermentation. Ethanol has been successfully generated by aerobic and anaerobic fermentation of crude glycerol with many microorganisms such as *Kluyvera cryocrescens*, *Enterobacter Aerogenes*, and *Escherichia coli* [17, 22]. Anaerobic fermentation is preferable as it consumes less energy (without aeration) and provides comparable ethanol yield [22, 23]. Equation 10.2 shows the stoichiometry of ethanol generation from glycerol by anaerobic fermentation.

Equation 10.2 $C_3H_8O_3 \rightarrow CH_3CH_2OH + CH_2O_2$

It has been reported that hydrogen was produced along with ethanol production (Equation 10.3) in anaerobic fermentation when certain microorganism such as *Enterobacter Aerogenes* was used [23]. Crude glycerol fermentation to produce ethanol with simultaneous hydrogen generation was considered to study the energy balance as it provided extra biofuel hydrogen. The schematic process is shown in Figure 10.4. Crude glycerol is fed to fermenter. The gas phase (hydrogen and CO_2) is collected and passed through a washing tank filled with NaOH to remove CO_2. After fermentation, the broth is distillated to recover ethanol and the biomass is concentrated for further utilization.

Equation 10.3 $C_3H_8O_3 \rightarrow CH_3CH_2OH + H_2 + CO_2$

10.4.2 Evaluation basics and definitions

The evaluation was based on 3.48 million L of 80% (w/v) crude glycerol utilization per year, which is around 10% of the total annul crude glycerol produced in Canada [24]. The items included in the study are defined as follows:

- Direct energy input = ∑energy containing in fuels, electricity, and steam used in the process;
- Indirect energy input = ∑energy used for producing chemicals that are used in the process;
- Total energy input = ∑direct and indirect energy input;
- Energy output = Energy content of biofuel produced;
- Energy credit = ∑ Energy content of by-products;
- Net energy input = total energy input-energy credit;
- Energy balance = energy output – net energy input;
- Energy ratio = energy output / net energy input.

10.5 Energy balance of biofuel production from crude glycerol

10.5.1 Biodiesel production

The process includes fermentation for lipid-rich cells production, cell disruption, lipid separation, transesterification, and biodiesel purification. The detailed calculations are shown in the following sections.

10.5.1.1 Oleaginous microorganism fermentation

Fermentation is the process to obtain lipid enriched cells by addition of nutrients. Carbon, nitrogen, and phosphorus are critical for cell growth. 80% crude glycerol was used as a carbon source in the study [25]. Other nutrients used are $(NH_4)_2SO_4$ (8.98 MJ/kg), $MgSO_4 \cdot 7H_2O$ (12.12 MJ/kg), and KH_2PO_4 (10.3 MJ/kg) with concentration of 0.7, 0.5, and 1 g/L, respectively [26, 27]. In addition, 1 M HCl (1.00 MJ/L) is used to adjust pH to 6.5 (from initial 9.0). The amount of 1 M HCl required to adjust the pH was 3.2 mL per liter crude glycerol. Pure glycerol has an energy content of 16.71 MJ/kg; however, the energy value of pure glycerol while diluted from 100% to 80% purity is reduced approximately by 6.52 MJ/kg [28]. Therefore, the energy content of the 80% crude glycerol is 10.19 MJ/kg.

Based on the studies conducted in our laboratory, isolated oleaginous fungus when grown in a medium containing 100 g/L crude glycerol (based on glycerol mass) accumulated 60% lipid w/w dry biomass (0.63 g biomass/g glycerol) in 72 h at 28 °C. Therefore, these data were used to estimate the energy balance. Two fermenters of 180 m3 each with 70% working volume equipped with aeration and agitation systems will be required for the lipid production. The agitation is performed by mixing (0.35 kWh/m^3) and aeration (0.5 VVM) is achieved by traditional fine pore aeration ceramic disc (4.26 kg air/kWh) [29, 30]. After fermentation, the broth is pumped with centrifugal pump with a capacity of 500 m3/h (32.6 kJ/m^3) to cell disruption unit [31]. The detailed energy used in the process is shown in Table 10.1.

10.5.2 Cell disruption and lipid separation

Lipid is an intercellular product of oleaginous cells. Cell disruption is the way to allow lipid release from the cells. Organic solvent extraction is generally used for lipid separation from

microorganisms [32]. Solvent addition pulls out the phospholipid (contained in the cell wall and the cellular membrane) and disrupts the cell. There are other approaches such as ultrasonication, homogenization, bead milling, and microwave for cell disruption. Comparing with organic solvent extraction, mechanical methods such as homogenization and bead milling are favorable as they are environmentally friendly. Bead milling as a method of cell disruption and release of lipids was chosen for this study as it provides similar efficiency and requires less energy input comparing with other cell disruption technologies such as ultrasonication and homogenization [33, 34]. The operation time is 20 min per pass and 3 passes are performed (totally one hour). DYNO®-MILL ECM bead miller with a capacity of 6 m^3/h and power consumption of 90 kW was used in this study [35]. Thus, the total energy used in the cell disruption process was 526315.79 kWh. The mixture of lipid, cell debris, and water is transferred to a phase separation tank for lipid and cell debris separation. After 45 min gravity settling, the lipid will float on the top layer of the tank. The lipid layer will then be collected and transferred (32.6 kJ/m^3) to transesterification reactor for biodiesel synthesis.

10.5.2.1 Transesterification

Transesterification is a chemical reaction where 1 mole of triglyceride reacts with 3 moles of short chain alcohols (methanol/ethanol) to form 3 moles of fatty acid esters and 1 mole glycerol. Alkaline and acidic catalytic transesterification is currently used for biodiesel synthesis. The selection of the alkaline or acidic catalyst is determined by the free fatty acid (FFA) content of the feedstock. Alkaline catalytic transesterification requires less time and amount of catalyst compared to acid catalyst; however, alkaline catalyst is not used when the FFA content of the feedstock oil is greater than 2% w/w. According to the studies conducted in our laboratory, it is found that the FFA content in the produced microorganism is less than 0.83% w/w. It indicates that alkaline catalyst can be used in transesterification of biodiesel synthesis.

After lipid extraction and recovery from the fungal biomass, the lipid was transferred to the reactor containing methanol (methanol to lipid molar ratio of 6:1) and catalyst, NaOH (2% w/w of the lipid). The transesterification reaction was conducted at 50 °C for an hour with mixing. The transesterification efficiency is assumed to be 99% [36]. In order to produce one kg of biodiesel, 96 g of methanol (22 MJ/kg) is required. The energy used in mixing and heating of the

reaction mixture is 0.03 kWh and 0.24 KJ per kg biodiesel produced, respectively [37]. The details of the energy used in the process are shown in Table 10.2.

10.5.2.2 Biodiesel purification

Since the biodiesel produced through transesterification is a mixture of methanol, catalyst, and by-product glycerol. It is required to be separated from the other components. Methanol recovery is normally performed by distillation (625 kW) at 1500 kg/h flow rate and its efficiency is assumed to be 96% [38]. The recovered methanol is mixed with fresh methanol and recycled to the transesterification reactor. The residue mixture (containing biodiesel, catalyst, salt, and glycerol) is allowed to separate from the biodiesel (top layer) and crude glycerol (bottom layer) by phase separation [39]. The biodiesel in the top layer is then dried by distillation to remove the residual water with an energy consumption of 313.50 kJ/kg biodiesel, and finally the pure biodiesel is obtained [37]. The total energy input in the purification step was 290.19 GJ. The bottom layer containing crude glycerol is stored and further used as carbon source for cultivation of oleaginous microorganism to produce lipid.

It is estimated that around 3.48 million L of 80% (w/v) crude glycerol is required to obtain 1 million kg lipid and 0.75 million kg of biomass is produced as residue (by-product) after extraction of lipids. There will be around 0.93 million kg biodiesel (37.8 MJ/kg) produced from the lipid generated. In addition, glycerol is generated as a by-product in a ratio of 0.14 g glycerol/g biodiesel produced. The total energy input is the sum of the energy input during fermentation, cell disruption, transesterification, and biodiesel purification, which is 34077.78 GJ (Table 10.3).

The residual biomass can be used as animal feed and assigned an energy value of 7.95 MJ/kg [40]. As the produced glycerol is mixed with catalyst and thus considered as crude glycerol. Every 13 g glycerol contains around 2 g catalyst, which leads to a glycerol concentration of 87% (w/w). Therefore, the energy content of the crude glycerol is 11.74 MJ/kg [28]. Produced crude glycerol and residual biomass contributes an energy credit of 7518.77 GJ. Therefore, the net energy input will be total energy input minus the energy credit, which is 26559.01 GJ. The

energy output is the energy content of the 0.93 million kg biodiesel which is 34989.57 GJ. Thus the net energy generated is 8430.56 GJ and the energy ratio is 1.32 (Table 10.3).

The highest energy input is in the form of crude glycerol (as raw material for lipid production), which counts for around 83% of the total energy input (Table 10.1 and 10.3). It suggests that lipid yield from glycerol (g lipid/g glycerol) has significant impact on the energy balance. In this study, the lipid yield is assumed to be 0.378 g lipid/g glycerol (=0.63 g biomass/g glycerol×60% lipid w/w biomass). High lipid yield leads to high net energy and energy ratio. For example, when the lipid content in biomass is increased to 70% with the same biomass yield (0.63 g biomass/g glycerol), the net energy and ratio will be increased from 8430.56 GJ to 12888.65 GJ and 1.32 to 1.58, respectively. While the net energy and ratio will be reduced from 8430.56 GJ to 2215.70 GJ and 1.32 to 1.07, when lipid content is 50%w/w of the biomass. There is almost no energy gain in the process when the lipid content is 47.5%, which suggests that the process is not feasible if the lipid yield is lower than 0.30 g lipid/g glycerol in terms of net energy obtained.

10.5.3 Hydrogen production

The process includes fermentation for hydrogen generation and hydrogen purification to remove CO_2. The detailed calculations are shown in the following sections. Apart from crude glycerol, other nutrients required for hydrogen production are yeast extract 1 g/L (6.46 MJ/kg) and KH_2PO_4 4.6 g/L (10.3 MJ/kg) [23, 41]. In addition, 1 M HCl (1.00 MJ/L) will be used to adjust pH of crude glycerol to 6.5 from initial 9.0; the required amount was 3.2 mL per L of crude glycerol.

High crude glycerol concentration was found to inhibit hydrogen production; therefore, glycerol concentration of 25 g/L was used in the calculation. [23]. As reported, general hydrogen (122 MJ/kg) yield was 5.4 - 43.5 mol/kg glycerol and the average value 24.25 mol/kg glycerol was used in the study [42, 43]. I It was assumed that the fermentation is conducted at 28 °C for 48 h with a final biomass concentration of 13.8 g/L [19]. The fermentation process was operated in eight fermenters with each volume 180 m^3 and working volume of 70% with agitation (0.35 kWh/m^3) [29, 30]. Heating is not required to keep the temperature (28 °C)

during fermentation as agitation generates heat which can maintain the temperature [44]. During fermentation, hydrogen is generated, collected, and passed through NaOH (18.5 MJ/kg) solution to remove CO_2 (1.89 g NaOH/g CO_2 produced). The detailed energy used in the process is shown in Table 10.4.

3.48 million L of 80% (w/v) crude glycerol could produce 135.06 tonne hydrogen and 1485.66 tonne CO_2. The total energy input, sum of the energy input during fermentation and CO_2 removal (hydrogen purification), is 86881.46 GJ.

The biomass, containing mainly protein can be used as animal feed after centrifuge (1 kWh/m^3) and has been assigned an energy value of 7.95 MJ/kg [31, 40]. CO_2 has a zero energy contribution. Only biomass is considered as energy credit and the energy credit will be 12220.55 GJ. Therefore, the net energy input will be total energy input minus energy credit, which is 74660.91 GJ. The net energy output is the energy content in the 135.06 tonne hydrogen, which is 16477.30 GJ. Thus the energy balance is -58183.60 GJ and the energy ratio is 0.22 (Table 10.4).

10.5.4 Biogas production

Many types of digester, mainly continuously stirred tank digester (CSTR), anaerobic filter (AF), and upflow anaerobic sludge blanket (UASB), have been utilized in biogas production. CSTR is the most common and simplest type of digester. It is employed in the study to investigate biogas production from crude glycerol. The process includes a digester and a water trap to remove vapor.

Research reports revealed that the optimal C/N ratio of biogas production was the range between 20:1 and 30:1 [45, 46]. It is known that wastewater sludge is rich in nitrogen and normally has a C/N ratio of 5:1 with available nitrogen concentration of 8 g/kg TS [47]. The optimal glycerol concentration for biogas production along with sludge was around 10 g/L [48, 49]. Therefore, the glycerol concentration 10 g/L was used to mix with the sludge (total solids concentration 50 g/L) to make a final C/N ratio of 23:1 [21]. The pH of the crude glycerol was adjusted to 6.5 from initial 9.0 using 1 M HCl (3.2 mL/L crude glycerol). As reported, methane content of biogas from the digestion of the mixture of sludge and glycerol was around 50% to

70% v/v and the biogas yield was around 500 m^3/tonne dry matter (sludge and glycerol); hence, the average methane content of 60% v/v corresponding to an energy density of 21.43 MJ/m^3 [21, 50] was assumed in the calculations. It was found that the yield from sludge was around 300 m^3/tonne dry matter degraded [51]; therefore, the biogas yield from glycerol would be 200 m^3/tonne glycerol.

It was assumed that the fermentation performed in twelve fermenters with 1000 m^3 each, sludge retention time 15 days under agitation [52, 53]. The fermentation was carried out under mesophilic condition (35 °C) with heating energy input 1.16 kWh/m^3/°C [21, 53]. The energy required for agitation is 0.4 kWh/m^3 [53]. The energy input for central pumping and others was assumed to be 0.2 and 0.05 kWh/m^3, respectively [53, 54]. Biogas contains toxic gas such as H_2S, and upgrading the process to remove H_2S would require and the energy input for this item is normally 11% of the energy content of the total biogas produced [54]. Digestion is the process to reduce solid matters and it was found that the solid matter reduction could be 70% to 90% [55-57]. In the study it was assumed that the sludge solids concentration was reduced from 50 to 10 g/L (80% reduction). It is known that sludge has value as fertilizer due to its richness in nitrogen (N), phosphorus (P) and potassium (K). Generally, each tonne of dry sludge is equivalent to around 16 kg fertilizer (1985650 kcal/ton) [58]. Thus, the sludge was assigned an energy content (from phosphorus) 0.13 MJ/kg dry sludge. The detailed energy used in the process is shown in Table 10.5.

3.48 million L of 80% (w/v) crude glycerol could produce 556947.93 m^3 of biogas corresponding to 11935.39 GJ. The total energy input is 43867.06 GJ. As the sludge after digestion is rich in K, N, and P, therefore, it was considered as credit using as phosphorus fertilizer. There was 44.56 tonne sludge (dry matter) produced in the calculation which has an energy density of 0.13 MJ/kg dry sludge. The credit would be 371.59 GJ, and thus the net energy input would be 43495.46 GJ. Subtracting the net energy input from energy output (biogas), the net energy would be then obtained to be -31560.07. The energy ratio was calculated to be 0.27.

10.5.5 Ethanol production

Several microbes such as *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* have been found to produce ethanol using crude glycerol as carbon source. E. coli is known to be highly amenable to utilize in industrial applications; therefore, it was employed in fermentation for ethanol production. During ethanol production, co-product hydrogen or formate is generated according to the pathway (Eq. 10.2 and 10.3). In the study, hydrogen was assumed to be produced along with ethanol production as hydrogen is a high value biofuel.

Studies have found that glycerol concentration 10 g/L was optimal for ethanol production (0.34 to 0.40 g/g glycerol) whereas the hydrogen production was optimal at glycerol concentration 20 to 25 g/L [21, 53]. As the goal is ethanol production; hence, glycerol concentration 10 g/L was used in the calculation. Ethanol and hydrogen yields were assumed to be 0.37 g/g glycerol and 1.11 mmol/g glycerol, respectively [21]. According to Eq. 10.3, one molar carbon dioxide is simultaneously produced in every molar hydrogen production. The fermentation media also contained yeast extract (5 g/L) and K_2HPO_4 (5 g/L) as nutrients and the pH was adjusted to 6.5 from initial 9.0 with 1M HCl (3.2 mL/L crude glycerol). The fermentation was performed in two 1000 m^3 fermenters at 37 °C under agitation (0.35 kWh/m^3) in 1000 m^3 fermenter. The gas phase (hydrogen and carbon dioxide) was passed through NaOH solution to remove carbon dioxide (as described in hydrogen production), and the fermentation broth was subjected to distillation (10.62 MJ/kg ethanol) for ethanol recovery followed by biomass harvesting by centrifugation (1 kWh/m^3) [54]. The detailed calculation was shown in Table 10.6.

3.48 million L 80% (w/v) crude glycerol could produce 1030.53 tonne ethanol, 1113.90 tonne biomass, 3091060.97 mole hydrogen, and 3091060.97 mole CO_2. The total energy input was 68788.89 GJ. The biomass containing mainly protein can be used as animal feed after centrifuge (1 kWh/m^3) and assign an energy value of 7.95 MJ/kg [30, 37]. Carbon dioxide has a zero energy contribution. Thus hydrogen and biomass were considered as energy credit and the energy credit was 9232.58 GJ. Therefore, the net energy input was 59556.31 GJ. The energy output (the energy content in the ethanol) was 30910.61 GJ. Thus the energy balance is -28645.70 GJ and the energy ratio is 0.52 (Table 10.6).

10.6 Discussion

It was observed that crude glycerol as carbon source for biodiesel production was energy gain (positive energy balance) process, while it was energy loss (negative energy balance) process when it was used for hydrogen, biogas and ethanol production. The energy ratio was 1.32, 0.22, 0.27, and 0.52 when crude glycerol was used as carbon source for the production of biodiesel, hydrogen, biogas, and ethanol, respectively. The low energy ratio of hydrogen production was due to the low yield of the product (48.5 kg/tonne glycerol) while high energy input for heating to maintain fermentation temperature of biogas (35 °C) and ethanol (37 °C) production was the cause of low energy ratio. The calculation showed that crude glycerol for biodiesel production was the most suitable application in terms of energy balance.

10.7 Conclusions

Crude glycerol showed greater potential for biodiesel production comparing to hydrogen, biogas, and ethanol production in terms of energy balance. Crude glycerol as substrate for oleaginous microorganism cultivation solves the large amount crude glycerol management pressure. In this study, it shows that 3.48 million L 80% (w/v) crude glycerol is reduced to 0.11 million L in using for biodiesel production, which reduced around 30 times. Crude glycerol for biodiesel production also provides energy gain (positive energy balance), 8430.56 GJ in per 0.93 million kilogram biodiesel produced.

10.8 Acknowledgements

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10.9 References

[1]. Annie D. Biofuels production, trade and sustainable development: emerging issues. Environmental Economics Programme/Sustainable Markets Group, September 2006 2006.

[2]. Hu S, Luo X, Wan C, Li Y. Characterization of crude glycerol from biodiesel plants. J. Agric. Food. Chem. 2012;60(23): 5915-5921.

[3]. Hansen CF, Hernandez A, Mullan BP, Moore K, Trezona-Murray M, King RH, Pluske JR. A chemical analysis of samples of crude glycerol from the production of biodiesel in Australia, and the effects of feeding crude glycerol to growing-finishing pigs on performance, plasma metabolites and meat quality at slaughter. Anim. Prod. Sci. 2009;49(2): 154-161.

[4]. Yang F, Hanna M, Sun R. Value-added uses for crude glycerol--a byproduct of biodiesel production. Biotechnol. Biofuels 2012;5(1): 1-10.

[5]. Easterling ER, French WT, Hernandez R, Licha M. The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of *Rhodotorula glutinis*. Bioresour. Technol. 2009;100(1): 356-361.

[6]. Papanikolaou S, Aggelis G. Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. Bioresour. Technol. 2002;82(1): 43-49.

[7]. Meesters PAEP, Huijberts GNM, Eggink G. High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. Appl. Microbiol. Biotechnol. 1996;45(5): 575-579.

[8]. Liang Y, Sarkany N, Cui Y, Blackburn JW. Batch stage study of lipid production from crude glycerol derived from yellow grease or animal fats through microalgal fermentation. Bioresour. Technol. 2010;101(17): 6745-6750.

[9]. Liu B, Christiansen K, Parnas R, Xu Z, Li B. Optimizing the production of hydrogen and 1.3-propanediol in anaerobic fermentation of biodiesel glycerol. International Journal of Hydrogen Energy 2013;38(8): 3196-3205.

[10]. Kivistö A, Santala V, Karp M. Closing the 1.3-propanediol route enhances hydrogen production from glycerol by Halanaerobium saccharolyticum subsp. saccharolyticum. International Journal of Hydrogen Energy 2011;36(12): 7074-7080.

[11]. Lo Y-C, Chen C-Y, Lee C-M, Chang J-S. Sequential dark–photo fermentation and autotrophic microalgal growth for high-yield and CO2-free biohydrogen production. International Journal of Hydrogen Energy 2010;35(20): 10944-10953.

[12]. Ngo TA, Kim M-S, Sim SJ. High-yield biohydrogen production from biodiesel manufacturing waste by Thermotoga neapolitana. International Journal of Hydrogen Energy 2011;36(10): 5836-5842.

[13]. Sabourin-Provost G, Hallenbeck PC. High yield conversion of a crude glycerol fraction from biodiesel production to hydrogen by photofermentation. Bioresource Technology 2009;100(14): 3513-3517.

[14]. Robra S, Serpa da Cruz R, de Oliveira AM, Neto JAA, Santos JV. Generation of biogas using crude glycerin from biodiesel production as a supplement to cattle slurry. Biomass and Bioenergy 2010;34(9): 1330-1335.

[15]. Castrillón L, Fernández-Nava Y, Ormaechea P, Marañón E. Optimization of biogas production from cattle manure by pre-treatment with ultrasound and co-digestion with crude glycerin. Bioresource Technology 2011;102(17): 7845-7849.

[16]. Johansson AE, Gustavsson AM, Öquist MG, Svensson BH. Methane emissions from a constructed wetland treating wastewater—seasonal and spatial distribution and dependence on edaphic factors. Water Research 2004;38(18): 3960–3970.

[17]. Choi W, Hartono M, Chan W, Yeo S. Ethanol production from biodiesel-derived crude glycerol by newly isolated Kluyvera cryocrescens. Applied Microbiology and Biotechnology 2011;89(4): 1255-1264.

[18]. Oh B-R, Seo J-W, Heo S-Y, Hong W-K, Luo LH, Joe M-h, Park D-H, Kim CH. Efficient production of ethanol from crude glycerol by a Klebsiella pneumoniae mutant strain. Bioresource Technology 2011;102(4): 3918-3922.

[19]. Sarma S, Brar S, Bihan Y, Buelna G. Bio-hydrogen production by biodiesel-derived crude glycerol bioconversion: a techno-economic evaluation. Bioprocess Biosyst Eng 2013;36(1): 1-10.

[20]. Argun H, Kargi F. Bio-hydrogen production by different operational modes of dark and photo-fermentation: An overview. International Journal of Hydrogen Energy 2011;36(13): 7443-7459.

[21]. Viana MB, Freitas AV, Leitão RC, Pinto GAS, Santaella ST. Anaerobic digestion of crude glycerol: a review. Environmental Technology Reviews 2012;1(1): 81-92.

[22]. Chaudhary N, Ngadi MO, Simpson B. Comparison of glucose, glycerol and crude glycerol fermentation by *Escherichia Coli* K12. Bioprocessing and Biotechniques 2012;1(1): 1-5.

[23]. Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N. Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. Journal of Bioscience and Bioengineering 2005;100(3): 260-265.

[24]. Valliyappan T. (2004) Hydrogen or syn gas production from glycerol using pyrolysis and steam gasification processes, In *Chemical Engineering*, p 107, University of Saskatchewan, Saskatoon.

[25]. Liu Y, Koh CMJ, Ji L. Bioconversion of crude glycerol to glycolipids in *Ustilago maydis*. Bioresource Technology 2011;102(4): 3927-3933.

[26]. US EERE. Agricultural chemicals: fertilizers. available at http://www1.eere.energy.gov/manufacturing/resources/chemicals/pdfs/profile_chap5.pdf accessed on April 2013 2002;141-174.

[27]. Alabi AO, Tampier M, Bibeau E. Microalgae technologies and processes for biofuelsbioenergy production in British Columbia. 2009;Report submitted to the British Columbia Innovaion Council, 2009.

[28]. Duque JAP. (2011) Design and analysis of technological schemes for glycerol conversion to added value products, In *Philosophy in Engineering*, p 224, Universidad Nacional de Colombia, Manizales.

[29]. Environmenal Dynamics Inc. Energy consuption and typical performance of various types of aeration equipment. Technical Bulletin of Columbia Missouri USA 2003;127(1-3.

[30]. Berk Z. Food process engineering and technology. 2008;180.

[31]. Jorquera O, Kiperstok A, Sales EA, Embiruçu M, Ghirardi ML. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresour Technol 2010;101(4): 1406–1413.

[32]. Ferraz TPL, Fiúza MC, dos Santos MLA, Pontes de Carvalho L, Soares NM. Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation. J Biochem Bioph Methods 2004;58(3): 187-193.

[33]. Dhanani T, Shah S, Gajbhiye NA, Kumar S. Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera. Arabian Journal of Chemistry 2013;0).

[34]. Prabakaran P, Ravindran AD. A comparative study on effective cell disruption methods for lipid extraction from microalgae. Lett. Appl. Microbiol. 2011;53(2): 150-154.

[35]. Willy AB. High-efficiency agitator bead mill. Avaiable at <http://www.wab.ch/fileadmin/redaktion/downloads/prospekt/EN_ECM_Leaflet.pdf>(accessed April 2013) 2011;1-6.

[36]. Singh SP, Singh D. Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: A review. Renewable Sustainable Energy Rev 2010;14(1): 200–216.

[37]. Batan L, Quinn J, Willson B, Bradley T. Net energy and greenhouse gas emission evaluation of biodiesel derived from microalgae. Environ Sci Technol 2010;44(20): 7975–7980.

[38]. Dhar B, Kirtania K. Excess methanol recovery in biodiesel production process using a distillation column: A simulation study. Chemical Engineering Research Bulletin 2009;13(2): 55–60.

[39]. Gonzalo A, Garcìla M, Luis Salnchez J, Arauzo J, Penìfa J. Water cleaning of biodiesel. effect of catalyst concentration, water amount, and washing temperature on biodiesel obtained from rapeseed oil and used oil. Ind Eng Chem Res 2010;49(9): 4436–4443.

[40]. Van Eys JE, Offner A, Bach A. Chemical analysis. manual of quality analysis for soybean products in the feed industry. American Soybean Association. Avaiable at <http//www.asa-europe. org/ Library/ library_e.htm> (accessed March. 2013) 2004.

[41]. Selembo PA, Perez JM, Lloyd WA, Logan BE. Enhanced hydrogen and 1.3-propanediol production from glycerol by fermentation using mixed cultures. Biotechnology and Bioengineering 2009;104(6): 1098-1106.

[42]. Sarma SJ, Brar SK, Sydney EB, Le Bihan Y, Buelna G, Soccol CR. Microbial hydrogen production by bioconversion of crude glycerol: A review. International Journal of Hydrogen Energy 2012;37(8): 6473-6490.

[43]. Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme and Microbial Technology 2006;38(5): 569-582.

[44]. Ghaly AE, Mahmoud NS. Heat generated by mechanical agitation and lactose metabolism during continuous propagation of *Kluyveromyces fragils* in cheese whey. Agricultural Engineering International 2003;4(1-21).

[45]. Achmad KTB, Hidayati YA, Fitriani D, Imanudin O. The effect of C/N ratio of a mixture of beef cattle feces and water hyacinth (*Eichornia crassipes*) on the quality of biomas and sludge Lucrari Stiintifice 2011;55(117-120.

[46]. Jayaweera MW, Dilhani JAT, Kularatne RKA, Wijeyekoon SLJ. Biogas production from water hyacinth (*Eichhornia crassipes (Mart.) Solms*) grown under different nitrogen concentrations. Journal of Environmental Science and Health, Part A 2007;42(7): 925-932.

[47]. Nourmohammadi D, Esmaeeli M-B, Akbarian H, Ghasemian M. Nitrogen removal in a full-scale domestic wastewater treatment plant with activated sludge and trickling filter. Journal of Environmental and Public Health 2013;2013(6.

[48]. Fountoulakis MS, Petousi I, Manios T. Co-digestion of sewage sludge with glycerol to boost biogas production. Waste Management 2010;30(10): 1849-1853.

[49]. Fountoulakis M, Sfendouraki M, Tselepidakis A, Terzakis S, Manios T. Enhanced biogas production of primary sludge co-digested with crude glycerol. Waste to Engergy Resreach and Technology, 2nd International Conference June 2009 2009.

[50]. Pöschl M, Ward S, Owende P. Evaluation of energy efficiency of various biogas production and utilization pathways. Applied Energy 2010;87(11): 3305-3321.

[51]. Sorensen J, Tholstrup G. Anaerobic digestion and thermal hydrolysis to reduce production of sludge in WWTPs. Vatten 1999;55(1): 45-51.

[52]. Nges IA, Liu J. Effects of solid retention time on anaerobic digestion of dewatered-sewage sludge in mesophilic and thermophilic conditions. Renewable Energy 2010;35(10): 2200-2206.

[53]. Moller HB, Nielsen AM, Murto M, Christensson K, Rintala J, Svensson M, Seppala M, Paavola T, Angelidaki I, Kaparaju PL. Manure and energy crops for biogas production. Status and barriers. Nordic council of Ministers 2008;1(1): 1-51.

[54]. Berglund M, Börjesson P. Assessment of energy performance in the life-cycle of biogas production. Biomass and Bioenergy 2006;30(3): 254-266.

[55]. Sugrue K, Kiely G, McKeogh E. A pilot plant study of the anaerobic digestion of a mixture of municipal sewage and industrial sludges. Water Science and Technology 1992;26(9-11): 2465-2468.

[56]. Mirzoyan N, Parnes S, Singer A, Tal Y, Sowers K, Gross A. Quality of brackish aquaculture sludge and its suitability for anaerobic digestion and methane production in an upflow anaerobic sludge blanket (UASB) reactor. Aquaculture 2008;279(1–4): 35-41.

[57]. Duan N, Dong B, Wu B, Dai X. High-solid anaerobic digestion of sewage sludge under mesophilic conditions: Feasibility study. Bioresource Technology 2012;104(0): 150-156.

[58]. Energy Information Document 1028. Appendix C: Units, Equivalents and Energy Constants. University of Florida 1990.

[59]. Thapa LP, Lee SJ, Yoo HY, Choi HS, Park C, Kim SW. Development of glycerol-utilizing Escherichia coli strain for the production of bioethanol. Enzyme and Microbial Technology 2013;In press(0).

[60]. Krochta MJ. Energy analysis for ethanol California Agriculture 1980;1(1): 9-11.

Table 10.1 Energy input in fermentation

Items	Quantity	Energy Kwh	Energy required/produced (GJ)	Energy contained in chemical used/produced (GJ)
$(NH_4)_2SO_4$ (tonne)	24.56			220.56
$MgSO_4 \cdot 7H_2O$ (tonne)	17.54			212.63
KH_2PO_4 (tonne)	35.09			361.40
Crude glycerol (million L)	3.48			28376.50
HCl (L)	11139			11.14
Agitation		12280.70	44.21	
Aeration		50448.89	18.16	
Pumping		3177.39	1.14	

Table 10.2 Energy input in transesterification

Inputs	Quantity required (tonne)	Energy required (kWh)	Energy required (GJ)	Energy contained in chemical used (GJ)
NaOH	20.00			370
Methanol	88.86			1954.97
Mixing		27769.50	99.97	
Heating			222.16	

Table 10.3 Energy balance of biodiesel production from crude glycerol

Inputs	Quantity (million kg)	Energy required (GJ)	Energy produced (GJ)
Fermentation		29245.75	
Cell disruption		1894.74	
Transesterification		2647.10	
Biodiesel purification		290.19	
Total energy input (a)		34077.78	
Credits			
Residual biomass (b)	0.75		5997.37
By-product glycerol (c)	0.13		1521.40
Total credit (d=b+c)			7518.77
Net energy input (e=a-d)		26559.01	
Biodiesel (f)	0.93		34989.57
Energy balance (g=f-e)			8430.56
Energy ratio (f/e)	1.32		

Table 10.4 Energy balance of biodiesel production from crude glycerol

Items	Quantity	Energy Kwh	Energy required/produced (GJ)	Energy contained in chemical used/produced (GJ)
Yeast extract (tonne)	111.39			719.58
KH_2PO_4 (tonne)	512.39			5277.64
Crude glycerol (million L)	3.48			28376.50
HCl (L)	11138			11.14
Water (tonne)	111389.59			5.57
NaOH (tonne)	2807.89			51946.05
Agitation		38986.35	140.35	
Centrifuge		111389.59	401.00	
Total energy input (a)				86881.46
Credits				
Biomass (tonne) (b)	1537.18			12220.55
By-product CO_2 (tonne) (c)	1485.66			0
Total credit (d=b+c)				12220.55
Net energy input (e=a-d)				74660.91
Hydrogen (tonne) (f)	135.06			16477.30
Net energy gain (g=f-e)				-58183.60
Energy ratio (f/e)	0.22			

Table 10.5 Energy balance of biogas production from crude glycerol

Items	Quantity	Energy Kwh	Energy required/produced (GJ)	Energy contained in chemical used/produced (GJ)
Crude glycerol (million L)	3.48			28376.50
HCl (L)	11138			11.14
Water (tonne)	1392369.81			69.62
Wastewater sludge (tonne)	13923.70 (based on dry matters)			1857.98
Agitation		111389.59	401.00	
Heating		3230297.97	11629.07	
Pumping		55694.79	200.50	
Others		13923.70	50.13	
Biogas upgrading				1312.89
Total energy input (a)				43867.06
Credits				
Residual sludge (tonne) (b)	44.56			371.59
Total credit (c=b)				371.59
Net energy input (d=a-c)				43495.46
Biogas (e)	556947.93 m^3			11935.39
Energy balance (f=e-d)				-31560.07
Energy ratio (e/d)	0.27			

Table 10.6 Energy balance of ethanol production from crude glycerol

Items	Quantity	Energy Kwh	Energy required/produced (GJ)	Energy contained in chemical used/produced (GJ)
Crude glycerol (million L)	3480.92			28376.50
HCl (L)	11138			11.14
Water (tonne)	278473.96			13.92
Yeast extract (tonne)	1392.37			8994.71
K_2HPO_4 (tonne)	1392.37			14341.41
NaOH (tonne)	2570.52			4755.47
Agitation		97465.89	350.88	
Distillation			10942.36	
Centrifugation		278473.96	1002.51	
Total energy input (a)				68788.89
Credits				
Biomass (tonne) (b)	1113.90			8855.47
Hydrogen (c)	3091060.97 mol			377.11
CO_2 (d)	3091060.97 mol			0
Total credit (e=b+c+d)				9232.58
Net energy input (f=a-e)				59556.31
Ethanol (g)	1030.53			30910.61
Net energy produced (h=g-f)				-28645.70
Energy ratio (g/f)	0.52			

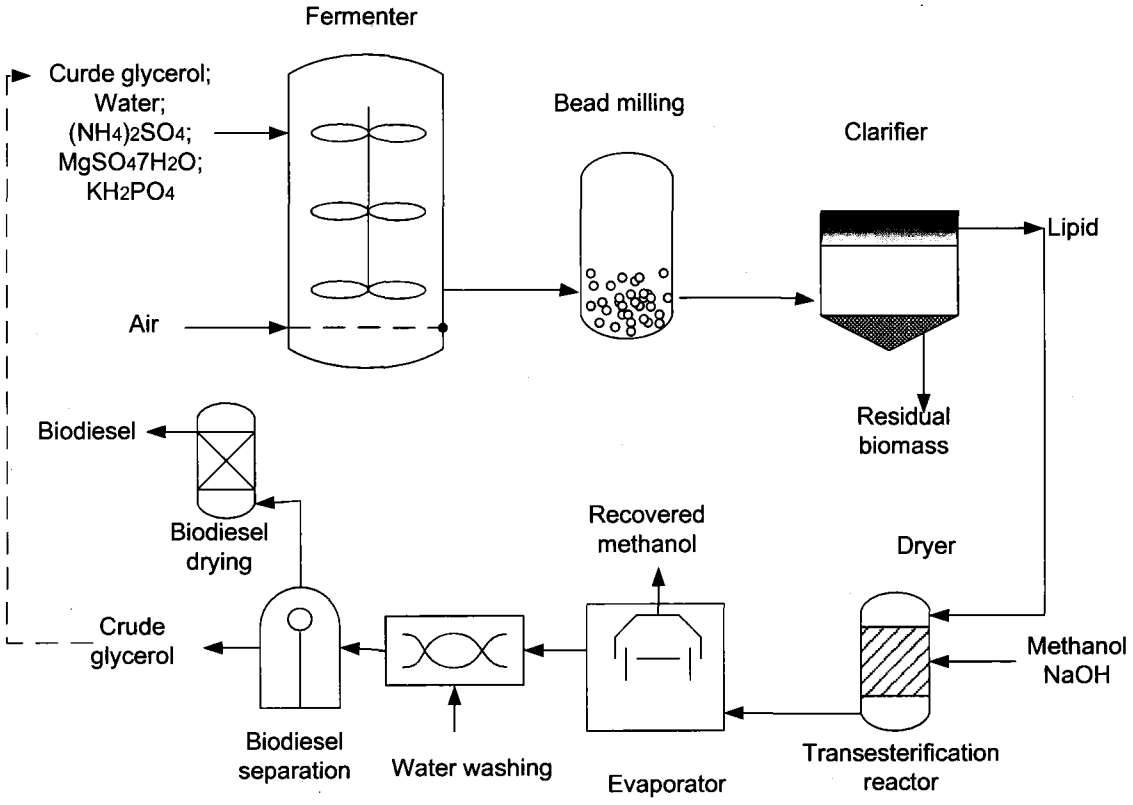

Figure 10.1 **Diagram of biodiesel production from crude glycerol**

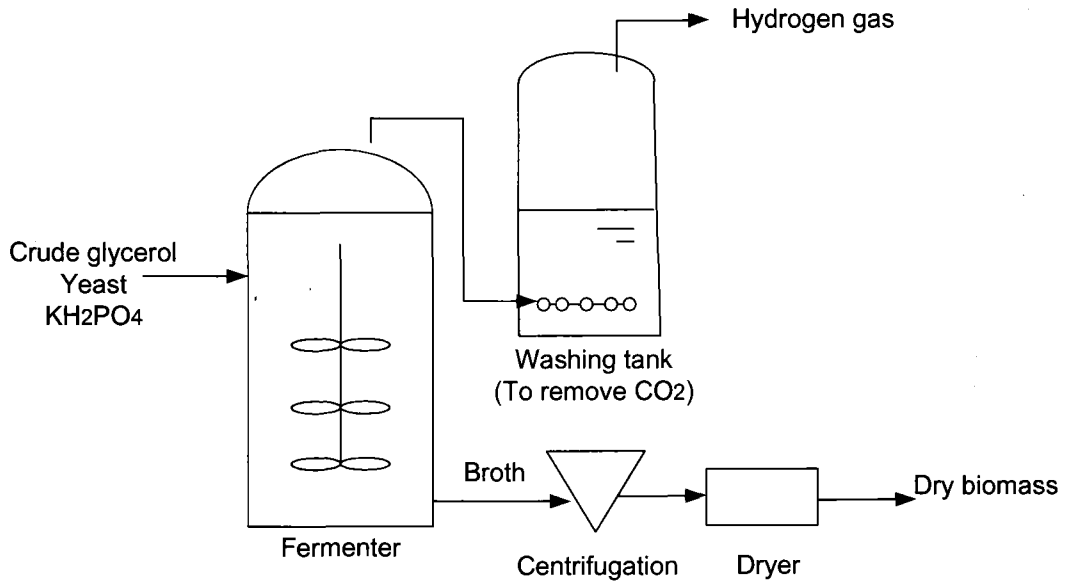

Figure 10.2 **Diagram of hydrogen production from crude glycerol**

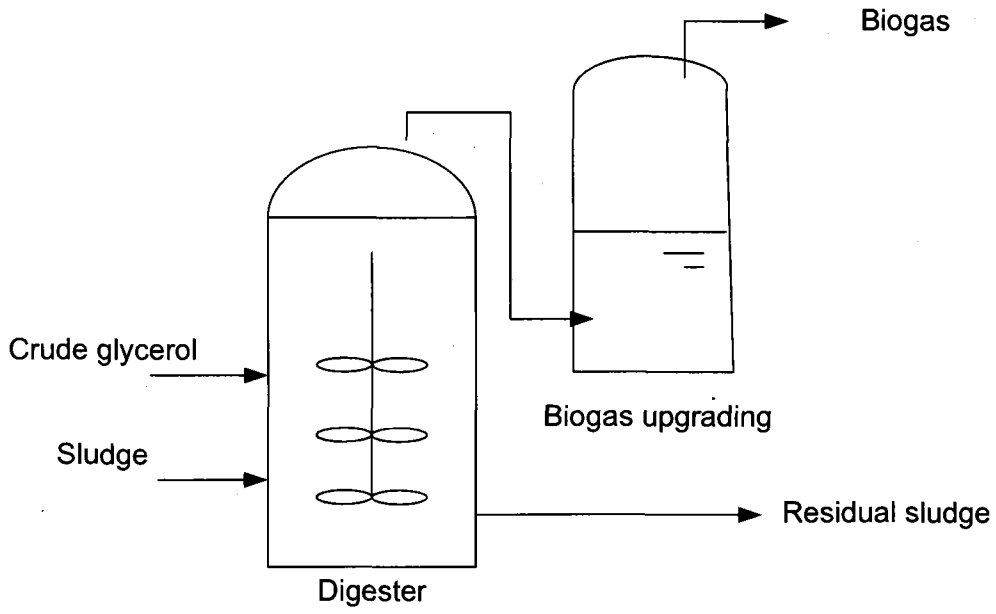

Figure 10.3 **Diagram of biogas production from crude glycerol**

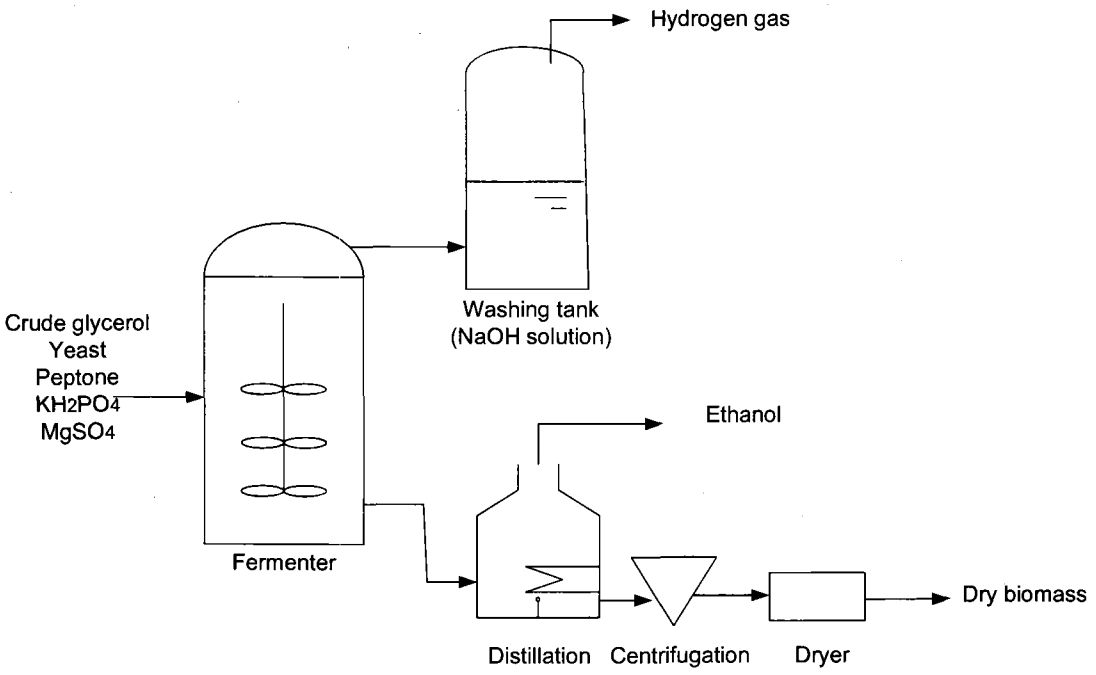

Figure 10.4 **Diagram of ethanol production from crude glycerol**

COST ESTIMATION OF LIPID PRODUCTION FROM CRUDE GLYCEROL

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BIOMASS AND BIOENERGY (UNDER REVIEW)

11 COST ESTIMATION OF LIPID PRODUCTION FROM CRUDE GLYCEROL

11.1 Résumé

Le glycérol brut, un sous-produit de la production de biodiesel, est une source de carbone utilisable pour certains microorganismes oléagineux. Dans notre laboratoire, une levure oléagineuse et des champignons ont été isolés et testés pour l'accumulation de lipides, en utilisant le glycérol brut comme source de carbone. Une forte teneur en lipides accumulés (jusqu'à 70% p/p de biomasse) a été observée. Cela suggère un fort potentiel de valorisation du glycérol brut comme matière première pour la production de biodiesel. Afin d'étudier la possibilité d'utiliser le glycérol brut pour produire des lipides, le coût du procédé a été évalué avec logiciel SuperPro Designer. Les paramètres y compris le temps de fermentation, la teneur en lipides, les sources d'éléments nutritifs pour la fermentation et la capacité de production ont un effet significatif sur le coût de production d'une unité de lipides. L'augmentation de la capacité de production de 1 million à 15 millions de litres de glycérol 80% (p/v), pourrait réduire le coût unitaire de 1.02 à 0.16 US$/kg de lipides produits. La teneur en lipides dans la biomasse produite joue également un rôle important sur le coût de production des lipides. Toute augmentation de la teneur de lipides de 10% aboutit à une diminution du coût de production de lipides de 0.20 US$ de l'unité. L'influence de la teneur en biomasse, du temps de fermentation et de la source de nutriments est moins importante sur le coût unitaire de production des lipides.

Mots clés : Biodiesel; coût; glycérol brut; lipide

11.2 Abstract

Crude glycerol, a by-product of biodiesel production, is valuable carbon source for some oleaginous microorganisms. In our lab, oleaginous yeast and fungi have been isolated and tested for lipid accumulation with crude glycerol as carbon source. High lipid content (up to 70% w/ biomass w) has been observed. It suggested that it could be a value added utilization of crude glycerol to produce lipid which is a great candidate of biodiesel production raw materials. To investigate the feasibility of utilizing crude glycerol for lipid production, cost is estimated by SuperPro Designer. The parameters including fermentation time, lipid content, and nutrient source for fermentation, and plant capacity effect on lipid production cost was investigated. Increase of the plant capacity from 1 million to 15 million liter 80% (w/v) glycerol, could reduce the unit cost from 1.02 to 0.16 US$/kg lipid produced. The lipid content of biomass produced also plays a significant role on the unit lipid production cost. Every 10% lipid increase gives around 0.20 US$ decrease in unit lipid production cost. Comparing to others, biomass yield, fermentation time and nutrient source is less affective in the unit cost.

Keywords: Biodiesel; cost; crude glycerol; lipid

11.3 Introduction

Large amount of crude glycerol is currently produced due to boom in biodiesel production industry. Purification of crude glycerol is becoming unaffordable due to high cost. Therefore, alternative method of crude glycerol management is highly required. Crude glycerol is a valuable carbon source for microorganisms to produce bio-products such as hydrogen, biogas, ethanol, and lipid. Use of crude glycerol as a raw material for lipid (subsequently converted to biodiesel) production is more attractive as it provides a green cycle (Figure 11.1). Lipid reacts with methanol to form biodiesel and glycerol. The glycerol then is recycled as carbon source to produce oleaginous microorganisms which is rich in lipid. After separation of lipid from biomass, the lipid will be then converted to biodiesel and glycerol again.

Oleaginous yeast and fungi have been isolated in our laboratory and tested for lipid production/accumulation with crude glycerol as carbon source. High lipid content (up to 70% lipid g/g biomass) has been observed. In order to investigate the economic feasibility of crude glycerol utilization as raw material to produce feedstock lipid for biodiesel production, cost estimations were made. Computer simulations to model and estimate the cost of production have been successfully used for many industrial processes such as bio-ethanol and bio-plastic (Kwiatkowski et al. 2006; Mudliar et al. 2008). In this study, SuperPro designer, widely used cost analysis software, was employed to estimate lipid production from crude glycerol.

11.4 Process model description

A simplified flow diagram of the process is shown in Figure 11.2. It includes seed fermentation (P-1/V-107) tank to produce seed culture, main fermentation (P-2/V-102) vessel to produce biomass rich in lipid, fermented broth storage (P-3/V-101) tank, cell disruption (P-4/BM-101) bead mill to release the lipid from biomass, and oil separation (P-5/OS-101) unit (phase separation) to separate lipid from residuals. The detailed information of each step of the process is given below.

Seed culture production: Seed fermenter (P-1/V-107) with a volume of 11 m^3 is used to produce seed culture. The medium used contains 100 g/L glycerol, 0.5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \bullet 7H_2O$, 0.2 g/L yeast extract, and 1 g/L of KH_2PO_4. The seed will be transferred into fermenter (P-2/V102) when the cells are in their exponential growth phase (approximately 24 h fermentation).

Main fermenter for biomass production: The seed culture is transferred to fermenter (P-2/V102) for biomass production. Two 120 m^3 fermenters were employed. The medium used contains 100 g/L glycerol and 5 g/L of wastewater sludge. After fermentation ended, the broth will be transferred to holding tank (P-3/V-101), which as a buffering tank before the broth is fed to bead milling.

Biomass storage tank: After fermentation, the broth is discharged to two 72 m^3 holding tank (P-3/V-101). The holding tank is used to temporarily store the broth before the broth is sent to bead milling.

Lipid extraction (bead mill): In order to separate lipids from cells, cell disruption is critical. Bead milling is a clean, efficient, and cheap cell disruption process, and suitable on cell disruption of spores, yeast and fungi (Shin et al. 1994; Klimek-Ochab et al. 2011). Therefore, bead milling was employed in the process for lipid separation from cells. The broth from holding tank is disrupted in two bead mill units (P-4/BM-101) with grinding volume of 0.45 m^3 for releasing lipid. After milling, the mixture is transferred to oil separation tank (P-5/OS-101). Lipids exist in all cells as energy storage molecular (cytoplasmic droplets, mainly triglycerides) and structural components (cell membranes, mainly phospholipids).

Lipid separation: After cell disruption, lipid droplets escape from cells and enter in water and tend to separate from water by flowing to the top (due to their low solubility in water and smaller density than water) which can be recovered as lipids (Haussard et al. 2003). The output of bead milling is sent to oil separation tank (P-5/OS-101) with a horizontal area of 1.64 m^2. The lipid is released out from the broken cells and float to the top. The cell debris settle in the bottom, and the water is in the middle. The lipid is collected for biodiesel production. The water

will be recycled to fermenter for dilution (Wang et al. 2012). The residual cells can be used as animal feed.

The assumptions and values of all operational parameters for each individual process are given in Table 11.1 and the mass balance of the process is presented in Table 11.2. The cost estimation was based on an annual lipid production rate of 1 million kg.

11.5 Economic evaluation

Capital investment is the sum of direct and indirect fixed capitals. For design purpose, the various items of direct fixed capital (DFC) and indirect fixed capital are estimated based on the total equipment purchase cost (PC) using several multipliers. Table 11.3 provides ranges and average values for the multipliers and a skeleton for the calculations. To calculate the capital investment, the equipment cost was the key.

The equipment purchase cost can be estimated from vendor quotations, equipment selling websites, published data, company data compiled from previous projects, and by using process simulators and other computer aids. Often, cost data for one or two discrete equipment sizes is available, but the cost for a different size piece of equipment has to be estimated. In such cases, the scaling law can be used as suggested in Equation 11.1:

Equation 11.1 $\mathbf{Cost_2=Cost_1\,(size2/size1)^I}$

Where the index ^{I} value normally falls between 0.5 and 1.0 with an average value for vessels of around 0.6. Generally 0.6 is applied when I value is unknown (Zhuang et al. 2007). In this study, the cost of the equipment was from equipment selling website (Table 11.4). Based on the equipment cost, capital cost was calculated and shown in Table 11.5.

The operating cost to run a biochemical plant is the sum of all expenses associated with raw materials, labor, utilities, overhead, etc. Dividing the annual operating cost by the annual production rate yields the unit production cost (in $/kg).

Raw materials account for the cost of all seed fermentation media. The price of a raw material can vary widely depending on its required purity. Various raw materials can be found in the Chemical Marketing Reporter. More recently, a number of websites have been created online where buyers pricing information can be found. In this study, fermentation media are wastewater sludge and crude glycerol. In order to obtain inoculum rapid growth, chemicals yeast extract, KH_2PO_4, $MgSO_4$, and $(NH_4)_2SO_4$ were assumed to be used as nutrients in seed fermentation. Current price of yeast extract, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ provide by ICIS (a world-leading chemical pricing and information service, offering unrivalled coverage of global chemical and energy markets) is 2000, 1200, 350, and 130 $/tonne. The total raw material cost is 3000 $/yr.

Labor cost is estimated based on the total number of operators required, which depends on the operation time. In a single product facility, the number of operators in each shift must be based on maximum demand during that shift. In general, smaller plants which are relatively less automatic tend to utilize a larger number of operators per processing step; however, in a large and highly automated plant a single operator may remotely handle the process alone. In addition, for the same automatic extent plant large scale plants is more labor cost efficient than the smaller plants. Because they may require the same number of operators for the process, but the production rate is different. Hence the labor cost contribution will be different in the unit production cost. In the study, the basic labor cost is 20 $/h. The labor cost was calculated by multiplying the basic labor cost and operation time per year (7920 h/yr), which is 158 000 $/yr.

Lab/QC/QA refers to the cost of off-line analysis, quality control (QC), and quality assurance (QA) costs. This cost is usually 10-20% of the operating labor cost. In this study, the average value 15% is taken to calculate lab/QC/QA cost and it is 24 000 $/yr.

Utilities include heating (steam) and cooling (cooling or chilled water) as well as electricity. The different utility costs are calculated as part of the material and energy balance. Fermenters and bead milling are major consumers of electricity but downstream processing equipment generally does not consume much electricity. In terms of unit cost, electricity costs is

0.06 $/kWh (Quebec, Canada). Cooling water and chilled water are 0.1 and 0.4 $/1000 kg, respectively. The utilities cost is 87 000 $/yr.

Equipment-dependent is from the depreciation of the fixed capital investment, maintenance of equipment, insurance, and local (property) taxes. For preliminary cost estimates, the entire fixed capital investment is usually depreciated linearly over a 10-year period. The annual equipment maintenance cost is normally estimated as a 10 per cent of the equipment's purchase cost (Petrides 2003). Insurance value for bioprocessing facilities is generally in the range of 0.5-1% of direct fixed capital cost (DFC). In this study 1% DFC is taken for insurance cost. The local tax is usually 2-5% of DFC and 2% is taken in this study. The factory expense represents overhead cost incurred by the operation of non-process-oriented facilities and organizations including accounting, payroll, fire protection, security, cafeteria, etc. A value of 5-10% of DFC is appropriate for these costs and 5% is taken in this study. Therefore, the equipment-dependent cost for this process is 269 000 $/yr.

The total annul operation cost is calculated as 541 000 $/yr, which is sum of the raw material, labor, utilities, lab/QA/QC, and equipment-dependent cost (Table 11.6). The unit cost of the product is thus calculated by dividing the annual operating cost (541 000 $ per year) by annual production rate (1 000 000 kg lipid per year). Thus, the unit production cost is 0.541 $/kg lipid. The breakdown of the unit cost is shown in Table 11.7. Equipment-dependent (49.78%) is the major fraction of the unit cost. Depreciation of the fixed capital investment and maintenance of the facility are the main contributors to this cost. Labor and utilities account for 29.31% and 16.05% of the overall cost, respectively. Lab/QC/QA cost lies in the fourth position and raw materials is the fifth. Normally, the raw material cost is around 50% of lipid production cost, while it is dramatically reduced in this process, which is due to the use of the wastewater sludge as nutrients.

There are two credits taken into consideration; the first one is the credit from biomass which is produced along with lipid (0.67 g biomass/ g lipid). The residual biomass is given a value of 75 $/ton (Alabi et al. 2009). Based on 0.67 kg residual biomass per kg lipid produced, the credit from residual biomass will be 0.050 $/kg lipid. The second credit is from the production of glycerol as a by-product. As the produced lipid will be used for biodiesel production; therefore,

0.096 kg of crude glycerol will be produced per kg of lipid, hence, around 96 000 kg extra glycerol will be generated annually. Then if the glycerol would be used to ferment by oleaginous microorganism, around 60480 kg lipid will be generated. The credit from this extra lipid generated (minus the operation cost for producing this extra lipid) will be 0.055 $/kg lipid. Thus, the net unit cost of lipid production will be 0.436$/kg lipids. The important information of the cost estimation is summarized in Table 11.8.

11.6 Sensitivity analysis

The sensitivity of the key process parameters including the plant capacity (0.4, 1, 1.8, and 5 million kg of lipid/year), glycerol concentration in fermentation (50 and 100 g/L), fermentation time (72, 48, and 36 h), biomass yield (0.63, 0.6, 0.5, and 0.4 g biomass/g glycerol), lipid content (50%, 60%, and 70% w/w biomass), crude glycerol composition (60 and 80% w glycerol/v), and crude glycerol cost (0.0 and 0.15 $/kg) was studied. The results are summarized in Table 11.9.

It can be seen that plant capacity has the greatest impact on unit lipid production cost (Table 11.9). Increase in plant capacity from 0.4 million to 5 million kg lipid production per year, reduced the calculated cost from 0.66 to 0.157 $/kg lipid produced. In addition, crude glycerol composition and crude glycerol cost also affected significantly the cost of lipid production. When methanol content is increased from approximately 2% to 15.8% w/v, the cost reduced from 0.44 to 0.3 $/kg lipid. Lipid production cost doubled when crude glycerol cost increased from 0.0 to 0.15 $/kg lipid. The lipid content of the biomass produced also plays a significant role on the unit lipid production cost. Every 10% lipid increase gives around 0.2 $ decrease in unit lipid production cost. Comparing to others, biomass yield, fermentation time and nutrient source affect the unit cost to a lesser extent.

From the calculation, it was observed that the unit production cost would be lower than 0.66 $/kg lipid as the plant is set on 1 million kg lipid produced or higher scale. The currently biodiesel feedstock is soybean oil, which sells at 0.85 $/kg. Therefore, it suggests that crude glycerol for lipid production is a promising alternative for biodiesel feedstock.

11.7 Conclusions

The cost lipid production using crude glycerol as carbon source and oleaginous microorganism to produce lipid was estimated. The study showed that the unit lipid production cost is 0.44 $/kg under the following conditions: 1 million kg lipid production per year, glycerol concentration 100 g/L, sludge as nutrient (2 g/L), biomass yield 0.63 g/g glycerol, lipid content 60% w/w biomass, and fermentation time 48 h. When the plant capacity and lipid content increased, the unit lipid production cost decreased. However, when fermentation time and crude glycerol price increased, the cost increased as well. The most profound parameter found was plant capacity and crude glycerol cost; therefore, the two parameters should be given the highest attention in building a practical plant.

11.8 Acknowledgements

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11.9 References

Alabi, A. O., Tampier, M., and Bibeau, E. (2009). "Microalgae technologies and processes for biofuelsbioenergy production in British Columbia." Report Seed Science Ltd. (Jan. 14, 2009).

Haussard, M., Gaballah, I., Kanari, N., de Donato, P., Barrès, O., and Villieras, F. (2003). "Separation of hydrocarbons and lipid from water using treated bark." Water Research, 37, 362-374.

Klimek-Ochab, M., Brzezińska-Rodak, M., Żymańczyk-Duda, E., Lejczak, B., and Kafarski, P. (2011). "Comparative study of fungal cell disruption—scope and limitations of the methods." Folia Microbiologica, 56, 469-475.

Kwiatkowski, J. R., McAloon, A. J., Taylor, F., and Johnston, D. B. (2006). "Modeling the process and costs of fuel ethanol production by the corn dry-grind process." Industrial Crops and Products, 23, 288-296.

Mudliar, S. N., Vaidya, A. N., Suresh Kumar, M., Dahikar, S., and Chakrabarti, T. (2008). "Techno-economic evaluation of PHB production from activated sludge." Clean Technologies and Environmental Policy, 10, 255-262.

Petrides, D. (2003). "Bioprocess design and economics." Oxford University Press, 2003.

Shin, J., Lee, G., and Kim, J. (1994). "Comparison of cell disruption methods for determining β-galactosidase activity expressed in animal cells." Biotechnology Techniques, 8, 425-430.

Wang, Y., Liu, W., and Bao, J. (2012). "Repeated batch fermentation with water recycling and cell separation for microbial lipid production." Frontiers of Chemical Science and Engineering, 6, 453-460.

Zhuang, J., Marchant, M. A., Nokes, S. E., and Strobel, H. J. (2007). "Economic analysis of cellulase production methods for bio-ethanol " American Society of Agricultural and Biological Engineers, 23, 679-687.

Table 11.1 Assumptions and operation parameters of lipid production from crude glycerol

Process	Details
Seed fermentation (P-1/V-107)	
Assumptions	Seed fermentation time: 24 h Density of 80% crude glycerol: 1.219 kg/L
Operation parameters	Temperature: 28 °C; Agitation energy: 0.5 kW/m^3 Aeration rate: 0.5 vvm Heat transfer: cooling water
Product fermentation (P-2/V102)	
Assumptions	Fermentation time: 48 h Inoculation volume: 5% seed v/v Biomass productivity: 0.63 g biomass/g glycerol Lipid content: 60% w/w biomass Density of 80% crude glycerol: 1.219 kg/L
Operation parameters	Temperature: 28 °C; Agitation energy: 0.5 kW/m^3 Aeration rate: 0.5 vvm Heat transfer: cooling water
Broth storage (P-3/V-101)	
Assumptions	Biomass concentration of output stream: 63 g/L
Operation parameters	Temperature: 25 °C;
Cell disruption (P-4/BM-101)	
Assumptions	Cell disruption efficiency: 96%
Operation parameters	Temperature: 25 °C; Processing rate: 2.2 m^3/h; Passes: 3 Processing time/pass: (20/3) min Cooling agent: chilled water
Oil separation (P-5/OS-101)	
Assumptions	Oil separation efficiency: 99%
Operation parameters	Temperature: 25 °C; Processing time: 45 min

Table 11.2 Mass balance of lipid production from crude glycerol

Process	Component	In (kg/year)	Out (kg/year)
Seed fermentation (P-1/V107)	KH_2PO_4	1 429.63	0
	Magne Sulfate	715.10	0
	Yeast	285.92	0
	Amm. Sulfate	714.82	0
	Crude glycerol	178 704.31	0
	Water	1 248 150.99	0
	Seed culture broth	0	1 430 000.77
	Total	1 430 000.77	1 430 000.77
Product fermentation (P-2/V102)	Crude glycerol	2 831 304.87	0
	Wastewater sludge	57 185.38	0
	Water	24 174 565.47	0
	Fermentation broth	0	27 063 055.72
	Total	27 063 055.72	27 063 055.72
Broth storage (P-3/V-101)	Fermentation broth	27 063 055.72	0
	Fermentation broth	0	27 063 055.72
	Total	27 063 055.72	27 063 055.72
Cell disruption (P-4/BM-101)	Biomass (Conc.=50g/L)	27 063 055.72	0
	Mixture (lipid, cell debris, and water)	0	27 063 055.72
	Total	27 063 055.72	27 063 055.72
Oil separation (P-5/OS-101)	Mixture (lipid, cell debris, and water	27063055.72	0
	Lipid	0	1 000 000
	Water	0	25 392 519.16
	Residual biomass	0	670 140.56
	Total	27 063 055.72	27 063 055.72

Table 11.3 Calculation information of capital investment

Items	Values
Year of analysis	2013
Depreciation	10 years
Salvage	5%
Total plant direct cost (TPDC)	
Equipment Purchase Cost (PC)	From references
Installation	0.40 x PC
Process Piping	0.2 x PC
Instrumentation	0.18 x PC
Insulation	0.03 x PC
Electrical Facilities	0.1 x PC
Unlisted equipment purchase cost (UEPC)	0.05 x PC
Unlisted equipment installation	0.30 x UEPCPC
TOTAL PLANT INDIRECT COST (TPIC)	
Engineering	0.10 x TPDC
TOTAL PLANT COST (TPC)	TPDC+TPIC
Contractor's fee	0.05 x TPC
Contingency	0.10 x TPC
Direct fixed capital (DFC)	TPC+ Contractor's fee+ Contingency
Start up and validation cost	5% DFC
Maintenance	1% DFC
Insurance	1% DFC
Local taxes	2% DFC
Factory expense	2% DFC
Basic labor cost (BLC)	20 $/h
Lab QC/QA	15%TLC
Electricity cost	
General load (such as office)	15% of total electricity utilization
Unlisted equipment	5% of total electricity utilization

Table 11.4 Major equipment specification and cost (2013 prices)

Equipment/ Quantity	Description	Unit cost ($)	Cost ($)	Ref.
Seed fermenter (P-1/V-107)/1	V=11 m^2 D=1.7 m	44 000	44 000	http://www.alibaba.com/product-gs/533365399/100_cbm_fermentation_tanks_industrial_fermentation.html
Production fermenter (P-2/V-102)/2	V=120 m^2 D=3.7 m	165 000	330 000	http://www.alibaba.com/product-gs/533365399/100_cbm_fermentation_tanks_industrial_fermentation.html
Storage tank (P-3/V-101)/3	V=72 m^2 D=3.1 m	68 000	204 000	http://www.alibaba.com/product-gs/559239746/NZS_9_Series_Mineral_Processing_Central.html
Bead milling (P-4/BM-101)/2	Grinding volume =0.45 m^3	49 000	98 000	http://www.alibaba.com/product-gs/670509134/Leather_Cemical_Vertical_Bead_Mill.html
Oil separation (P-5/OS-101)/1	Horizontal Area = 1.64 m^2	9 000	9 000	http://www.alibaba.com/product-gs/532503026/YSFL_COMMON_OIL_AND_WATER_SEPARATION.html
Unlisted equipment			36 000	
Total			722000	

Table 11.5 Fixed capital estimate summary (2013 prices)

A. TOTAL PLANT DIRECT COST (TPDC) (cost $)

1. Equipment Purchase Cost 722 000
2. Installation 306 000
3. Process Piping 144 000
4. Instrumentation 130 000
5. Insulation 22 000
6. Electricals 72 000

TPDC = 1 397 000

B. TOTAL PLANT INDIRECT COST (TPIC)

7. Engineering 140 000

TPIC = 140 000

C. TOTAL PLANT COST (TPDC+TPIC) TPC = 1 536 000

8. Contractor's fee 77 000
9. Contingency 123 000

(8+9) = 200 000

D. DIRECT FIXED CAPITAL (DFC) TPC+8+9 = 1 736 000

Table 11.6 Annual operation cost summary (2013 prices)

Cost items	$/year	%
Raw materials	3 000	0.46
Labor	158 000	29.31
Equipment-dependent	269 000	49.78
Lab/QC/QA	24 000	4.40
Utilities	87 000	16.05
Total	541 000	1000

Table 11.7 Unit production cost break down

Cost item	$/kg	Fraction (%)
Raw materials	0.003	0.46
Labor-dependent	0.159	29.31
Equipment-dependent	0.269	49.78
Lab/QA/QC	0.024	4.40
Utilities	0.087	16.05
Unit production cost	0.541	100

Table 11.8 The summary of the cost estimation of 1 million kg lipid production

Scale	1 million kg lipid production per year
80% crude glycerol utilization amount (liter/per yr)	3110009
Glycerol concentration for fermentation	100 g/L
Nutrient of production fermentation	Wastewater sludge
Biomass productivity	0.63 g biomass/g glycerol
Lipid content	60 %
Fermentation time	48 h
Plant operation time/yr	7920 h (330 d)
Labor shift /d	3 (every 8 h for shift)
Electricity installed capacity	134 Kw
Electricity utilization	1055110 kWh/yr
Capital investment	1845000 $
Equipment cost	269000 $/yr
Raw material cost	3000 $/ yr
Labor cost	158400 $/ yr
Utilities cost	86757 $/ yr
Lab/QA/QC	23760 $/ yr
Operation cost	540000 $/ yr
Production rate	1000000 kg/yr
Unit production cost (C1)	0.541 $/ kg
Credit from residual biomass (C2)	0.050 $/kg
Credit from extra glycerol production (C3)	0.055 $/kg
Net production cost (C1-C2-C3)	0.436 $/kg

Table 11.9 Parameter effect on unit lipid production cost

Cases	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Lipid prod. rate (million kg/yr)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	1	1	1	1	1	1	1	1.8	5
Crude glycerol purity (w/v)	80%	80%	80%	80%	80%	80%	80%	80%	80%	80%	80%	80%	80%	80%	80%	80%	80%
Methanol content (w/v)	<2%	<2%	<2%	<2%	<2%	<2%	<2%	<2%	<2%	<2%	<2%	<2%	15.8% (20% v/v)	15.8% (20% v/v)	31.6% (40% v/v)	<2%	<2%
Crude glycerol cost ($/kg)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.15	0.15	0.0	0.0
Fermentation time (h)	72	72	72	72	48	48	36	48	48	48	48	48	48	48	48	48	48
Nutrients	Chem.	Chem.	Chem.	Slu.	Chem.	Slu.	Chem.	Slu.	Slu.	Slu.	Slu.	Slu.	Slu.	Slu.	Slu.	Slu.	Slu.
Glycerol concentration (g/L)	50	50	50	50	50	50	50	100	100	100	100	100	100	100	100	100	100
Biomass yield (g/g glycerol)	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.60	0.50	0.40	0.63	0.63	0.63	0.63	0.63
Lipid content (%w/w biomass)	50	60	70	70	70	70	70	60	60	60	60	60	60	60	60	60	60
Unit lipid cost ($/kg)	1.56	1.23	1.104	1.008	0.994	0.879	0.934	0.765	0.545	0.558	0.576	0.604	0.405	0.765	0.665	0.412	0.267
Credits ($/kg)	0.13	0.105	0.084	0.087	0.084	0.089	0.084	0.105	0.105	0.105	0.105	0.105	0.105	0.105	0.105	0.109	0.110
Net unit lipid cost ($/kg)	1.43	1.19	1.02	0.921	0.910	0.790	0.850	0.660	0.440	0.453	0.471	0.499	0.300	0.660	0.560	0.303	0.157

Chem.=chemicals; Slu.=sludge

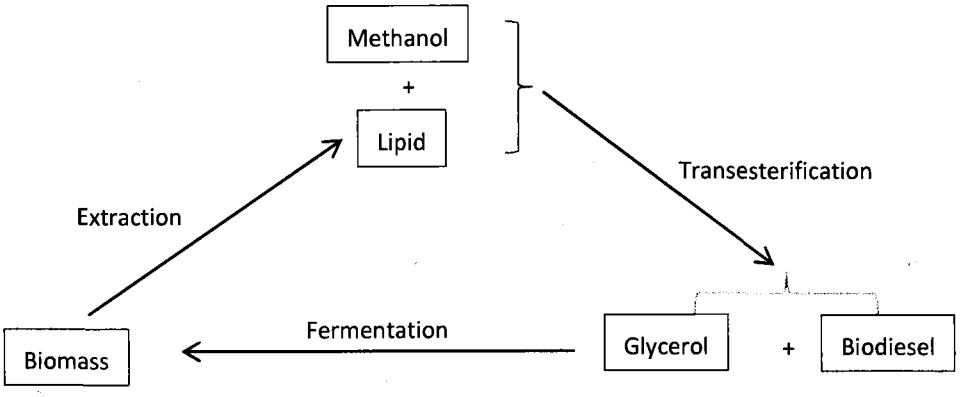

Figure 11.1 **Crude glycerol utilization in biodiesel production**

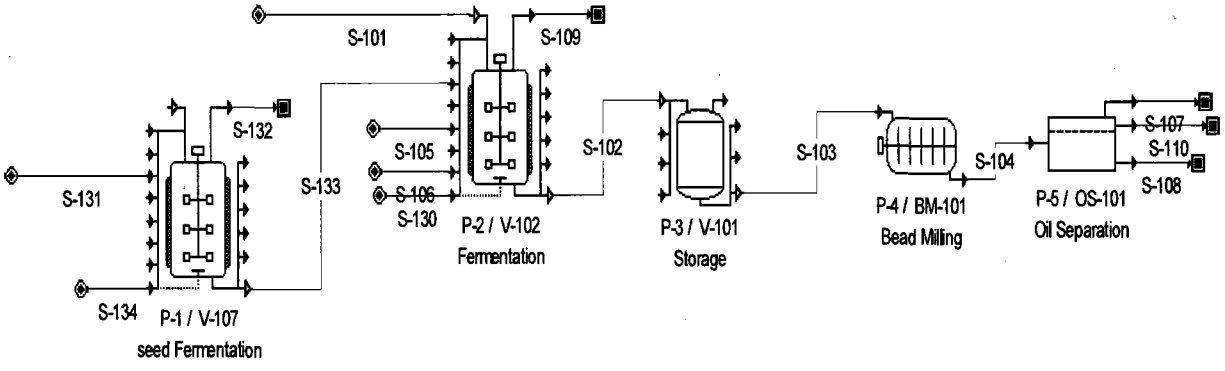

Figure 11.2 **Process flow sheet of lipid production from crude glycerol**

ULTRASONICATION ASSISTED LIPID EXTRACTION FROM OLEAGINOUS MICROORGANISMS

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BIORESOURCE TECHNOLOGY (SUBMITTED)

12 ULTRASONICATION ASSISTED LIPID EXTRACTION FROM OLEAGINOUS MICROORGANISMS

12.1 Résumé

Différents solvants, y compris l'eau, l'hexane, le méthanol et le chloroforme/méthanol (1:1 v/v), ont été testés pour l'extraction des lipides à partir de *Trichosporon oleaginosus* et SKF-5 sous ultrasons (520 KHz 40 W et 50 Hz 2800 W). L'efficacité d'extraction par ultrasons a été comparée avec la méthode d'extraction conventionnelle par chloroforme/méthanol (2:1 v/v). Des taux de récupération maximums de lipides de 10.2% et de 9.3% avec de l'eau, 43.2% et 33.2% à l'hexane, 75.7% et 65.1% avec du méthanol, 100% et 100% p/p de biomasse avec du chloroforme/méthanol ont été obtenus à partir de *Trichosporon oleaginosus* et SKF-5, respectivement, avec ultrason ultilisation à 50 Hz 2800 W. L'extraction par chloroforme/méthanol et ultrasons a permis la récupération du contenu total en lipides en peu de temps (15 minutes) et à basse température (25 °C) tandis que la récupération du contenu total en lipides par extraction conventionnelle avec chloroforme/méthanol nécessite un temps de 12 h à 60 °C. L'extraction par chloroforme/méthanol et ultrasons serait une méthode prometteuse pour l'extraction des lipides des microorganismes.

Mots clés : Extraction des lipides ; ultrasons ; microorganisme oléagineux; biodiesel

12.2 Abstract

Various solvents, including water, hexane, methanol, and chloroform/methanol (1:1 v/v), were tested to identify the efficiency of lipid extraction from *Trichosporon oleaginosus* and an oleaginous fungal strain SKF-5 under ultrasonication (520 kHz 40 W and 50 Hz 2800 W) and compared with the conventional chloroform methanol (2:1 v/v) extraction method. The highest lipid recovery 10.2% and 9.3% with water, 43.2% and 33.2% with hexane, 75.7% and 65.1% with methanol, 100% and 100% w/w biomass with chloroform/methanol were obtained from *Trichosporon oleaginosus* and SKF-5 strain, respectively, at ultrasonication frequency 50 Hz and power input 2800 W. Ultrasonication chloroform/methanol extraction recovered total lipid in a short time (15 min) and low temperature (25 °C). Whereas the conventional chloroform methanol extraction to achieve total lipid recovery required 12 h and 60 °C. Ultrasonication chloroform/methanol extraction would be a promising method of lipid extraction from the microorganisms.

Keywords: Lipid extraction; ultrasonication; oleaginous microorganism; biodiesel

12.3 Introduction

Demand for alternative fuels has grown significantly due to the fact that traditional fuel is depleting, petroleum prices are increasing, and control of greenhouse gas emissions is getting more important than ever before. Biodiesel, fatty acid methyl esters (FAMEs), has grabbed great attention due to the advantages that it is renewable, sustainable, environment friendly (burns much cleaner than petroleum diesel), compatible with current commercial diesel engines, as well as has excellent lubricity and could provide similar energy density to diesel (Canakci and Sanli 2008). Biodiesel is derived from oils or fats, which are contained in plants seeds, microorganisms, or animals. Most of plants seed oil and animal fats are essentially required in food production industry and kitchens. Oleaginous microorganisms are promising feedstock of biodiesel production due to their impressive lipid content up to 80 % w/w on its dry biomass (Koutb and Morsy 2011; Gao et al. 2013).

Biodiesel production from microorganisms includes three steps, microorganism cultivation (lipid accumulation), lipid extraction (lipid separation from biomass), and biodiesel synthesis. Lipid extraction is the critical step in biodiesel production. Chloroform and methanol mixture is currently employed on lipid extraction from microorganisms and found efficient (Vicente et al. 2009; Cheirsilp et al. 2011; Cheng et al. 2011; Boyd et al. 2012). The concerns on flammability and high toxicity of chloroform lead to seeking the technologies with less threat to the human being and the environment. In addition, lipid extraction from microorganisms with chloroform and methanol requires long time (4 to 12 h) at moderate temperature (50 to 60 °C). Therefore, to lower the amount or to completely eliminate the use of toxic solvent and reduce the lipid extraction time and temperature becomes the key solution of the problem.

Cell disruption with bead milling, homogenizer, microwave, or ultrasonication prior to solvent extraction could reduce the solvent utilization as well as decrease the process time (Ranjan et al. 2010; Araujo et al. 2013). A study has been conducted on lipid extraction with ultrasonication without addition of organic solvent (Adam et al. 2012). Biomass concentration and extraction time were varied in the study and it was found that with 50 g/L microbial biomass concentration and 30 min process time provide the highest lipid recovery (5% w/w total lipid).

However, literature is devoid of the data on ultrasonication treatment conditions (such as solvent type, cell concentration, ultrasonication frequency and power input, etc.) impact on lipid extraction. Therefore, the aim of this research work is to demonstrate the potential of ultrasonication aided lipid extraction from oleaginous yeast and fungus employing various solvents at different operating conditions.

12.4 Methods

12.4.1 Strain, culture and harvesting conditions

Oleaginous yeast *Trichosporon oleaginosus* (ATCC20509) was grown in (per liter): 1 g $(NH_4)_2SO_4$, 1g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g yeast extract, 20 g glucose, 0.04 $CaCl_2 \cdot 2H_2O$, 0.0055 $FeSO_4 \cdot 7H_2O$, 0.0052 citric acid$\cdot H_2O$, 0.001 $ZnSO_4 \cdot 7H_2O$, and 0.00076 $MnSO_4 \cdot H_2O$ (Zheng et al. 2012). Fungus SkF-5 (isolated in our lab) was grown in the medium containing (per liter): 1 g $(NH_4)_2SO_4$, 1g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g yeast extract, and 50 g glycerol. The crude glycerol (by-product of biodiesel production) was obtained from an industrial biodiesel production company in Quebec of Canada. The purification of the crude glycerol was performed by lowering its pH to 2 followed by removal of the FFA and salt by centrifugation. The purified glycerol thus obtained was used to grow lipid producing microorganisms.

The experiments on cultivation of oleaginous microorganisms were performed in shake flasks using a shaking incubator at 200 rpm 28 °C. After 72 h fermentation, biomass was harvested by centrifugation at 5000 rpm for 15 min. The biomass was washed twice with distilled water to remove the residual nutrients and glycerol. Part of the biomass was dried by lyophilisation and then stored for further study. The other part of the biomass was suspended in distilled water to achieve desired biomass concentration for further study.

12.4.2 Conventional lipids extraction methods

The standard chloroform and methanol extraction procedure with minor modification was employed to determine the lipid content in the biomass (Folch et al. 1957; Vicente et al. 2009). 200 mg dry biomass (after lyophilisation) was mixed with 4 ml solvent mixture of chloroform

and methanol (2:1 v/v), and then subjected to 60 °C for 4 h. The mixture was then centrifuged at 5000 rpm for 15 min and the lower solvent phase was withdrawn and transferred into a pre-weighed glass vial (W_1). The extraction procedure was repeated two times. Afterwards, the vial containing the total volume of the solvent collected from each extraction was evaporated at 60 °C and then weighed (W_2). The lipid amount was calculated by the difference of W_2 and W_1. The lipid content in the biomass was calculated as (W_2-W_1)/200 mg ×100%. The obtained lipid was then converted to biodiesel through transesterification.

12.4.3 Ultrasonic assisted lipid extraction

High frequency low power ultrasonication (520 kHz, 40 W) extraction was carried out in an ultrasonic set-up as shown in Figure 12.1. The ultrasonic system consists of an ultrasonic transducer installed on the bottom of a double-walled (jacket) glass reactor, an amplifier (T&C power Conversion, Inc.) for power control, a Hewlett Packard Model 3300A function generator for frequency control, and a temperature control device (Poly Stat, Cole Parmer), which circulates water in the jacket of the glass reactor.

Low frequency high power ultrasonication (50 Hz, 2800 W) extraction was performed in an ultrasonication bath (Fisher Scientific, FB15069).

12.4.3.1 Water as solvent

One liter of fresh biomass with 30, 50, and 70 g/L suspended solids concentration (suspended in distilled water) was transferred to the ultrasonication reactor/bath. The solution was subjected to ultrasonication for lipid extraction at 25, 35, 45, and 55 °C and samples were taken for analysis at 5, 10, 15, 20, and 30 min. After extraction, NaCl was added to bring the final NaCl concentration of 5% w/v sample solution for demulsifying lipid/water emulsion, and a few drops of hexane were added followed by centrifugation at 9000 rpm for 15 min. The supernatants were collected in burettes and allowed to stand for 24 h. The organic phase (the top layer) was collected in a pre-weighed glass tube and heated to 60 °C until its weight becomes constant. Then the lipid content was calculated. Thereafter, the lipid was converted to biodiesel through transesterification.

12.4.3.2 Hexane, methanol, and chloroform/methanol as solvent

The process was performed similar to that of lipid extraction with water as solvent. The dried biomass obtained from lyophilisation was mixed with solvent hexane, methanol, or chloroform/methanol (1:1 v/v) (50 g biomass/L solvent). The mixtures were then subjected to ultrasonication for desired time (5, 10, 15, and 20 min) at different temperature (25, 35, 45, and 55 °C). After ultrasonication, the mixture was centrifuged at 9000 rpm for 15 min and the supernatant solvent phase was withdrawn and transferred into a pre-weighed glass vial. After solvent evaporation at 60 °C in an oven, the weight of glass vial was recorded. The lipid amount was calculated by the difference in weight of the vial before and after solvent evaporation. The lipid was converted to biodiesel through transesterification.

12.4.4 Transesterification

Lipids obtained from different extraction methods were first dissolved in hexane (50 mL hexane/g lipid), then mixed with methanol. Lipid to methanol molar ratio was 1:6 (0.3 mL methanol per gram lipid). Sodium hydroxide was used as catalyst with concentration of 1% w/w (NaOH/oil). The mixture was then heated at 55 °C for 2 h. After reaction, 5% NaCl solution was added with a concentration of 100 mL per gram lipid, and then FAMEs was extracted by two times washing with hexane (100 mL per gram lipid). After washing, the mixture was allowed to stand for phase separation, and then hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% (w/v) sodium bicarbonate solution (20 mL per gram lipid), and the top layer was then dried at 60 °C in an oven (Halim et al. 2011)

The FAMEs in hexane were analyzed using a Gas Chromatography linked to Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500). The dimensions of the column used were 30 m × 0.25 mm, with a phase thickness of 0.2 μm. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1.3-dichlorobenzene was also used as internal standard with a concentration of 50 ppm.

All experiments were performed in triplicates, and average results were reported with standard deviation less than 5%.

12.5 Results and discussion

Lipids exist in all cells as energy storage molecular (cytoplasmic droplets, mainly triglycerides) and structural components (cell membranes, mainly phospholipids). Mechanical pressing and organic solvent extraction are the two widely applied methods for lipid extraction from lipid bearing substances while the disadvantages of the methods are the low lipid yield and high toxicity, respectively (Bamgboye and Adejumo 2007; Cheng et al. 2011). Therefore, clean and efficient alternatives are required. Ultrasonification provides cavitation phenomena. Microscopic bubbles at various nucleation sites in the fluid were formed during ultrasonification, which has two phases, rarefaction and compression. The bubbles grow during the rarefaction and are compressed during compression phase which cause the collapse of the bubbles. A violent shock wave is formed by the collapse of the bubbles, and then tremendous heat, pressure, and shear is generated, which induces the cell disruption (Benov and Al-Ibraheem 2002; Da Porto et al. 2013). After the disruption, cells open to release intercellular products (such as lipids droplets). Water as a green solvent is preferable to use for recovery of lipids. When lipid droplets escape from cells, they enter in water (as solvent) and tend to separate from water by flowing to the top (due to their low solubility in water and smaller density than water), and thus, it can be recovered as lipids.

12.5.1 Water as solvent

The Scanning Electron Microscope (SEM) images of the two strains before and after ultrasonication are shown in Figure 12.2. It has been observed that the cells disruption occurred after ultrasonication in case of both the microbial strains.

For both ultrasonication systems, extraction time is a critical parameter. The lipid recovery increased as the time of sonication increased from 5 to 20 min (Figures 12.3 and 12.4). After 20 min, the lipid recovery was near to maximum. Biomass concentration also showed effect on lipid extraction, and 50 and 70 g/L biomass provided a similar performance (Figures 12.3 and 12.4). Biomass concentration mainly impact on energy transfer on the surface of the cells. With higher biomass concentration, less energy is received by every single cell, and thus made the cell disruption harder. However, the lower biomass concentration will reduce the chances of

collision among the cells, and thus reduces the shear between the cells, which deceases the cell disruption. Therefore optimum concentration of biomass for ultrasonication to achieve maximum oil extrcation efficiency was 50 g/L.

Low frequency high power (50 Hz and 2800 W) ultrasonication was observed to perform slightly better in the extraction process than that of high frequency low power (520 kHz and 40 W). High operating frequency of the ultrasonic system provides gently and evenly distributed shear across the surface of the cells, while low frequencies produce more aggressive shear than the high one (Chanamai et al. 2000). Therefore, low frequency high power ultrasonication will be more likely to generate higher cell disruption than that of high frequency low power. It could also be observed from Figure 12.2 that ultrasonication at 50 Hz 2800 W provided better cell disruption than that at 520 kHz 40 W.

In the study, it was assumed that conventional chloroform and methanol (2:1 v/v) lipid extraction extracted the total lipid from biomass, which means 100% lipid in biomass was obtained. The lipid obtained from conventional chloroform and methanol (2:1 v/v) was 0.122 and 0.069 g per 0.2 g biomass of *Trichosporon oleaginosus* and SkF-5, respectively. Therefore, it is considered that the total lipid was 0.122 and 0.069 g per 0.2 g biomass of Trichosporon oleaginosus and SkF-5, respectively. Lipid recovery (%) was defined as the amount of lipid extracted out of the total lipid. Lipid recovery of ultrasonication extraction with water as solvent was 10.2% and 9.3% g lipid/ g total lipid for *Trichosporon oleaginosus* and SkF-5, respectively. The results were not consistent with the hypothesis that free lipid droplets would escape from the cells and flow up to the top (as described before). It indicates that lipid drops did not only simply exist inside the cells but also must be associated with other compounds, which are soluble in water. Some researchers reported that there could be a monolayer of phospholipids (embedding) proteins surrounding the lipid droplets (Brown 2001; Natter et al. 2005). Monolayer phospholipid with their polar head towards outer environment (water solution) and non-polar tails towards the neutral TAG core leads to the overall lipid droplets in water soluble form. As it is soluble in water, therefore, the lipid droplets separation from water by phase separation was not possible. Thus, it resulted in the low lipid recovery with water as solvent. Higher temperature showed increased lipid recovery. Temperature determines the

movement of the solutes in the system, and high temperature promotes the movements and increases the shear between the solutes. The lipid droplets surrounded by monolayer phospholipid collide with one another or other solutes such as cell residues, and could break the layer. Non-polar lipid droplets would be free and contact with water and thus be recovered from the mixture. It indicates that high temperature could enhance the lipid recovery; therefore, study on high temperature cooperating with ultrasonication lipid extraction with water should be further studied. In addition, pressure provides compression on molecules, and thus leads to high movement of the molecules and increases the shearing between solutes. Similarly as high temperature, pressure could also be used to increase lipid recovery in ultrasonication lipid extraction with water and it should be investigated.

12.5.2 Hexane, methanol, and chloroform/methanol as solvent

As water didn't provide comparable lipid recovery as conventional chloroform methanol extraction; therefore, organic solvents hexane, methanol, and chloroform/methanol were used in ultrasonication aided lipid extraction. Solvent type showed great impact on the extraction efficiency of lipids. Chloroform/methanol was the best solvent followed by methanol, then hexane for both the strains. The maximum lipid recovery from *Trichosporon oleaginosus* with chloroform/methanol, methanol, and hexane was 100%, 75.7%, and 43.2% w/w total lipid in 20 min extraction time with a biomass concentration of 50 g/L (Figure 12.5). Chloroform/methanol aided by ultrasonication was also the best one for lipid extraction from fungus SKF-5. The highest lipid recovery was 99.7% w/w total lipid in 20 min extraction time with 50 g/L biomass concentration.

As mentioned above, the lipid droplets were surrounded by monolayer of phospholipids; therefore, high polarity solvent such as methanol was required to solubilize the phospholipid layer. Chloroform and hexane are non-polar solvents and cannot accomplish the task. Ultrasonication aided extraction employing hexane mainly depended on the mass transfer of hexane to the monolayer. Methanol provides high physical attraction to the polar head of the phospholipids, and pulls or could even tear out the layer. When the damage on the phospholipids layer was large enough, the lipid droplets would escape out as free lipids, which could be the mechanism of ultrasonication aided methanol extraction. During the

ultrasoncation aided chloroform/methanol system, methanol played the role to disrupt the phospholipids layer and provided the chance for non-polar solvent chloroform to approach and dissolve the lipid droplets, and eventually recover the lipid. This would be the reason that chloroform/methanol gave the highest lipid recovery for both strains and no significant effect on extraction was observed with temperature change.

A slightly higher lipid recovery for each strain was observed during extraction with ultrasonication 50 Hz 2800 W than 520 kHz 40 W for methanol and hexane (Figures 12.5 and 3.8.6). As discussed before, it could be due to a stronger shear generated at low frequency ultrasonication treatment than at higher frequency. For chloroform/methanol extraction, they have the ability to extract the total lipids even without ultrasonication which plays the role to disrupt the cells and speed up the lipid extraction process.

It was observed that ultrasonication broke the cell wall of yeast *Trichosporon oleaginosus* and fungus SFK-5 (Figure 12.2). It suggested that ultrasonication is suitable in use for cell disruption of yeast and fungus. Within 10 min, around 90% lipid recovery was obtained in yeast but to get the similar lipid recovery (90%) it required around 15 min in fungus (Figures 12.5 and 12.6). It would be due to the difference of the cell wall structure of yeast and fungus. Yeast cell wall mainly contains glucan, manan, protein, and a small amount of chitin (1-2% w/w cell wall) while chitin, glucan, protein are the major component of fungus cell wall (Northcote and Horne 1952; Bowman and Free 2006). It was revealed that chitin was locating near the plasma membrane in layer form, and glucan, manan, and protein extended throughout the cell wall (Bowman and Free 2006). Chitin is long chain polymer and very tough to be broken. As fungus contains high chitin, it would be the reason that longer time was required to recover similar lipid from fungus than that of yeast (Figures 12.5 and 12.6).

Chloroform/methanol (1:1 v/v) with ultrasonication aided lipid extraction recovered total lipids in 15 min with lesser addition (requirement) of high toxic solvent chloroform while conventional chloroform methanol (2:1 v/v) needed 12 h to achieve the similar results. Moreover, a complete lipid extraction was achieved at room temperature 25 °C with ultrasonication instead of 60 °C for conventional chloroform methanol extraction. It suggested that ultrasonication aided lipid extraction with chloroform/methanol (1:1 v/v) would be a

promising alternative of conventional chloroform methanol extraction, which is time and energy consuming.

12.5.3 Profile of biodiesel obtained from lipid extracted with different solvents

For all types of extraction, biodiesel obtained from the extracted lipids mainly contains C16 and C18 (Tables 12.1 and 12.2). There was no difference in fatty acid compositions extracted using conventional chloroform methanol (2:1 v/v) or ultrasonication aided chloroform/methanol (1:1 v/v). It indicates ultrasonication doesn't affect the properties of the final product (composition of fatty acids). Comparing with other solvents, methanol exaction led to high percentage of C18:2. C18:2, which is mainly from phospholipids (Meesters et al. 1996); therefore, it indicates that lipid extracted with methanol has high content of phospholipids. This is due to the fact that methanol (polar) and phospholipids (head is polar) has the same polarity and soluble in each other.

Both strains have high saturation rate which is around 35 to 45% w/w total lipids (Tables 12.1 and 12.2). It suggested that the biodiesel will have high oxidation stability than soybean biodiesel (less than 20% w saturated lipids /w the total lipids). The high saturation tends to increase melting point and viscosity. While Jatropha biodiesel possesses around 30% w saturated lipids /w the total lipids (Kumar Tiwari et al. 2007), which (has similar saturation degrees as that of *Trichosporon oleaginosus* and SKF-5 lipids) is currently being used in diesel engine and performs well. It is thus concluded that the lipids produced from *Trichosporon oleaginosus* and SKF-5 are suitable in utilization of biodiesel production.

12.6 Conclusions

Type of solvent and concentration has significant impact on ultrasonication aided lipids extraction from oleaginous microorganisms. The highest lipid recovery was obtained using chloroform/methanol (1:1 v/v). The lipids recovery was of the same order as that of conventional chloroform methanol (2:1 v/v) extraction. Ultrasonication reduced extraction time to 15 min from 12 h used in the conventional method without affecting fatty acids profile.

Water as solvent with ultrasonication extracted around 10% w/w total lipids. It wasn't as efficient as organic solvent mediated extraction; however, it is a green technology and should be further studied to further increase the efficiency.

12.7 Acknowledgements

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12.8 References

Adam, F., Abert-Vian, M., Peltier, G., and Chemat, F. (2012). ""Solvent-free" ultrasound-assisted extraction of lipids from fresh microalgae cells: A green, clean and scalable process." *Bioresource Technology*, 114, 457-465.

Araujo, G. S., Matos, L. J. B. L., Fernandes, J. O., Cartaxo, S. J. M., Gonçalves, L. R. B., Fernandes, F. A. N., and Farias, W. R. L. (2013). "Extraction of lipids from microalgae by ultrasound application: Prospection of the optimal extraction method." *Ultrasonics Sonochemistry*, 20, 95-98.

Bamgboye, A. I., and Adejumo, A. O. D. (2007). "Development of a sunflower oil expeller." *International Commission of Agricultural Engineering*, 9, 1-7.

Benov, L., and Al-Ibraheem, J. (2002). "Disrupting escherichia coli: A comparison of methods." *Journal of Biochemistry and Molecular Biology*, 35, 428-431.

Boyd, A. R., Champagne, P., McGinn, P. J., MacDougall, K. M., Melanson, J. E., and Jessop, P. G. (2012). "Switchable hydrophilicity solvents for lipid extraction from microalgae for biofuel production." *Bioresource Technology*, 118, 628-632.

Brown, D. A. (2001). "Lipid droplets: proteins floating on a pool of fat." *Current Biology*, 11, R446-R449.

Canakci, M., and Sanli, H. (2008). "Biodiesel production from various feedstocks and their effects on the fuel properties." *J. Ind. Microbiol. Biotechnol.*, 35, 431-441.

Chanamai, R., Herrmann, N., and McClements, D. J. (2000). "Probing floc structure by ultrasonic spectroscopy, viscometry, and creaming measurements." *Langmuir*, 16, 5884-5891.

Cheirsilp, B., Suwannarat, W., and Niyomdecha, R. (2011). "Mixed culture of oleaginous yeast Rhodotorula glutinis and microalga Chlorella vulgaris for lipid production from industrial wastes and its use as biodiesel feedstock." *New Biotechnology*, 28, 362-368.

Cheng, C.-H., Du, T.-B., Pi, H.-C., Jang, S.-M., Lin, Y.-H., and Lee, H.-T. (2011). "Comparative study of lipid extraction from microalgae by organic solvent and supercritical CO_2." *Bioresour Technol*, 102, 10151-10153.

Da Porto, C., Porretto, E., and Decorti, D. (2013). "Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (Vitis vinifera L.) seeds." *Ultrasonics Sonochemistry*, 20, 1076-1080.

Folch, J., Lees, M., and G.H., S. S. (1957). "A simple method for the isolation and purification of total lipides from animal tissues." *Journal of Biological Chemistry*, 226, 497-509.

Gao, D., Zeng, J., Zheng, Y., Yu, X., and Chen, S. (2013). "Microbial lipid production from xylose by Mortierella isabellina." *Bioresource Technology*, 133, 315-321.

Halim, R., Gladman, B., Danquah, M. K., and Webley, P. A. (2011). "Oil extraction from microalgae for biodiesel production." *Bioresource Technology*, 102, 178-185.

Koutb, M., and Morsy, F. M. (2011). "A potent lipid producing isolate of Epicoccum purpurascens AUMC5615 and its promising use for biodiesel production." *Biomass and Bioenergy*, 35, 3182-3187.

Kumar Tiwari, A., Kumar, A., and Raheman, H. (2007). "Biodiesel production from jatropha oil (Jatropha curcas) with high free fatty acids: An optimized process." *Biomass and Bioenergy*, 31, 569-575.

Meesters, P. A. E. P., Huijberts, G. N. M., and Eggink, G. (1996). "High-cell-density cultivation of the lipid accumulating yeast <i>Cryptococcus curvatus</i> using glycerol as a carbon source." *Applied Microbiology and Biotechnology*, 45, 575-579.

Natter, K., Leitner, P., Faschinger, A., Wolinski, H., McCraith, S., Fields, S., and Kohlwein, S. D. (2005). "The spatial organization of lipid synthesis in the yeast *Saccharomyces cerevisiae* derived from large scale green fluorescent protein tagging and high resolution microscopy." *Molecular & Cellular Proteomics*, 4, 662-672.

Ranjan, A., Patil, C., and Moholkar, V. S. (2010). "Mechanistic Assessment of Microalgal Lipid Extraction." *Industrial & Engineering Chemistry Research*, 49, 2979-2985.

Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., Sádaba, I., Ruiz-Vázquez, R. M., Torres-Martínez, S., and Garre, V. (2009). "Biodiesel production from biomass of an oleaginous fungus." *Biochemical Engineering Journal*, 48, 22-27.

Zheng, Y., Chi, Z., Ahring, B. K., and Chen, S. (2012). "Oleaginous yeast Cryptococcus curvatus for biofuel production: Ammonia's effect." *Biomass and Bioenergy*, 37, 114-121.

Table 12.1 **Comparison of fatty acid profiles of biodiesel produced from the lipid of *Trichosporon oleaginosus***

Fatty acids	Relative amount of total fatty acid (% w/w)				
	Conventional chloroform methanol (2:1 v/v)	Water +ultra.	Hexane +ultra.	Methanol +ultra.	Chloroform/methanol +ultra.
C14:0	0.3	ND	ND	0.4	0.7
C15:0	0.6	0.3	0.8	0.6	0.7
C16:0	23.6	32.2	30.4	28.6	22.1
C16:1	0.5	ND	0.4	0.8	0.9
C18:0	12.4	11.1	10.3	12.1	11.9
C18:1	53.1	45.4	48.6	36.4	53.8
C18:2	8.3	11.0	9.5	20.9	8.5
C20:0	1.2	ND	0.3	0.2	1.5

ND= not detected;

Ultra.= ultrasonication;

The extraction with water was performed at ultrasonication 50 Hz 2800 W temperature 55 °C for 20 min at biomass concentration 50 g/L;

The extractions with hexane, methanol, and chloroform/methanol were performed at ultrasonication 50 Hz 2800 W temperature 25 °C for 15 min.

Table 12.2 Comparison of fatty acid profiles of biodiesel produced from the lipid of SKF-5

Fatty acids	Relative amount of total fatty acid (% w/w)				
	Conventional chloroform methanol (2:1 v/v)	Water +ultra.	Hexane +ultra.	Methanol +ultra.	Chloroform/methanol +ultra.
C14:0	ND	ND	ND	ND	ND
C15:0	0.4	0.1	0.3	0.4	0.4
C16:0	33.7	31.5	33.6	35.2	33.1
C16:1	2.2	1.9	1.9	1.8	2.3
C18:0	8.9	10.4	9.6	10.3	8.7
C18:1	42.1	41.0	44.5	29.2	43.0
C18:2	12.7	15.1	10.1	23.1	11.5
C20:0	ND	ND	ND	ND	ND

ND= not detected;

Ultra.= ultrasonication;

The extraction with water was performed at ultrasonication 50 Hz 2800 W temperature 55 °C for 20 min at biomass concentration 50 g/L;

The extractions with hexane, methanol, and chloroform/methanol were performed at ultrasonication 50 Hz 2800 W temperature 25 °C for 15 min.

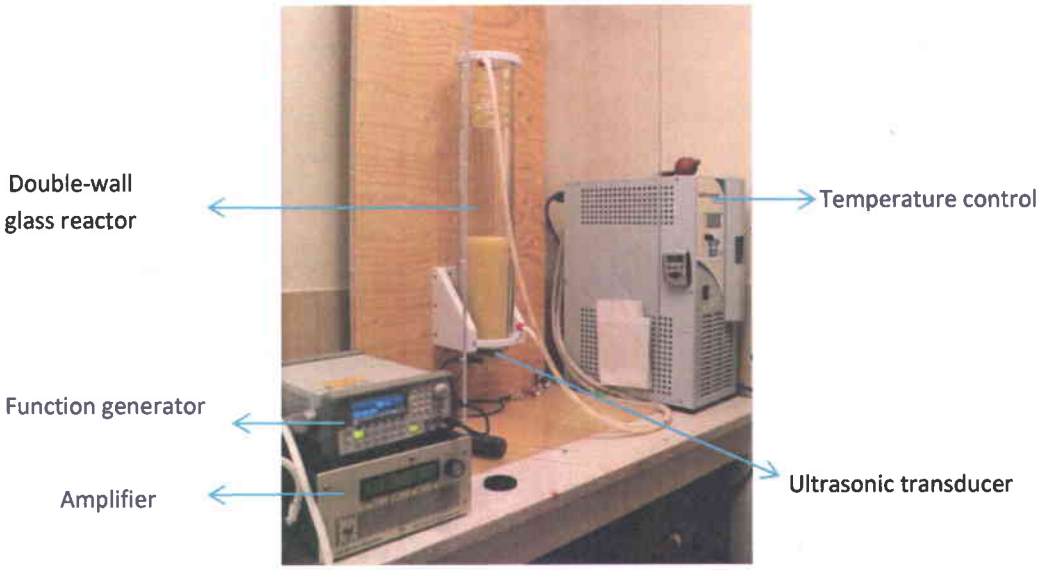


Figure 12.1 **Ultrasonication reactor (520 kHz, 40 W) for lipid extraction**

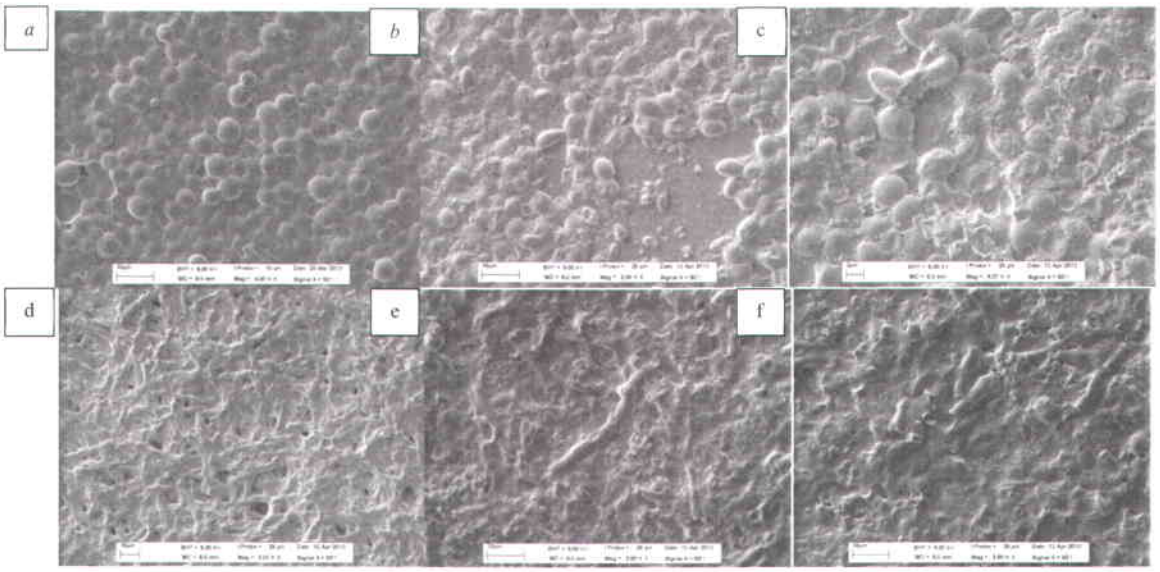


Figure 12.2 **SEM images of *Trichosporon oleaginosus* and SkF-5 before and after ultrasonication for 20 min at 25 °C (a= *Trichosporon oleaginosus* before ultrasonication; b= *Trichosporon oleaginosus* after ultrasonication at 520 kHz 40 W; c= *Trichosporon oleaginosus* after ultrasonication at 50 Hz 2800 W; d= SkF-5 before ultrasonication; e= SkF-5 after ultrasonication at 520 kHz 40 W; f= SkF-5 after ultrasonication at 50 Hz 2800 W)**

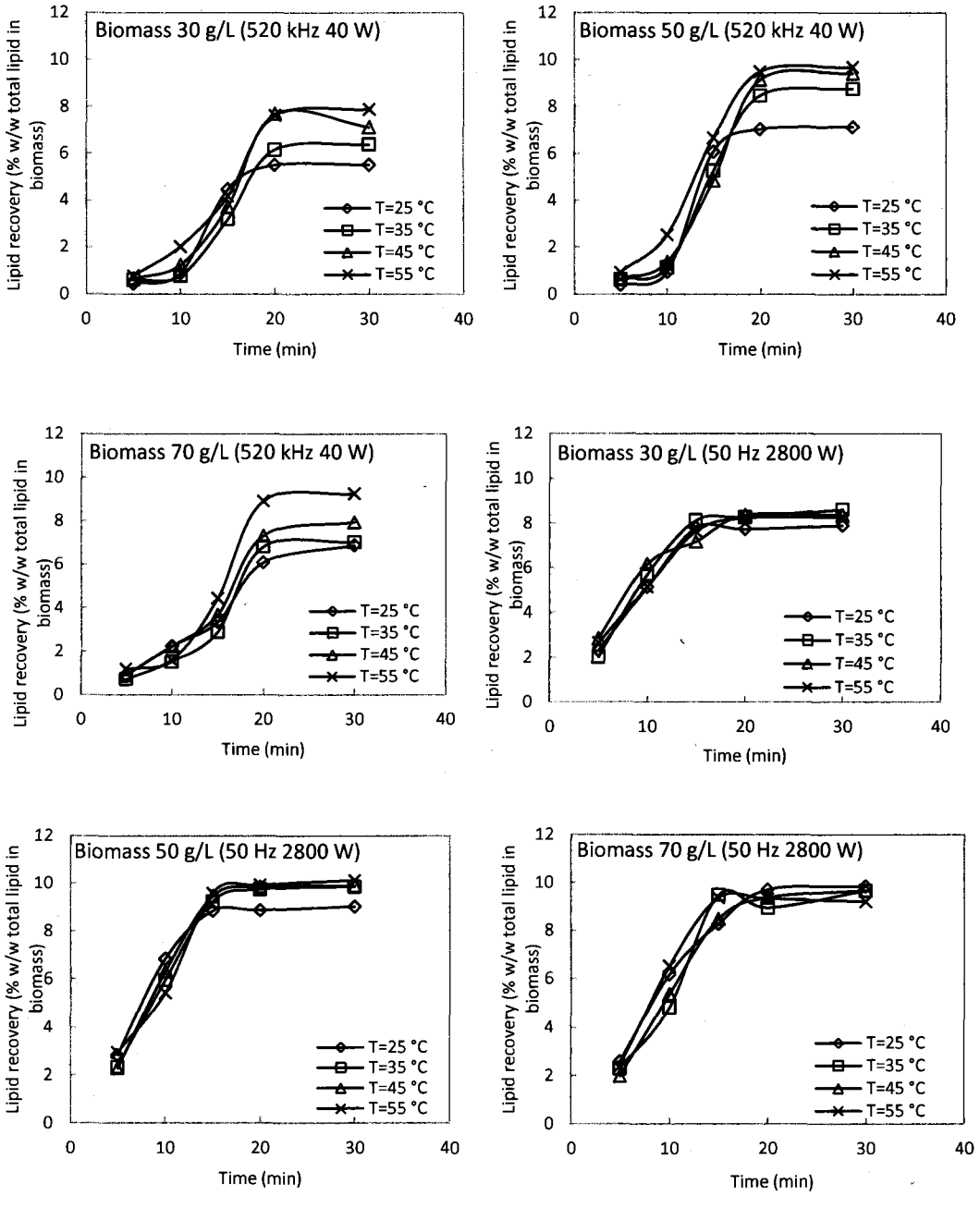


Figure 12.3 **Ultrasonication lipid extraction from *Trichosporon oleaginosus* with water as solvent; standard deviation was less than 5%**

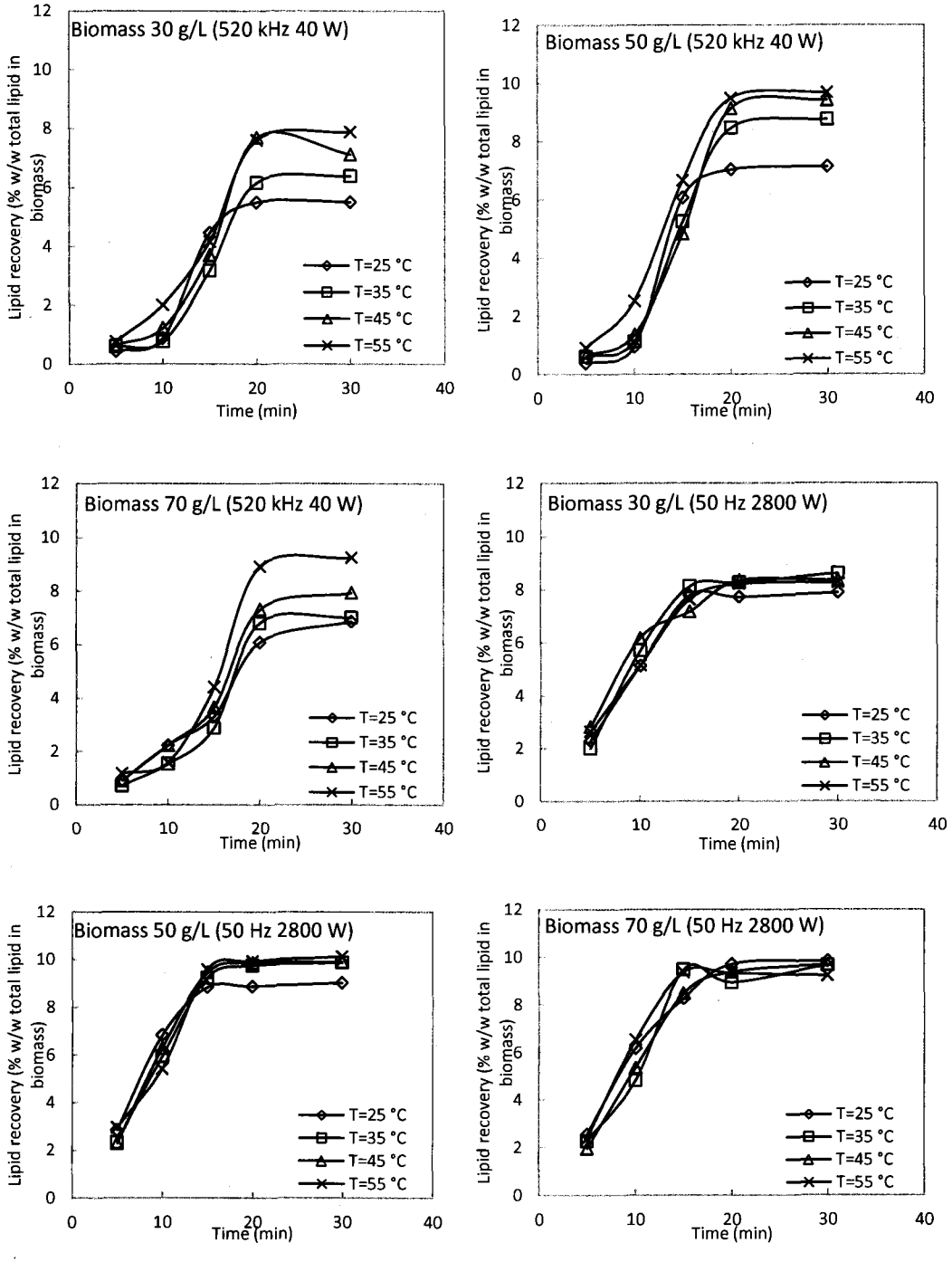
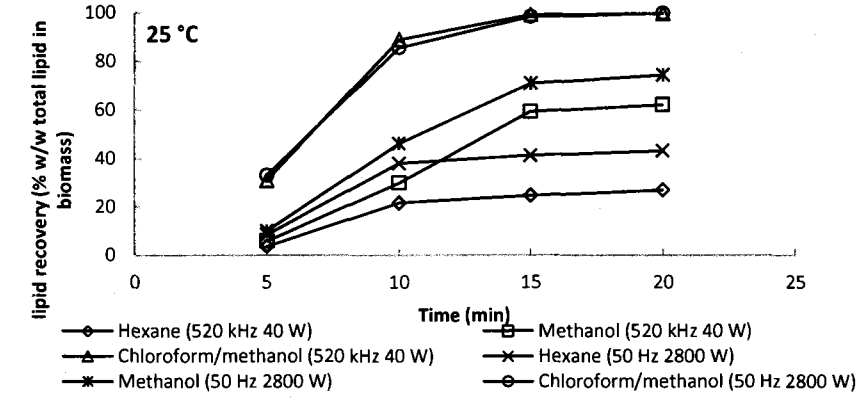
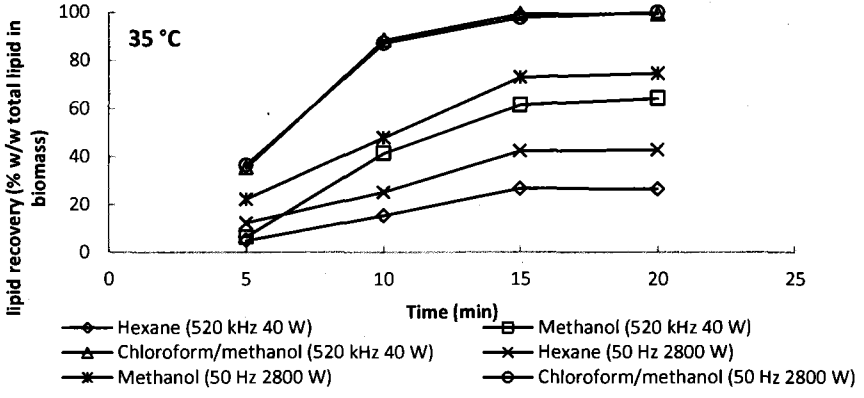
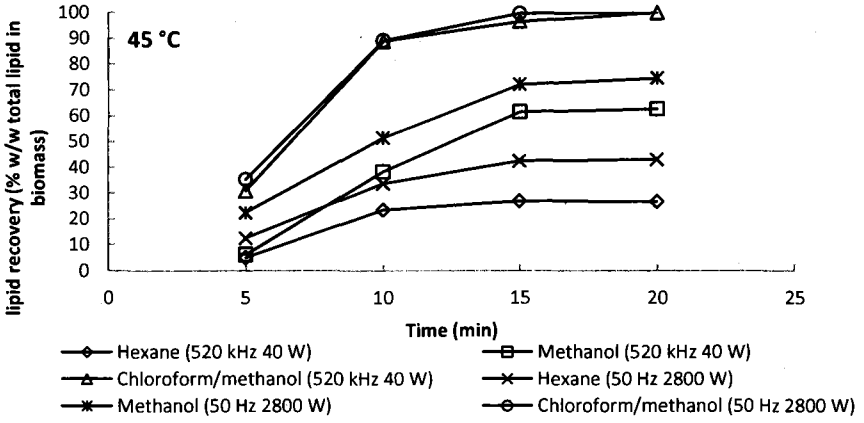


Figure 12.4 **Ultrasonication lipid extraction from SKF-5 with water as solvent; standard deviation was less than 5%**


25 °C
lipid recovery (% w/w total lipid in biomass)
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)


35 °C
lipid recovery (% w/w total lipid in biomass)
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)


45 °C
lipid recovery (% w/w total lipid in biomass)
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)

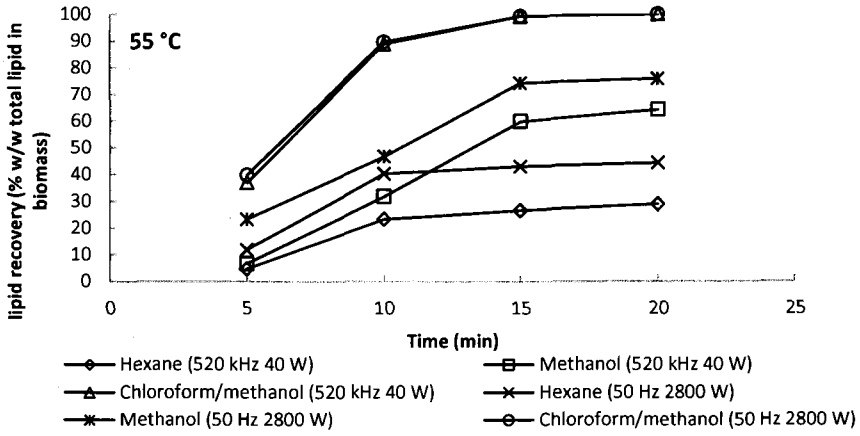
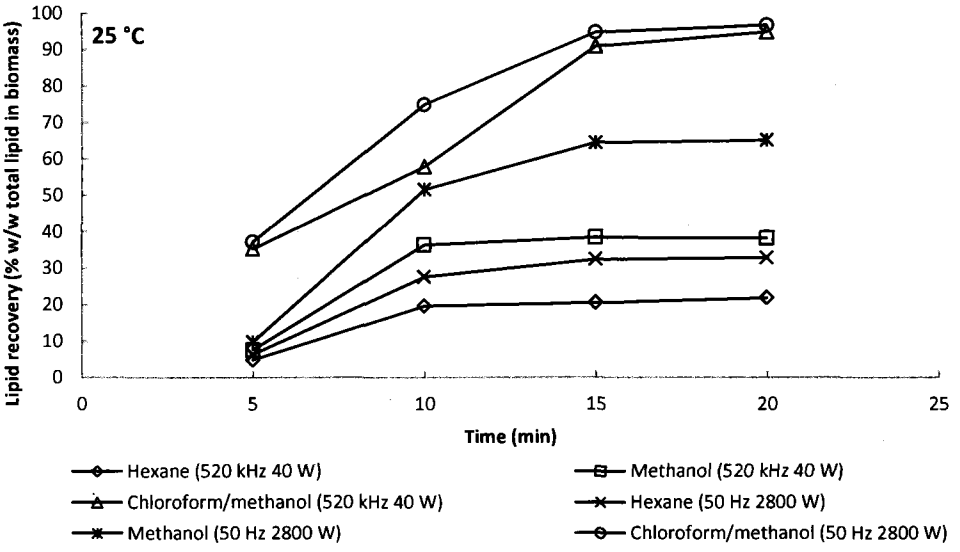
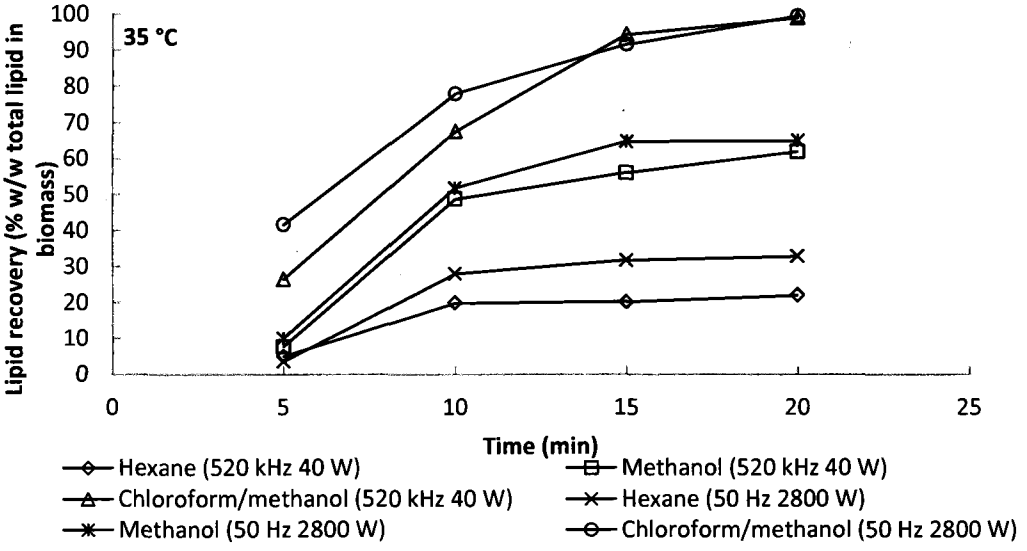


Figure 12.5 **Ultrasonication lipid extraction from *Trichosporon oleaginosus* with organic solvents; standard deviation was less than 5%**


25 °C
Lipid recovery (% w/w total lipid in biomass)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)


35 °C
Lipid recovery (% w/w total lipid in biomass)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)

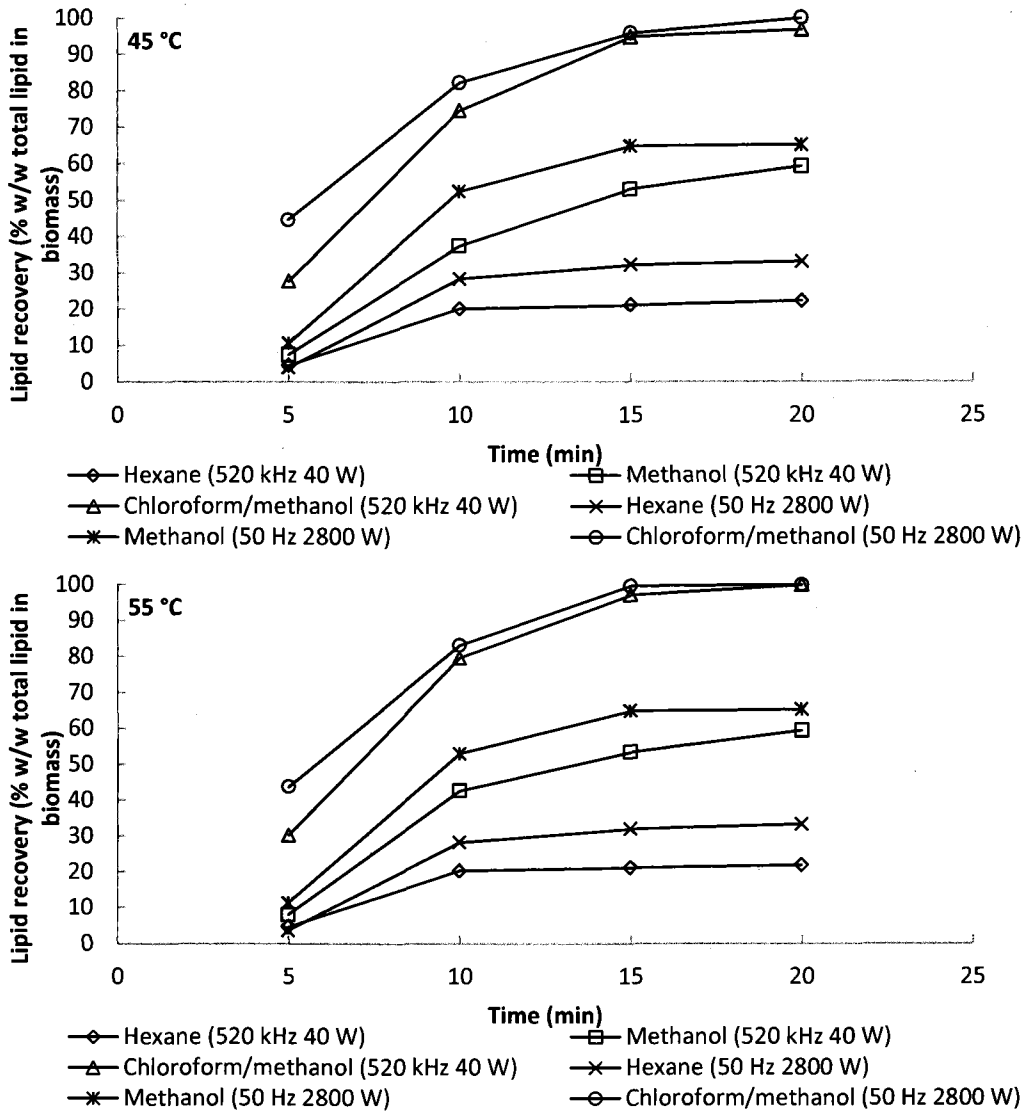


Figure 12.6 **Ultrasonication lipid extraction from SKF-5 with organic solvents; standard deviation was less than 5%**

ULTRASONICATION AIDED IN-SITU TRANSETSERIFICATION OF MICROBAIL LIPID TO BIODIESEL

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FUEL (SUBMITTED)

13 ULTRASONICATION AIDED IN-SITU TRANSESTERIFICATION OF MICROBIAL LIPID TO BIODIESEL

13.1 Résumé

La conversion des lipides des microorganismes en biodiesel a été largement étudiée. Une attention croissante a été accordée à la trans-estérification *in-situ* afin d'éviter l'extraction des lipides, qui est une étape nécessaire dans le procédé à deux étapes (l'extraction des lipides et la trans-estérification). Pour améliorer encore la possibilité de la trans-estérification *in-situ*, l'ultrasons a été utilisée pour réduire la grande quantité de méthanol requise et le long temps de réaction. Les résultats ont montré que l'utilisation d'ultrasons pourrait aboutir à un rendement de production de FAMEs élevé de 92.1% (p/p lipides) avec un ratio molaire méthanol/lipide de 60:1 et une quantité de NaOH ajoutée de 1% (p/p de lipides) durant 20 min. En comparaison, pour obtenir un rendement similaire par trans-estérification in situ sans ultrasons, il faut utiliser un ratio molaire méthanol/lipide de 360:1, et une teneur de NaOH 1% (p/p de lipides) et un temps de réaction de 12 h. Les compositions de FAMEs obtenues par ultrasons et trans-estérification *in-situ* étaient similaires à celles obtenues par trans-estérification en deux étapes.

Mots clés : Lipides des microorganismes; trans-estérification *in-situ*; ultrasons ; biodiesel

13.2 Abstract

In-situ transesterification of microbial lipid to biodiesel has been paid substantial attention due to the fact that the lipid extraction and transesterification can be conducted in one stage process. To improve the feasibility of in-situ transesterification, ultrasonication was employed to reduce methanol requirement and reaction time. The results showed that the use of ultrasonication could achieve high conversion of lipid to FAMEs (92.1% w lipid conversion/w total lipids) with methanol to lipid molar ratio 60:1 and NaOH addition 1% w/w lipid in 20 min, while methanol to lipid molar ratio 360:1, NaOH addition 1% w/w lipid, and reaction time 12 h was required to obtain similar yield in in-situ transesterification without ultrasonication. The compositions of FAMEs obtained from ultrasonication aided in-situ transesterification were similar as that of two stage extraction and transesterification processes.

Keywords: Microbail lipid; in-situ transesterification; ultrasonication; biodiesel

13.3 Introduction

Biodiesel production from microbial oil, also called single cell oil, has grabbed great attention due to unaffordable cost of traditional oils such as vegetable oils which are used for biodiesel production. Microorganisms grow faster and accumulate higher lipid content (up to 80% w/w) as compared to crops (several months and 30% w/w oil content) [1-2]. Numerous studies have successfully transferred microbial oil to biodiesel [3-4]. The process chain includes microorganism cultivation, harvesting, lipid extraction, and transesterification. Lipid extraction from microorganism requires large amount of organic solvent generally chloroform and methanol [1, 5]. Chloroform has adverse impact on the environment and requires extra attention in manipulation. Hexane/isopropanol has also been applied for lipid extraction but the extraction efficiency is lower as compared to chloroform and methanol as solvent [6]. Terpenes, green solvents obtained from plants, is a great selection to extract microbial oil and yields similar efficiency as chloroform/methanol, yet the cost is high [7]. Therefore, extraction becomes an obstacle in biodiesel production from microbial sources.

In-situ transesterification has been reported in biodiesel production rom microorganisms. The method simultaneously achieved extraction and transesterification of the lipid in microorganism. It thus eliminated lipid extraction step. In previous studies, in-situ transesterification on soy flakes and wastewater sludge have accomplished high yield of biodiesel (up to 97%) [8-10]. However, methanol addition was around a hundred times higher in one step or in-situ transesterification (methanol to lipid molar ratio around 300: 1) than two stage conversion (methanol to oil ratio 6:1 to 12: 1). Moreover, long reaction time was required (around 12 h for in-situ and 2 h for two stage transesterification).

The objective of this work is to investigate ultrasonication aided in-situ transesterification for biodiesel production from oleaginous microorganisms. Ultrasonication was used to investigate its effect on the methanol requirement as well as reaction time. Parameters including ultrasonication time, catalysts concentration, and different methanol to lipid molar ratios were examined. The transesterification without ultrasonication aid was also conducted to compare

the results. The impact of ultrasonication aided in-situ transesterification on FAMEs composition was also investigated.

13.4 Methods

13.4.1 Strain, culture and harvesting conditions

Oleaginous yeast *Trichosporon oleaginosus* (ATCC20509) was grown in a glycerol medium containing (per liter): 1 g $(NH_4)_2SO_4$, 1g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g yeast extract, 50 g purified glycerol, and minerals 0.04 $CaCl_2 \cdot 2H_2O$, 0.0055 $FeSO_4 \cdot 7H_2O$, 0.0052 citric acid·H_2O, 0.001 $ZnSO_4 \cdot 7H_2O$, and 0.00076 $MnSO_4 \cdot H_2O$ were added (Zheng et al. 2012). The purified glycerol was derived from crude glycerol, which was from an industrial biodiesel production company in Quebec. The purification was performed by lowering the pH of the crude glycerol to 2 [11], and then the FFA (on the top) and salt (in the middle) were removed by centrifugation. The experiment was performed in shake flask at 200 rpm 28 °C. After 72 h fermentation, biomass was harvested by centrifugation at 5000 rpm for 15 min. The biomass was washed twice with distilled water to remove the residual nutrients and glycerol. Part of the biomass was dried by lyophilisation and then stored for further study.

13.4.2 Lipids extraction methods

The standard chloroform and methanol extraction procedure with minor modification was employed to determine the lipid content in the biomass [12-13]. 200 mg dry biomass (after lyophilisation) was mixed with 4 ml solvent mixture of chloroform and methanol (2:1 v/v), and then subjected to 60 °C for 4 h. The mixture was then centrifuged at 5000 rpm for 15 min and the solvent phase was withdrawn and transferred into a pre-weighed glass vial (W_1). The extraction procedure was repeated two times. Afterwards, the vial containing the total volume of the supernatant collected from each extraction was subjected to 60 °C in an oven to evaporate the solvents and then weighed (W_2). The lipid amount was calculated by the difference of W_2 and W_1. The lipid content in the biomass is $(W_2-W_1)/200$ mg ×100%. The obtained lipid was stored in dark at 4 °C for further transesterification study.

13.4.3 Lipid transesterification

Lipid obtained from solvent extraction from *Trichosporon oleaginosus* was first dissolved in hexane (25 mL hexane per gram lipid), then mixed with methanol. Lipid to methanol molar ratio is 1:6 (0.3 mL methanol for per gram lipid). Sodium hydroxide was used as catalyst with the addition amount of 1 % w/w (NaOH/ oil). The mixture was then subjected to 55 °C for 2 hours. After reaction, 5% w/v NaCl solution was added (100 mL NaCl solution per gram lipid), and then FAMEs was extracted by two times washing with hexane (100 mL per gram lipid). After phase separation by settling, the hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% sodium bicarbonate solution (20 mL per gram lipid) and allowed the mixture to stand for 15 min for phase separation, and the top layer was collected and dried at 60 °C in an oven [14].

The FAMEs was then re-dissolved in hexane (10 mL/mg lipid) and analyzed using a Gas Chromatography linked with Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500). The dimensions of the column used are 30 m × 0.25 mm, with a phase thickness of 0.2 µm. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1.3-dichlorobenzene was used as internal standard with a concentration of 50 ppm.

13.4.4 *In-situ* transesterification

0.2 gram of biomass was mixed with methanol containing NaOH and then the mixture was subjected to 55 °C for 2 to 12 h. The different molar ratios of methanol to oil investigated were 6:1, 60:1, 120:1, 240:1, and 360:1 corresponding to 0.08, 0.8, 1.6, 3.2, 6.4 mL methanol, respectively, with addition of 1%, 2%, and 5% w/w (NaOH/oil). 5 mL of hexane was added to each sample to increase the lipid solubility in the mixture. After reaction, 5% (w/v) NaCl solution was added to biomass (1 mL per gram of biomass), and then the FAMEs were extracted by two times washing with hexane (10 ml per gram biomass), after 15 min settling, phase separation was achieved. Then the hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% w/v sodium bicarbonate solution (2 ml per gram biomass), and allowed the mixture to stand for 15 min for phase separation, and then the top layer was

collected and dried at 60 °C in an oven [14]. The resulting FAMEs were analyzed by the method described in Lipid transesterification section.

13.4.5 *In-situ* transesterification with ultrasonication

Ultrasonication was conducted with ultrasonic processor CPX 750 (Cole-Parmer Instrument IL) at 20 kHz (Figure 13.1). Methanol with NaOH was added to 0.2 gram of dry biomass and then ultrasonication horn directly immersed in the solution in a beaker placed in a water bath to control temperature at around 25 °C for a desired time. A plastic cover with a hole, to introduce sonication horn, was placed on top of the flask to minimize the loss of methanol and hexane. The sonication time varied from 1 to 30 min, and methanol to biomass ratio was set at 6:1, 60:1, 120:1, 240:1, and 360:1 corresponding to 0.08, 0.8, 1.6, 3.2, 6.4 mL methanol, respectively, with 1%, 2%, and 5% w/w (NaOH/oil). Co-solvent hexane addition amount was 5 mL per 0.2 g biomass.

After reaction, 5% w/v NaCl solution was added (1 mL per gram biomass) followed by FAMEs extraction by two times washing with hexane (10 mL per gram biomass), Settling, centrifugation (9000 rpm, 20 min), and filtration (Whatman Filter Paper) processes were performed, respectively, to achieve phase separation. Then the hexane phase (upper layer) was collected and allowed the mixture to stand for 15 min for phase separation, and then the top layer was collected and dried at 60 °C [14]. The FAMEs were analyzed described before in Lipid transesterification section.

All experiments were performed in triplicates, and average results were reported with standard deviation less than 5%.

13.5 Results and discussion

13.5.1 *In-situ* transesterification

According to the equilibrium reaction 1 g lipid could produce approximately 1 g of biodiesel (Equation 13.1), which suggested that the FAME yield is 100% w FAME/w lipid. The total lipid content in the biomass was 47.3 ± 0.9% w/w dry biomass determined by conventional chloroform methanol (2:1 v/v) extraction method. Lipid conversion yield (g lipid converted/g total lipids) is the lipid converted to FAMEs out of the total lipids.

Therefore, the lipid conversion yield is calculated by determining amount of FAMEs by GC-MS and dividing that with total lipids (g FAMEs/g total lipids).

For an equilibrium reaction, reactant concentration (methanol to lipid molar ratio for transesterification), amount of catalyst added, reaction time, and temperature have great impact on lipid conversion to FAMEs. For transesterification, normally 50 to 60 °C temperature is employed, high temperature (beyond methanol boiling point) has been found to decrease the FAMEs yields due to saponification of FAMEs and loss of methanol due to evaporation [15-16]. Therefore, in this study, the reaction temperature was fixed to investigate the effect of different methanol to lipid molar ratios, amount of NaOH added, and reaction time on transesterification.

The lipid conversion yield during in-situ transesterification of biomass as a function of reaction time at various methanol to lipid molar ratios and catalyst concentration used are shown in Figure 13.2. The lipid conversion increased with the reaction time. It was observed that the reaction equilibrium reached in a shorter time with high methanol to lipid molar ratio than for that with low methanol to lipid molar ratio. For all the experiments, the lipid conversion increased from 0 to 6 h and then either increased slowly or did not increase at all. The decrease of the reaction rate was due to the reduction of reactants concentration (lipids and methanol) in the reaction mixture and the increase of product concentration (FAMEs and glycerol).

Equation 13. 1 Triglycerides (890 g) + 3 Methanol (32 g) → 3 FAMEs (298 g) + Glycerol (92 g)

At any given concentration of catalyst (NaOH), the lipid conversion increased with methanol to lipid molar ratio. At 1% w/v NaOH concentration, the conversion of lipids was 15.2 ± 0.3% w/w total lipids for 6:1 and 72.3 ± 1.2% w/w total lipids for 60:1, which increased around 4 times. However, from methanol to lipid molar ratio 120:1 (81.9 ± 1.7% w/w total lipids) to 360:1 (86.5 ± 2.0% w/w total lipids), the lipid conversion didn't increase much. This established that the methanol wasn't limiting the reaction degree when methanol to lipid molar ratio was higher than 120:1. It indicates that increased lipid conversion will not be realized by simply increasing the concentration of methanol without changing the other parameters.

At a constant methanol to lipid molar ratio, higher concentration of NaOH catalyst resulted in higher conversion of lipids to FAMEs. Increasing the concentration of catalyst (NaOH) from 1% to 5% w/w (NaOH/lipid) increased the conversion of lipids to FAMEs from 15.2 ± 0.3% to 36.7 ± 0.8% w/w (FAMEs/total lipid) at methanol to lipid molar ratio of 6:1. The lipid conversion efficiency was greatly different at methanol to lipid molar ratio 60:1 than at 120:1, 240:1, and 360:1 with catalyst concentration (NaOH) of 1% w/w (NaOH/lipid). The difference became smaller as concentration of the catalyst (NaOH) increased. Moreover, the reaction time to reach the equilibrium was also reduced from 10 to 6 h when the catalyst concentration (NaOH) increased from 1% to 5% w/w (NaOH/lipid).

In two stage transesterification which converts the extracted lipid to FAMEs, lipid conversion of 93.8 ± 1.7% w lipid/w total lipids) was achieved with methanol to lipid molar ratio 6:1 and NaOH addition 1% w/w lipid in 2 h, yet the lipid conversion was only 3.2 ± 0.2% w/w total lipids in in-situ transesterification under similar conditions. The highest lipid conversion 90.4 ± 2.0% w/w total lipids was obtained at methanol to lipid molar ratio 360:1, NaOH addition 5% w/w lipid, and reaction time 12 h. It displayed that in-situ tranesterification required much larger amount of methanol and NaOH addition and far longer time to achieve similar lipid conversion yield than two stage transesterification (Table 13.1). Transesterification occurs when lipid gets in contact with methanol. Lipid is an intercellular product of microorganism, thus reaction becomes difficult in in-situ transesterification due to the presence of cell wall which separates lipid from methanol. In order to react, large amount of methanol has to be added to act as solvent (to weaken/disrupt and penetrate the cell walls) and as reactant to form FAMEs. It

would be great obstacle of in-situ transesterification application in practice biodiesel production in which high lipid conversion is preferable to attain with short time and low wasting on the material and energy.

13.5.2 Ultrasonication assisted in-situ transesterification

As described above, the biggest issue of in-situ transesterification would be the requirement of high methanol to lipid molar ratio and the reaction time [8, 17], which would result in large amount of methanol loss (if no methanol recovery) or large energy consumption (with methanol recovery). Study has found that stirring enhanced lipid conversion of in-situ transesterification due to the improvement in mass transfer [18]. Ultrasonication generates microscopic bubbles which will later collapse during compression from surrounding environment. The collapse of bubbles induces violent shock waves, which provides a good mixing, and hence increases the mass transfer.

With variation on methanol to lipid molar ratio and NaOH amount added, in-situ transesterification was performed under ultrasonication 20 kHz 700 W for 1 to 30 min with hexane as co-solvent. After reaction, another portion of hexane was used to extract FAMEs from the mixture by hand shaking. In in-situ transesterification without ultrasonication, the phase separation after hexane extraction was rapidly completed in 15 min; however, in the case of ultrasonication aided in-situ transesterification phase separation was not observed even after 12 h (the mixture remained in emulsion). This would be due to that ultrasonication finely breaks cells and well blends the methanol, hexane (co-solvent), formed FAMEs, and cell residues to emulsion. When hexane was again added to extract FAMEs it is involved into the emulsion, and thus, phase separation was not formed by simple settling. In order to separate FAMEs in hexane from the mixture, centrifugation and filtration were tested. After centrifugation, the top layer (hexane containing FAMEs, both are clear liquid) was not clear as original hexane. It suggested that impurities are present in the hexane phase. With filtration, the filtrate (methanol, hexane, and FAMEs) was collected and then allowed to stand for 15 min. Thereafter, two layers were observed and the top layer (FAMEs in hexane) was collected for FAMEs quantification.

The results of lipid conversion in ultrasonication aided in-situ transesterification were given in Figure 13.3. It was observed that with the utilization of ultrasonication, a reaction time of 20 min could achieve high lipid conversion (> 92% w/w total lipids) for all different concentrations of NaOH and methanol to lipid molar ratio except 6:1. It suggested that high lipid conversion could be achieved with low methanol and NaOH addition with short time in presence of ultrasonication.

The highest lipid conversion of 90.4% w/w total lipids of in-situ transesterification was obtained at methanol to lipid ratio 360:1 NaOH addition 5% w/w lipid and reaction time 12 h, while a higher lipid conversion (92.4% w/w total lipids) was attained in ultrasonication in-situ transesterification with methanol to lipid molar ratio 60:1 and 1% NaOH w/w lipid in 20 min (Table 13.1). In addition, the lipid conversion with methanol to lipid molar ratio 6:1 was doubled in ultrasonication presence. It indicates that when ultrasonication was used, methanol and NaOH requirement and reaction time was largely reduced. Ultrasonication creates pressure and shear on the cell walls and results in a rapid cell disruption, and hence methanol requiremenmt and reaction time was reduced as compared to that without ultrasonication. Formation of free radicals of reacting species led by ultrasonication cavitation could also contribute to high reaction rate and thus decreases the reaction time [19].

Lipid conversion was 93.8% w/w total lipids after 2 h reaction in two stage transesterification process. The similar lipid conversion efficiency was reached in ultrasonication assisted in-situ transesterification (94.1% w/w total lipids) with methanol to lipid molar ratio 60:1, 1% NaOH w/w lipid in 30 min. Even though higher methanol addition is still required, the energy consumed by methanol recovery could be compensated by the energy (to maintain temperature and mixing) saved due to the reduction of reaction time. It suggests that ultrasonication in-situ transesterification could be feasible to use at large scale biodiesel production process.

13.5.3 Comparison of composition of FAMEs from different transesterification

The composition of FAMEs obtained in different processes was presented in Table 13.2. With decrease of methanol to lipid molar ratio, C18:2 concentration increases and C16:0 and

C18:1 decreased in in-situ transesterification. It suggested that more FAMEs were produced from phospholipids at low methanol to lipid molar ratio than at high ratio [20]. Phospholipids are mainly present in cell membrane. It indicates that low concentrate methanol couldn't disrupt cells to react with lipid droplets inside the cell (mainly containing C16:0, C18:0, and C18:1) but contacted and reacted with membrane phospholipids to form FAMEs. As methanol concentration increased, it could penetrate the cell membrane and react with lipid droplets inside the cells to generate FAMEs. Variation of NaOH addition amount didn't impact on the composition of FAMEs.

Comparing the fatty acid profiles, the results revealed that ultrasonication didn't impact on biodiesel composition. The compositions of FAMEs from two stage transesterification, in-situ transesterification, and ultrasonication in-situ transesterification were similar.

13.6 Conclusions

From the results obtained in this study, it was shown that it was feasible to reduce the large amount of methanol required in in-situ transesterification assisted by ultrasonication. Compared to two-stage transesterification process, an increase in lipid conversion yield (w/w total lipids) at less reaction time was also achieved with ultrasonication aided in-situ transesterificationt. In addition, FAMEs from ultrasonication in-situ transesterification revealed similar composition to that of two-stage transesterification. Overall, ultrasonication in-situ transesterification could be a promising alternative of current two-stage transesterification process.

13.7 Acknowledgements

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13.8 References

Dejoye Tanzi, C., Abert Vian, M., and Chemat, F. (2013). "New procedure for extraction of algal lipids from wet biomass: A green clean and scalable process." *Bioresource Technology*, 134, 271-275.

Eevera, T., Rajendran, K., and Saradha, S. (2009). "Biodiesel production process optimization and characterization to assess the suitability of the product for varied environmental conditions." *Renewable Energy*, 34, 762-765.

Ehimen, E. A., Sun, Z. F., and Carrington, C. G. (2010). "Variables affecting the in situ transesterification of microalgae lipids." *Fuel*, 89, 677-684.

Ferraz, T. P. L., Fiúza, M. C., dos Santos, M. L. A., Pontes de Carvalho, L., and Soares, N. M. (2004). "Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation." *Journal of Biochemical and Biophysical Methods*, 58, 187-193.

Folch, J., Lees, M., and G.H., S. S. (1957). "A simple method for the isolation and purification of total lipides from animal tissues." *Journal of Biological Chemistry*, 226, 497-509.

Gao, D., Zeng, J., Zheng, Y., Yu, X., and Chen, S. (2013). "Microbial lipid production from xylose by *Mortierella isabellina*." *Bioresour. Technol.*, 133, 315-321.

Giroud, S., Frare, C., Strijkstra, A., Boerema, A., Arnold, W., and Ruf, T. (2013). "Membrane phospholipid fatty acid composition regulates cardiac SERCA activity in a hibernator, the Syrian Hamster (Mesocricetus auratus)." *PLoS ONE*, 8, 23-28.

Haas, M., and Scott, K. (2007). "Moisture Removal Substantially Improves the Efficiency of in Situ Biodiesel Production from Soybeans." *Journal of the American Oil Chemists' Society*, 84, 197-204.

Haas, M., Scott, K., Marmer, W., and Foglia, T. (2004). "In situ alkaline transesterification: An effective method for the production of fatty acid esters from vegetable oils." *Journal of the American Oil Chemists' Society*, 81, 83-89.

Halim, R., Gladman, B., Danquah, M. K., and Webley, P. A. (2011). "Oil extraction from microalgae for biodiesel production." *Bioresource Technology*, 102, 178-185.

Leung, D. Y. C., and Guo, Y. (2006). "Transesterification of neat and used frying oil: Optimization for biodiesel production." *Fuel Processing Technology*, 87, 883-890.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., and Xian, M. (2009). "Biodiesel production from oleaginous microorganisms." *Renewable Energy*, 34, 1–5.

Mondala, A., Liang, K., Toghiani, H., Hernandez, R., and French, T. (2009). "Biodiesel production by in situ transesterification of municipal primary and secondary sludges." *Bioresour. Technol.*, 100, 1203-1210.

Pimentel, D., and Patzek, T. W. (2005). "Ethanol production using corn, switchgrass, and wood; biodiesel production using soybean and sunflower." *Nat Resour Res*, 14, 65–76.

Rokhina, E. V., Lens, P., and Virkutyte, J. (2009). "Low-frequency ultrasound in biotechnology: state of the art." *Trends in Biotechnology*, 27, 298-306.

Ruan, Z., Zanotti, M., Wang, X., Ducey, C., and Liu, Y. (2012). "Evaluation of lipid accumulation from lignocellulosic sugars by *Mortierella isabellina* for biodiesel production." *Bioresour. Technol.*, 110, 198-205.

Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., Sádaba, I., Ruiz-Vázquez, R. M., Torres-Martínez, S., and Garre, V. (2009). "Biodiesel production from biomass of an oleaginous fungus." *Biochemical Engineering Journal*, 48, 22-27.

Zheng, Y., Chi, Z., Ahring, B. K., and Chen, S. (2012). "Oleaginous yeast *Cryptococcus curvatus* for biofuel production: Ammonia's effect." *Biomass and Bioenergy*, 37, 114-121.

Table 13.1 FAMEs yield of in situ transesterification

Exp.	Two stage transesterification	In-situ transesterification (biomass to FAMEs)			
		Variables			FAMEs yield (% w/w lipid)
	FAMEs yield (% w/w lipid)	methanol: lipid molar ratio	Catalyst amount (% w/w oil)	Ultrasonication	
1	93.8 ± 1.7				
2		6:1	1	No	15.2 ± 0.4
3		60:1	1	No	73.2 ± 1.1
4		120:1	1	No	81.9 ± 0.9
5		240:1	1	No	84.2 ± 1.4
6		360:1	1	No	86.5 ± 1.2
7		6:1	2	No	28.3 ± 0.5
8		60:1	2	No	78.6 ± 0.9
9		120:1	2	No	83.4 ± 0.6
10		240:1	2	No	85.1 ± 1.3
11		360:1	2	No	86.7 ± 1.4
12		6:1	5	No	36.7 ± 1.0
13		60:1	5	No	83.3 ± 1.7
14		120:1	5	No	87.6 ± 1.2
15		240:1	5	No	89.2 ± 2.1
16		360:1	5	No	90.4 ± 1.3
17		6:1	1	Yes	24.1 ± 0.5
18		60:1	1	Yes	92.1 ± 1.1
19		120:1	1	Yes	92.4 ± 2.0
20		240:1	1	Yes	92.4 ± 1.4
21		360:1	1	Yes	92.8 ± 1.6
22		6:1	2	Yes	26.2 ± 1.0
23		60:1	2	Yes	93.6 ± 2.2
24		120:1	2	Yes	94.2 ± 1.9
25		240:1	2	Yes	94.5 ± 0.3
26		360:1	2	Yes	94.7 ± 1.1
27		6:1	5	Yes	26.9 ± 1.1
28		60:1	5	Yes	94.1 ± 1.7
29		120:1	5	Yes	95.4 ± 0.8
30		240:1	5	Yes	95.4 ± 1.1
31		360:1	5	Yes	95.6 ± 1.9

The FAMEs yields of in-situ transesterification without ultrasonication are from 12 h reaction time.

The FAMEs yields of in-situ transesterification with ultrasonication are from 20 min reaction time.

Table 13.2 Comparison of fatty acid profiles of biodiesel produced though different processes

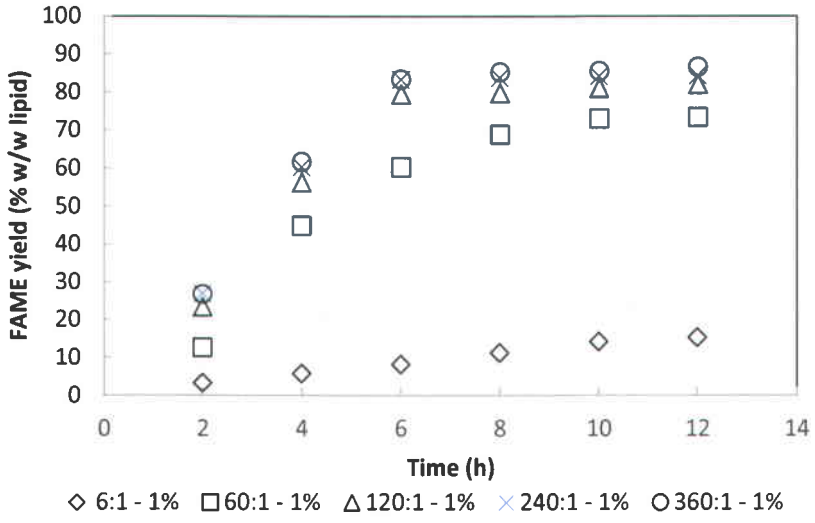
Fatty acids	Transesterification (lipid to FAMEs)	In-situ transesterification[a]					Ultrasonication in-situ transesterification[b]				
		6:1	60:1	120:1	240:1	360:1	6:1	60:1	120:1	240:1	360:1
C14:0	0.5	ND	0.4	0.4	0.5	ND	ND	0.4	0.5	0.5	0.5
C15:0	0.5	ND	0.5	0.5	0.5	0.5	0.4	0.5	0.5	0.5	0.5
C16:0	28.1	22.1	23.6	25.3	27.7	28.4	25.7	28.7	28.5	28.3	28.5
C16:1	1.1	0.4	0.8	0.5	0.9	0.7	1.1	0.9	1.1	1.1	1.0
C18:0	10.3	9.0	9.7	9.6	9.9	10.5	9.3	9.9	10.1	10.3	10.2
C18:1	49.6	39.4	43.1	46.3	48.2	48.5	44.1	49.3	49.5	49.5	49.3
C18:2	8.9	28.5	21.2	16.2	11.8	10.3	18.1	9.1	9	8.8	8.9
C20:0	1.0	0.6	0.7	1.2	1.0	1.1	1.3	1.2	0.8	1.0	1.1

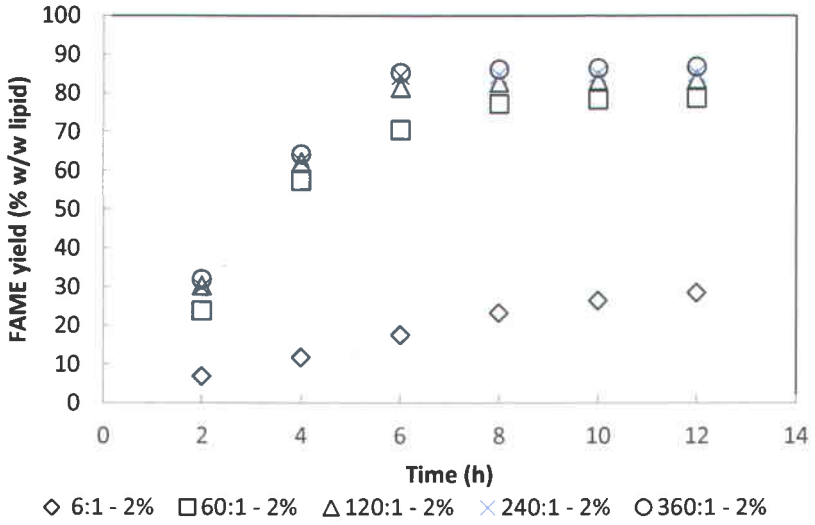
[a]: NaOH addition 1% w/w lipid for 12 h reaction;

[b]: NaOH addition 1% w/w lipid for 20 min reaction;

ND: not detected.

Figure 13.1 **Ultrasonication device used in the experiment**


FAME yield (% w/w lipid)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Time (h)
6:1 - 1%
60:1 - 1%
120:1 - 1%
240:1 - 1%
360:1 - 1%


FAME yield (% w/w lipid)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Time (h)
6:1 - 2%
60:1 - 2%
120:1 - 2%
240:1 - 2%
360:1 - 2%

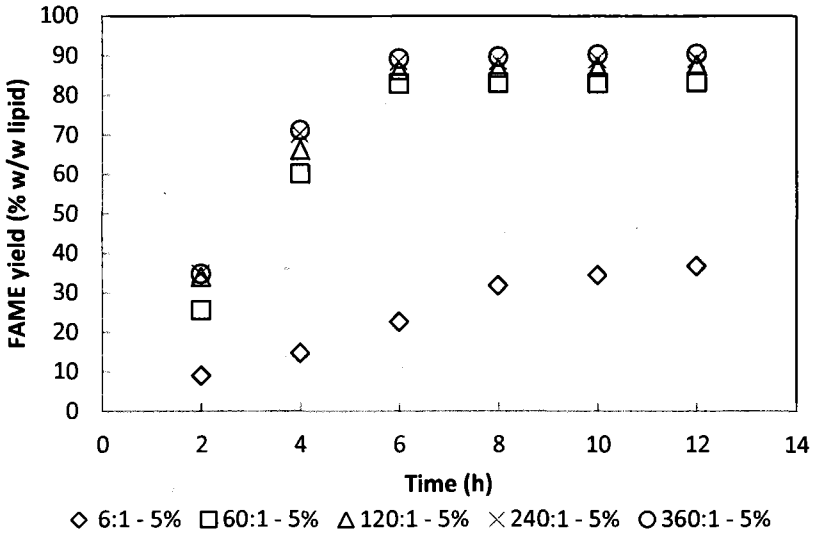
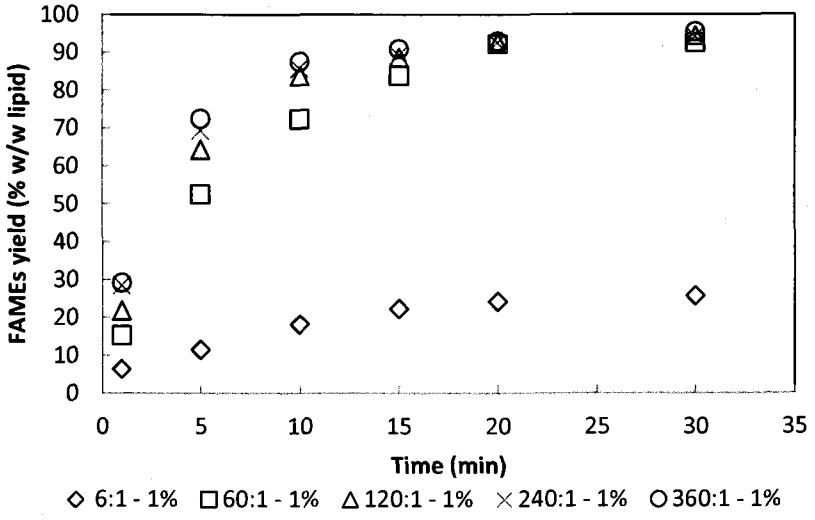
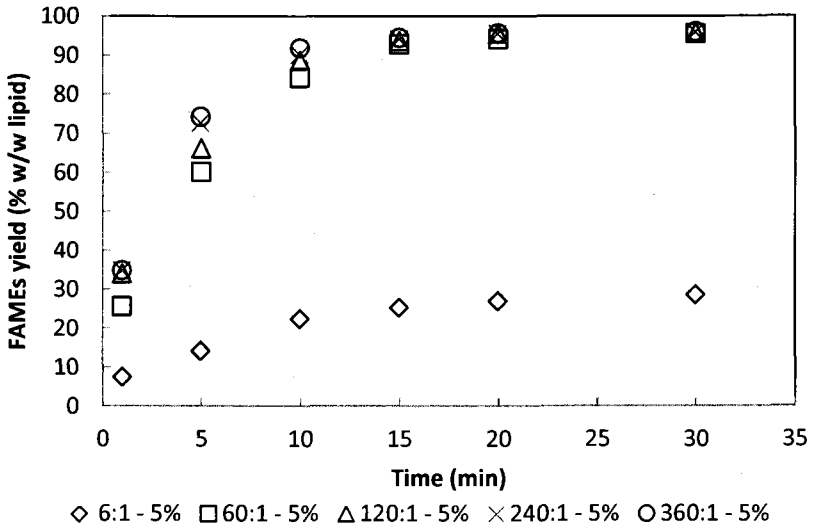


Figure 13.2 **FAME yields versus reaction time for the in-situ transesterification; standard deviation is less than 5%**


FAMEs yield (% w/w lipid)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Time (min)
6:1 - 1%
60:1 - 1%
120:1 - 1%
240:1 - 1%
360:1 - 1%


FAMEs yield (% w/w lipid)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Time (min)
6:1 - 5%
60:1 - 5%
120:1 - 5%
240:1 - 5%
360:1 - 5%

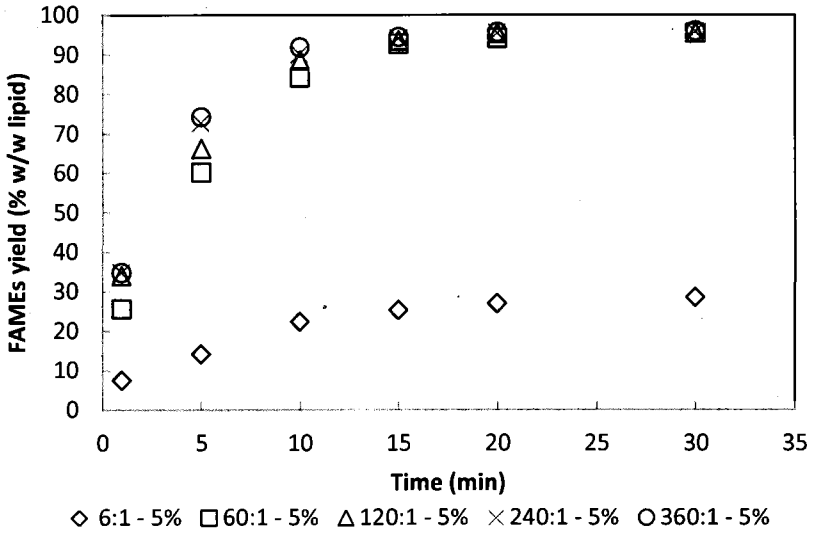


Figure 13.3 **FAME yields versus reaction time for the in-situ transesterification under ultrasonication assistance; standard deviation is less than 5%**

ULTRASONICATION APPLICATION IN EXTRACTION AND IN-SITU TRANSESTERIFICATION OF LIPID DERIVED FROM SLUDGE

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FUEL (UNDER REVIEW)

14 ULTRASONICATION APPLICATION IN EXTRACTION AND IN-SITU TRANSESTERIFICATION OF LIPID DERIVED FROM SLUDGE

14.1 Résumé

Différents solvants, y compris l'eau, l'hexane, le méthanol et le chloroforme/méthanol (1:1 v/v), ont été testés pour l'extraction des lipides à partir de *Trichosporon oleaginosus* et SKF-5 sous ultrasons (520 KHz 40 W et 50 Hz 2800 W). L'efficacité d'extraction par ultrasons a été comparée avec la méthode d'extraction conventionnelle par chloroforme/méthanol (2:1 v/v). Des taux de récupération maximums de lipides de 11.8%, 35.3%, 62.0%, et 95.3% p/p de biomasse avec de l'eau, à l'hexane, du méthanol, et du chloroforme/méthanol ont été obtenus à partir de *Trichosporon oleaginosus*, respectivement, par ultrasons à 50 Hz 2800 W. L'extraction par chloroforme/méthanol et ultrasons a permis la récupération du 95% lipides en peu de temps (20 minutes) et à basse température (45 °C) tandis que la récupération du contenu total en lipides par extraction conventionnelle avec chloroforme/méthanol nécessite un temps de 12 h à 60 °C. La conversion des lipides des microorganismes en biodiesel a été largement étudiée. Une attention croissante a été accordée à la trans-estérification *in-situ* afin d'éviter l'extraction des lipides, qui est une étape nécessaire dans le procédé à deux étapes (l'extraction des lipides et la trans-estérification). Les résultats ont montré que l'utilisation d'ultrasons pourrait aboutir à un rendement de production de FAMEs élevé de 95% (p/p lipides) durant 60 min. En comparaison, pour obtenir un rendement similaire par trans-estérification *in-situ* sans ultrasons, il faut utiliser un temps de réaction de 12 h. Les compositions de FAMEs obtenues par ultrasons et trans-estérification *in-situ* étaient similaires à celles obtenues par trans-estérification en deux étapes.

Mots clés : Extraction des lipides; trans-estérification *in-situ*; ultrasons; boues; biodiesel

14.2 Abstract

Various solvents, including water, hexane, methanol, and chloroform/methanol (1:1 v/v), were tested to identify the efficiency of ultrasonication (520 kHz 40 W and 50 Hz 2800 W) lipid extraction from original sludge and *Trichosporon oleaginosus* cultivated with sludge, and compared with the conventional chloroform methanol (2:1 v/v) extraction. The highest lipid recovery 11.8%, 35.3%, 62.0%, and 95.3% with water, hexane, methanol, and chloroform/methanol was obtained from *Trichosporon oleaginosus*, occurred at ultrasonication 50 Hz 2800 W. Ultrasonication chloroform/methanol extraction recovered 95% lipid with short time (20 min) and lower temperature (45 °C) while conventional chloroform methanol extraction to achieve total lipid recovery required 12 h and 60 °C. As organic solvents are toxic, to reduce or eliminate their utilization is significantly important. Ultrasonication in-situ tranesterification which converts the lipid in biomass to biodiesel without lipid extraction was studied. The results showed that use of ultrasonication could achieve high FAMEs yield 95 % w/w lipid in 60 min, while 24 h was required to obtain similar yield in in-situ transesterification without ultrasonication. The compositions of FAMEs obtained from in ultrasonication in-situ transesterification were similar as that of two-stage transesterification (lipid extraction followed by transesterification).

Keywords: Lipid extraction; in-situ transesterification; ultrasonication; wastewater sludge; biodiesel

14.3 Introduction

Currently, biodiesel is derived from oils or fats which are contained in plants seeds, or animals. Most of plants seed oil and animal fat are demanded in food production industry and kitchens. Oleaginous microorganisms are promising feedstock of biodiesel production due to their impressive lipid content up to 80 % w/w biomass (Koutb and Morsy 2011; Gao et al. 2013). Organic waste such as wastewater sludge is rich in nutrients and have been studied for growth of microorganism such as *Acidithiobacillus ferrooxidans, Lipomyces starkeyi, Sinorhizobium meliloti*, and *Bacillus thuringiensis* (Picher et al. 2002; Vidyarthi et al. 2002; Angerbauer et al. 2008; Zhao et al. 2009). When wastewater sludge is used as medium for the growth of oleaginous microorganisms it would reduce the cost of lipid production and mitigate the sludge disposal pressure.

Biodiesel production from microorganism includes three steps, microorganism cultivation (lipid accumulation), lipid extraction (lipid separation from biomass), and biodiesel synthesis. Lipid extraction as the central step in the production is critical. Chloroform and methanol mixture is currently employed on lipid extraction from microorganisms and found efficient (Vicente et al. 2009; Cheirsilp et al. 2011; Cheng et al. 2011; Boyd et al. 2012). The concerns on flammability and high toxicity of chloroform lead to the seeking on technologies with less threat to the human being and environment. In addition, to extraction lipid from microorganisms with chloroform and methanol requires long time (4 to 12 h) and high temperature (50 to 60 °C). Therefore, to lower or eliminate the toxic solvent utilization amount and reduce the extraction time and temperature becomes the key solution of the problem.

Cell disruption with bead milling, homogenizer, microwave, or ultrasonication prior to solvent extraction could reduce the solvent utilization amount as well as proceeding time (Ranjan et al. 2010; Araujo et al. 2013).

In-situ transesterification is the method simultaneously achieved extraction and transesterification of the lipid in microorganism. It thus eliminated lipid extraction step. In previous studies, in-situ transesterification on soy flask and wastewater sludge have accomplished high yield of biodiesel (up to 97%) (Haas et al. 2004; Haas and Scott 2007;

Mondala et al. 2009). However, methanol addition was around a hundred times higher in the in-situ transesterification (methanol to lipid molar ratio around 300: 1) than two stage conversion (methanol to oil ratio 6:1 to 12: 1). Moreover, long reaction time was required (around 12 h for in-situ and 2 h for two stage transesterification).

The aim of the work is to demonstrate the potential of ultrasonication for extraction and in-situ transesterification from the lipid in oleaginous yeast cultivated with sludge. Water, hexane, methanol, chloroform/methanol were employed as solvent for the extraction. Operating parameters including temperature and time were evaluated on effect of lipid recovery. In in-situ transesterification study, parameters including ultrasonication time, catalysts, and methanol to lipid molar ratio were examined. The transesterification without ultrasonication assistance was also conducted to compare with that in the presence of ultrasonication. Profiles of biodiesel converted from ultrasonication chloroform methanol extraction, conventional chloroform methanol extraction, in-situ transesterification, and ultrasonication in-situ transesterification were compared.

14.4 Methods

14.4.1 Strain, culture and harvesting conditions

Oleaginous yeast *Trichosporon oleaginosus* (ATCC20509) was grown in sterilized (121 °C for 15 min) secondary wastewater sludge (30 g/L suspended solids (SS) concentration). The secondary wastewater sludge was obtained from a municipal wastewater treatment plant, Communauté Urbain de Québec (CUQ), Quebec, Canada. The experiment was performed at 200 rpm 28 °C. After 48 h fermentation, sludge-biomass was harvested by centrifugation at 9000 rpm for 20 min. Two times distilled water washing on sludge-biomass was conducted to remove the residual nutrients. Part of the sludge-biomass was dried by lyophilisation and then stored for further study. The other part of the sludge-biomass was dissolved to distilled water to desired biomass concentration for further study.

14.4.2 Conventional lipids extraction methods

The standard chloroform and methanol extraction procedure with minor modification was employed to determine the lipid content in the sludge-biomass (Folch et al. 1957; Vicente et al. 2009). 200 mg dry sludge-biomass (after lyophilisation) was mixed with 4 ml solvent mixture of chloroform and methanol (2:1 v/v), and then subjected to 60 °C for 4 h. The mixture was then centrifuged at 5000 rpm for 15 min and the supernatant solvent phase was withdrawn and transferred into a pre-weighed glass vial (W_1). The extraction procedure was repeated two times. Afterwards, the vial containing the total volume of the supernatant collected from each extraction was put under evaporation and then weighed (W_2). The lipid amount was calculated by the difference of W_2 and W_1. The lipid content in the biomass is $(W_2-W_1)/200$ mg $\times 100\%$. The obtained lipid was then converted to biodiesel through transesterification.

The original sludge (without fermentation) was treated similarly as control to check the lipid content in natural sludge.

14.4.3 Ultrasonic assisted extraction

For original sludge and sludge-biomass, high frequency low power ultrasonication (520 kHz, 40 W) extraction was carried out in an ultrasonic set-up as shown in Figure 14.1. The global ultrasonic system consists of an ultrasonic transducer installed on the bottom of a double-wall glass reactor, an amplifier (T&C power Conversion, Inc.) for power control, a Hewlett Packard Model 3300A function generator for frequency control, and a temperature control device (Poly Stat, Cole Parmer) which circulates the water in the double-wall glass reactor.

Low frequency high power ultrasonication (50 Hz, 2800 W) extraction was performed in an ultrasonication bath (Fisher Scientific, FB15069).

14.4.3.1 Water as solvent

500 mL of fresh sludge or sludge-biomass with desired concentration (dissolved in distilled water) was transferred to the ultrasonication reactor/bath. The solution was subjected to ultrasonication for lipid extraction and samples were taken for analysis. After extraction, 5% NaCl (w/v) for demulsifying lipid/water emulsion, and a few drops of hexane were added

followed by centrifugation at 9000 rpm for 15 min. The supernatants were collected in burettes and allowed to stand for 24 h. The organic phase (the top) was collected in a pre-weighed glass tube and subjected to 60 °C till weight constant. Then the lipid obtained was used to calculate lipid recovery. Thereafter, the lipid was converted to biodiesel through transesterification. The parameters varied in the extraction are biomass concentration (30, 50, and 70 g/L), operation time (5, 10, 15, 20, and 30 min,) and operation temperatures (25, 35, 45, and 55 °C).

14.4.3.2 Hexane, methanol, and chloroform/methanol as solvent

The process was performed similarly as lipid extraction with water as solvent. The dried original sludge or sludge-biomass obtained from lyophilisation was mixed with solvent hexane, methanol, or chloroform/methanol (1:1 v/v) (50 g/L solvent). The mixtures were then subjected to ultrasonication for desired time (5, 10, 15, and 20 min) at different temperature (25, 35, 45, and 55 °C). After ultrasonication, the mixture was centrifuged at 9000 rpm for 15 min and the supernatant solvent phase was withdrawn and transferred into a pre-weighed glass vial. After solvent evaporation, the weight of glass vial was taken. The lipid amount was calculated by the difference of the vial before and after. Then the lipid obtained was used to calculate lipid recovery. The lipid was converted to biodiesel through transesterification.

14.4.4 Transesterification

Lipid obtained from extraction in vials was first dissolved in hexane (5 mL), then mixed with methanol. Lipid to methanol molar ratio is 1:60 (18 mL methanol for per gram lipid). Sulfuric acid was used as catalyst with the addition amount of 1% v/v methanol. The mixture was then subjected to 55 °C for 12 h. After reaction, 5% NaCl was added with the amount of 100 mL per gram lipid, and then FAMEs was extracted by two times washing with hexane (100 mL per gram lipid), then the hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% sodium bicarbonate (20 mL per gram lipid), and the top layer was then dried over 60 °C oven (Halim et al. 2011)

The FAMEs in hexane were analyzed using a Gas Chromatography Linked to Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500). The dimensions of the column used were 30 m × 0.25 mm, with a phase thickness of 0.2 μm. The calibration curve was prepared with a mixture comprising

37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1.3-dichlorobenzene was also used as internal standard with a concentration of 50 ppm.

14.4.5 *In-situ* transesterification

0.2 gram of original sludge and sludge-biomass was mixed with methanol containing H_2SO_4 and then the mixture was subjected to 55 °C for 2 to 24 h. The methanol to oil molar was 6:1, 60:1, 120:1, 240:1, and 360:1 corresponding to 0.08, 0.8, 1.6, 3.2, 6.4 mL methanol, respectively, with addition of 1%, 2%, and 5% H_2SO_4 v/v methanol. 5 mL of hexane was added to increase the lipid solubility in the mixture. After reaction, 5% NaCl was added with the amount of 1 mL per gram biomass, and then FAMEs was extracted by two times washing with hexane (10 ml per gram biomass), then the hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% sodium bicarbonate (2 ml per gram biomass), and the top layer was then dried over 60 °C oven (Halim et al. 2011). Then the FAMEs were analyzed with the same method as described in Lipid transesterification section.

14.4.6 Ultrasonication aided in-situ transesterification

Ultrasonication was conducted with ultrasonic processor CPX 750 (Cole-Parmer Instrument IL) working at 20 kHz (Figure 14.2). The ultrasonic electrical generator converts the standard electricity to high frequency (20 kHz) electrical energy which will be transferred to mechanical vibrations by transducer. The resulting vibrations are transmitted to the horn. In order to avoid high temperature occurring during ultrasonication, cooling water bath (plastic beaker) was used to control the temperature to be around 20 to 25 °C. Methanol with H_2SO_4 was added to 0.2 gram of dry original sludge or sludge-biomass and then directly immersed to ultrasonication horn for a desired time. A cover with a hole to introduce sonication horn was placed on the top of the flask to minimize the loss of methanol and hexane. The experiment time varied from 5 to 60 min, and methanol to biomass ratio was set at 6:1, 60:1, 120:1, 240:1, and 360:1 corresponding to 0.08, 0.8, 1.6, 3.2, 6.4 mL methanol, respectively, with 1%, 2%, and 5% H_2SO_4 v/v methanol. Co-solvent hexane addition amount was 5 mL per 0.2 g biomass.

After reaction, 5% NaCl was added with the amount of 1 mL per gram dry matter, and then FAMEs was extracted by two times washing with hexane (10 mL per gram dry matter), then the

hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% sodium bicarbonate (2 mL per gram dry matter), and the top layer was then dried over 60 °C oven (Halim et al. 2011). Then the FAMEs were analyzed with the same method as described in Transesterification section.

All experiments were performed in triplicates and average results were reported with standard deviation less than 5%.

14.5 Results and discussion

The lipid content obtained with conversional chloroform and methanol (2:1 v/v) extraction was 38.2 ± 1.07% w/w dry matters for sludge-biomass (48 h fermentation broth) and 6.3 ± 0.13% w/w dry matters for original sludge, respectively. It was considered as total lipid in the dry matters, and the lipid recovery was calculated as the lipid obtained from the ultrasonication extraction divided by the total lipid (38.2 and 6.3 w/w dry matters for sludge-biomass and original sludge)

14.5.1 Water as solvent

After 48 h fermentation, the SS concentration was 22.3 g/L. After centrifugation, it became 168.1 g/L. Then, the harvested sludge-biomass was mixed with distill water to obtain SS concentration of 30, 50, and 70 g/L and then subjected to ultrasonication 520 kHz 40 W or 50 Hz 2800 W for 5, 10, 15, 20 and 30 min at 25, 35, 45, or 55 °C. For both ultrasonication system, extraction time is critical parameter and the lipid recovery increased as the time increase from 5 to 20 min (Figure 14.3). After 20 min, the lipid recovery nearly stopped. SS concentration also showed effect on lipid extraction, and 50 g/L provided the highest lipid recovery. The SS concentration mainly impact on energy transfer on the surface of the cells. With higher SS concentration, less energy is received by every single cell, and thus caused cell disruption harder. However, the lower SS concentration will reduce the chance of bumping between the cells, and thus reduces the shear between the cells, which deceases the cell disruption.

Low frequency high power (50 Hz and 2800 W) ultrasonication was observed to perform better in the extraction than that of high frequency low power (520 kHz and 40 W). High operating frequency of the ultrasonic system provides gently and evenly distributed shear across the surface of the cells, while low frequencies produce more aggressive shear than the high one (Chanamai et al. 2000). Therefore, low frequency high power ultrasonication will be more likely leading to cell disruption than that of high frequency low power. In addition, high energy input (high power) could also be the cause of high lipid recovery in 50 Hz and 2800 W ultrasonication system (Gunduz 2009).

Comparing with the conventional chloroform and methanol (considering that 100% lipid was recovered), lipid yield of ultrasonication extraction with water as solvent was low in which the highest lipid yield was around 4.5% (11.8% lipid recovery) w/w dry matters. The lipid recovery was higher than that obtained in our previous study in which similar experiment was performed to pure biomass of *Trichosporon oleaginosus*. Lipid in sludge biomass was not only from cells but also the lipid (from wastewater) attached on sludge. When ultrasonication was applied, the lipid (not degraded by cells) bounding with sludge could be easily dissociated from sludge due to the shearing force generated by ultrasonication. Thus high lipid recovery was observed.

Higher temperature showed more lipid recovery. Temperature determines the movements of the solutes in the system, and high value promotes the movements and increases the shear between the solutes. As mentioned, ultrasonication could break the cells and release intercellular products such as lipid droplets. It was reported that lipid droplets was surrounded by monolayer phospholipid embedding proteins (Brown 2001; Natter et al. 2005). Monolayer phospholipid polar head towards outer environment and non-polar tails towards the neutral TAG core leads to the overall lipid droplets in a water soluble form. Higher temperature leads to stronger movements of lipid droplets surrounded by monolayer phospholipid and residual cells. The conflicts between one lipid droplets with another or with cell residues could break the layer. Non-polar lipid droplets would be free and contact with water and thus be recovered from the mixture.

Due to the low lipid content in original sludge, lipid was not detected in ultrasonication extraction with water as solvents.

14.5.2 Hexane, methanol, and chloroform/methanol as solvent

As water didn't provide comparable lipid yield as conventional chloroform methanol extraction, organic solvent hexane, methanol, and chloroform/methanol were used in ultrasonication lipid extraction. Solvent type showed great impact on the extraction. Chloroform/methanol was the best solvent followed by methanol, then hexane for original sludge and sludge-biomass. The maximum lipid recovery from sludge-biomass with chloroform/methanol, methanol, and hexane was 95.3%, 62.0%, and 35.3% w/w dry matters, respectively (Figure 14.4).

As mentioned above, the lipid droplets were surrounded by monolayer phospholipid; therefore, to break the layer requires high polarity solvent such as methanol. Chloroform and hexane are non-polar solvents and cannot to accomplish the task. Ultrasonication hexane extraction mainly counted on the mass transfer of hexane to the monolayer. Methanol provides high physical attraction to the polar head of the phospholipid, and pulls or could even tear out the layer. When the damage on the layer was large enough, the lipid droplets would escape out as free lipid which could be the mechanism of ultrasonication methanol extraction. In the ultrasoncation chloroform/methanol system, methanol played the role to disrupt the layer and provided the chance for non-polar solvent chloroform to approach and dissolve the lipid droplets, and eventually recover the lipid. This would be the reason that chloroform/methanol gave the highest lipid recovery and no significant effect on extraction was observed with temperature change.

Similar results were observed in lipid recovery from original sludge and sludge-biomass with ultrasonication frequency and power 50 Hz 2800 W than 520 kHz 40 W (Figures 14.4 and 14.5). Ultrasonication plays the role to disrupt the cells and speed up the extraction. When cell was disrupted, solvents access to pull out lipid.

Chloroform/methanol (1:1 v/v) ultrasonication lipid extraction recovered total lipid in 15 min with less addition amount of high toxicity solvent chloroform while conventional chloroform methanol (2:1 v/v) required 12 h to achieve the similar result. Moreover, 92% lipid recovery was achieved at 25 °C with ultrasonication instead of 60 °C for conventional chloroform methanol extraction. It suggests that ultrasonication lipid extraction with chloroform/methanol

(1:1 v/v) would be a promising alternative of conventional chloroform methanol extraction which is time and energy consuming.

For original sludge ultrasonication lipid extraction, chloroform/methanol was the best one, and the highest lipid recovery was 93.3% w/w dry matters. With hexane extraction, lipid was not detected due to the low lipid content in original sludge. With methanol extraction, the lipid recovery was observed after 15 min extraction. The results were shown in Figure 14.5.

14.5.3 *In-situ* transesterification without or with ultrasonication

According to the equilibrium reaction 1 g lipid could produce approximately 1 g of biodiesel (Equation 14.1). The total lipid in the biomass was obtained being 38.2 ± 1.07% and 6.3 ± 0.13% w/w dry matters for sludge-biomass (48 h fermentation broth) and original sludge by conventional chloroform methanol (2:1 v/v) extraction, respectively. Therefore, biodiesel yield was calculated based on the lipid amount (% FAMEs w/w lipid). For an equilibrium reaction, reactant concentration (methanol to lipid molar ratio for transesterification), catalyst addition amount, and reaction time have great impact on the product yield.

Equation 14.1 **Triglycerides (890 g) + 3 Methanol (32 g) → 3 FAMEs (298 g) + Glycerol (92 g)**

Without or with ultrasonication, the biodiesel yield increased as methanol to lipid molar ratio, catalyst, and reaction time increased in-situ transesterification (Figure 14.6). With ultrasonication (around 40 min), the reaction equilibrium was reached with shorter time than that without ultrasonication (12 h). Ultrasonication creates pressure and shear on the cell walls and results in rapid cell disruption, and hence methanol addition amount and reaction time were reduced comparing with that without ultrasonication. Formation of free radicals of reacting species led by ultrasonication cavitation could also contribute to high reaction rate and thus decreases reaction time (Rokhina et al. 2009).

Comparing with two stage transesterification (12 h) in which lipid was first extracted and then converted to FAMEs, to obtain similar FAMEs yield (95% w/w lipid) ultrasonication in-situ

transesterification (50 min) required shorter reaction time for ultrasonication. The problem of ultrasonication in-situ transesterification was the high methanol to lipid molar ratio requirement (60:1 for ultrasonication in-situ transesterification and 360:1 for two stage transesterification). Even though higher methanol addition is still required, the energy consumed by methanol recovery could be compensated by the energy (to maintain temperature and mixing) saved due to the reduction on reaction time. It suggests that ultrasonication in-situ transesterification could be feasible in use in large scale biodiesel production.

14.5.4 Profile of biodiesel obtained from lipid extracted with different solvents

Comparing with the lipid recovery amount calculated based on lipid extraction, the lipid recovery from GC-MS quantification (amount sum of FAMEs) was lower. The highest lipid recoveries from sludge-biomass and original sludge with ultrasonication chloroform methanol extraction were 95.3 and 93.3% w/w total lipid, respectively. After converting the extracted lipid to biodiesel (1 g lipid produces 1 g biodiesel), biodiesel analyzed with GC-MS and the quantification results showed that the lipid amount was 85.2 and 80.7% w/w total lipid for sludge-biomass and original sludge, respectively. Considering that the conversion efficiency was 95% (according to the result of transesterification of lipid extracted with conventional chloroform methanol), the lipid recovery was supposed to be 90.5 and 88.6% w/w total lipid for sludge-biomass and original sludge, respectively. The difference of lipid recovery with different methods (weight difference and GC-MS quantification) was 5.3 and 7.9% w/w total lipid for sludge-biomass and original sludge, respectively. In fact, if the extraction time increased, higher lipid recovery could be reached, and even the recovery value will be higher than 100% w/w total lipid as the presence of impurities in lipid.

That the higher lipid recovery was observed with calculation by weight difference than GC-MS quantification would be due to the impurities in the extracted lipid. While for the case of lipid extracted with conventional chloroform methanol, the lipid recovery was around 5% difference between calculation from weight difference and GC-MS quantification which was considered due to that transesterification efficiency was 95%. High impurity content was observed with ultrasonication lipid extraction, it was predicated due to ultrasonication dissociated the

impurities bounding with sludge and extracted along with lipid. It suggested that ultrasonication could not be suitable for lipid extraction from complex matrix such as sludge.

For all types of extraction, biodiesel obtained from the extracted lipid mainly contains C16 and C18 (Tables 14.1). No much difference on the fatty acid compositions from conventional chloroform methanol (2:1 v/v) and ultrasonication chloroform/methanol (1:1 v/v) extraction was observed. In addition, in-situ transesterification and ultrasonication in-situ transesterification provided similar FAMEs profile as two stage transesterification. It indicates ultrasonication doesn't impact on the properties of the final product.

14.6 Conclusions

Solvent has significant impact on the efficiency of ultrasonication lipid extraction from oleaginous microorganisms. The highest lipid recovery was from chloroform/methanol (1:1 v/v) extraction compared to other solvents, and the recovery was the same as that of conventional chloroform methanol (2:1 v/v) extraction. The addition of ultrasonication reduced the extraction time to 50 min and temperature to 25 °C from 12 h and 60 °C used in conventional method and has no effect on fatty acid profiles. However, FAMEs obtained from ultrasonication extraction lipid had high impurity content which was up around 5% for sludge-biomass and 8% for original sludge, respectively. The FAMEs profile from ultrasonication extracted lipid was similar as that from conventional extracted lipid. It indicates that ultrasonication didn't impact on the FAMEs profiles.

Ultrasonication in-situ tranesterification largely reduced reaction time and obtained FAMEs were similar as that of two stage transesterification. It suggested that ultrasonication in-situ tranesterification could be a promising alternative of biodiesel production method over conventional two stage transesterification.

14.7 Acknowledgements

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14.8 References

Angerbauer, C., Siebenhofer, M., Mittelbach, M., and Guebitz, G. M. (2008). "Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production." *Bioresource Technology*, 99, 3051-3056.

Araujo, G. S., Matos, L. J. B. L., Fernandes, J. O., Cartaxo, S. J. M., Gonçalves, L. R. B., Fernandes, F. A. N., and Farias, W. R. L. (2013). "Extraction of lipids from microalgae by ultrasound application: Prospection of the optimal extraction method." *Ultrasonics Sonochemistry*, 20, 95-98.

Boyd, A. R., Champagne, P., McGinn, P. J., MacDougall, K. M., Melanson, J. E., and Jessop, P. G. (2012). "Switchable hydrophilicity solvents for lipid extraction from microalgae for biofuel production." *Bioresource Technology*, 118, 628-632.

Brown, D. A. (2001). "Lipid droplets: proteins floating on a pool of fat." *Current Biology*, 11, R446-R449.

Chanamai, R., Herrmann, N., and McClements, D. J. (2000). "Probing floc structure by ultrasonic spectroscopy, viscometry, and creaming measurements." *Langmuir*, 16, 5884-5891.

Cheirsilp, B., Suwannarat, W., and Niyomdecha, R. (2011). "Mixed culture of oleaginous yeast Rhodotorula glutinis and microalga Chlorella vulgaris for lipid production from industrial wastes and its use as biodiesel feedstock." *New Biotechnology*, 28, 362-368.

Cheng, C.-H., Du, T.-B., Pi, H.-C., Jang, S.-M., Lin, Y.-H., and Lee, H.-T. (2011). "Comparative study of lipid extraction from microalgae by organic solvent and supercritical CO_2." *Bioresour Technol*, 102, 10151-10153.

Folch, J., Lees, M., and G.H., S. S. (1957). "A simple method for the isolation and purification of total lipides from animal tissues." *J. Biol. Chem.*, 226, 497-509.

Gao, D., Zeng, J., Zheng, Y., Yu, X., and Chen, S. (2013). "Microbial lipid production from xylose by Mortierella isabellina." *Bioresource Technology*, 133, 315-321.

Gunduz, C. (2009).Ultrasonic disintegration of sewage sludge. In *Graduate School of Natural and Applied Sciences*, Vol. Master, 72 Izmir: Dokuz Eylul University.

Haas, M., and Scott, K. (2007). "Moisture Removal Substantially Improves the Efficiency of in Situ Biodiesel Production from Soybeans." *Journal of the American Oil Chemists' Society*, 84, 197-204.

Haas, M., Scott, K., Marmer, W., and Foglia, T. (2004). "In situ alkaline transesterification: An effective method for the production of fatty acid esters from vegetable oils." *Journal of the American Oil Chemists' Society*, 81, 83-89.

Halim, R., Gladman, B., Danquah, M. K., and Webley, P. A. (2011). "Oil extraction from microalgae for biodiesel production." *Bioresour. Technol.*, 102, 178-185.

Koutb, M., and Morsy, F. M. (2011). "A potent lipid producing isolate of Epicoccum purpurascens AUMC5615 and its promising use for biodiesel production." *Biomass and Bioenergy*, 35, 3182-3187.

Mondala, A., Liang, K., Toghiani, H., Hernandez, R., and French, T. (2009). "Biodiesel production by in situ transesterification of municipal primary and secondary sludges." *Bioresour. Technol.*, 100, 1203-1210.

Natter, K., Leitner, P., Faschinger, A., Wolinski, H., McCraith, S., Fields, S., and Kohlwein, S. D. (2005). "The spatial organization of lipid synthesis in the yeast *Saccharomyces cerevisiae* derived from large scale green fluorescent protein tagging and high resolution microscopy." *Molecular & Cellular Proteomics*, 4, 662-672.

Picher, S., Drogui, P., Guay, R., and Blais, J. F. (2002). "Wastewater sludge and pig manure used as culture media for bioleaching of metal sulphides." *Hydrometallurgy*, 65, 177-186.

Ranjan, A., Patil, C., and Moholkar, V. S. (2010). "Mechanistic Assessment of Microalgal Lipid Extraction." *Industrial & Engineering Chemistry Research*, 49, 2979-2985.

Rokhina, E. V., Lens, P., and Virkutyte, J. (2009). "Low-frequency ultrasound in biotechnology: state of the art." *Trends in Biotechnology*, 27, 298-306.

Vicente, G., Bautista, L. F., Rodríguez, R., Gutiérrez, F. J., Sádaba, I., Ruiz-Vázquez, R. M., Torres-Martínez, S., and Garre, V. (2009). "Biodiesel production from biomass of an oleaginous fungus." *Biochem. Eng. J.*, 48, 22-27.

Vidyarthi, A. S., Tyagi, R. D., Valero, J. R., and Surampalli, R. Y. (2002). "Studies on the production of B. thuringiensis based biopesticides using wastewater sludge as a raw material." *Water Research*, 36, 4850-4860.

Zhao, Y. Q., Zhao, X. H., and Babatunde, A. O. (2009). "Use of dewatered alum sludge as main substrate in treatment reed bed receiving agricultural wastewater: Long-term trial." *Bioresource Technology*, 100, 644-648.

Table 14.1 **Comparison of fatty acid profiles of biodiesel produced from the lipid of *Trichosporon oleaginosus***

Fatty acids	Relative amount of total fatty acid (% w/w)							
	Original sludge				Sludge-biomass			
	Conven. Ch: Me	Ch:Me +ultra.	In-situ	Ultra. in-situ	Conven. Ch: Me	Ch:Me +ultra.	In-situ	Ultra. in-situ
C14:0	0.56	0.54	0.66	0.27	1.9	1.21	0.82	1.53
C14:1	0.67	0.57	0.62	0.62	3.68	2.95	2.39	3.09
C15:0	0.74	0.61	0.83	0.54	3.17	3.46	3.76	2.77
C15:1	0.51	0.50	0.58	0.52	1.11	0.82	0.64	0.74
C16:0	31.82	33.15	35.09	33.28	27.41	29.39	31.73	30.91
C16:1	2.17	1.95	2.37	2.72	18.23	19.14	16.29	18.30
C17:0	4.28	4.66	3.15	2.09	2.39	1.72	2.04	1.82
C18:0	12.24	10.87	9.46	11.3	12.18	12.03	13.18	12.78
C18:1	32.39	33.01	35.29	34.02	20.82	21.77	23.44	21.39
C18:2	6.12	5.62	3.74	5.74	2.57	2.09	1.31	1.74
C18:3	5.09	5.29	6.13	5.88	1.09	0.95	1.26	0.88
C20:0	1.19	1.00	0.31	0.67	1.82	1.92	1.09	1.32
C20:1	0.56	0.52	0.69	0.83	1.12	0.83	0.75	0.97

The fatty acid content is less than 0.5% was not given;

Conven. Ch:Me= conventional chloroform methanol extraction;

Ultra.= ultrasonication;

The extractions with chloroform/methanol were performed at ultrasonication 50 Hz 2800 W temperature 55 °C for 20 min.

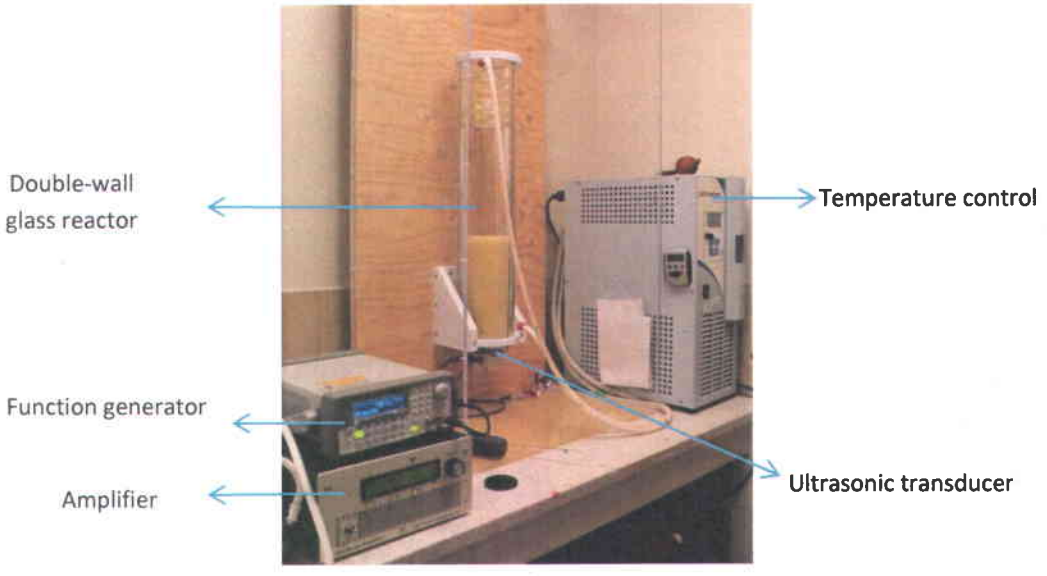


Figure 14.1 **Ultrasonication reactor (520 kHz, 40 W) for lipid extraction**

Figure 14.2 **Ultrasonication device used in the experiment**

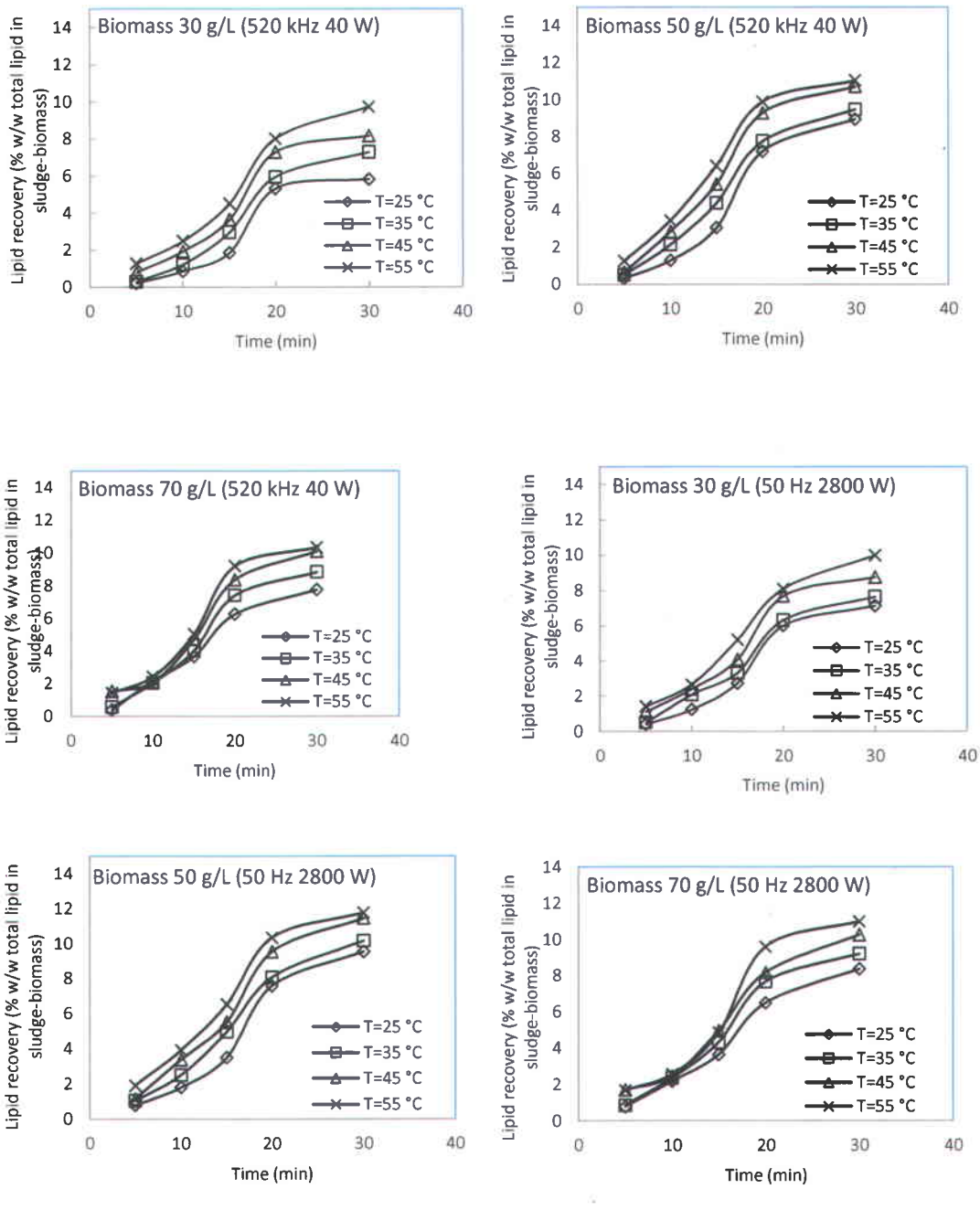
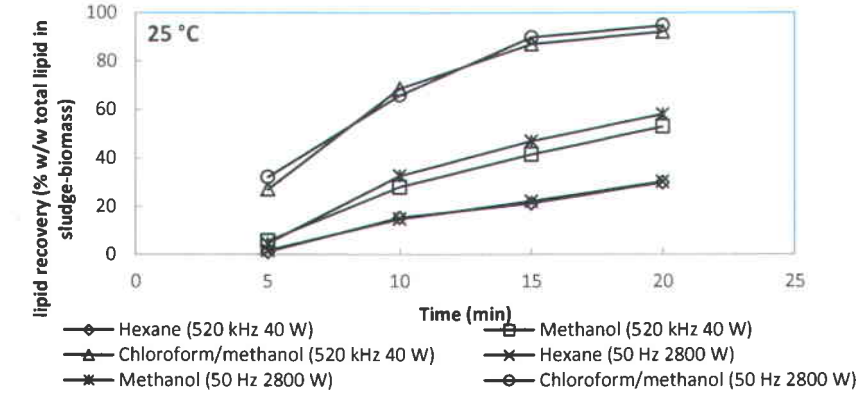
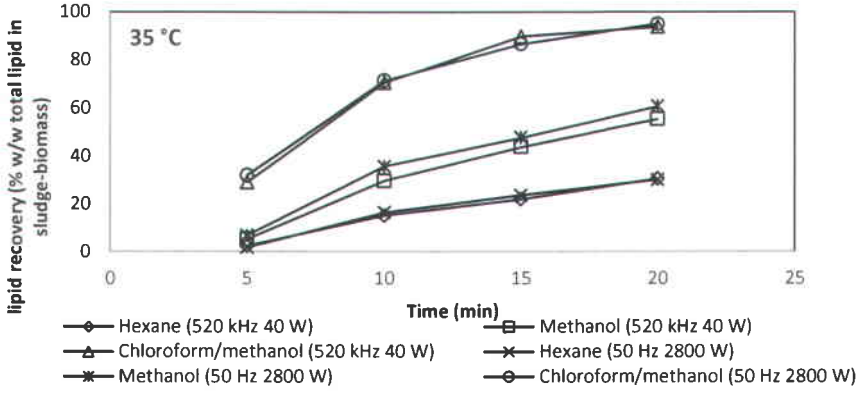
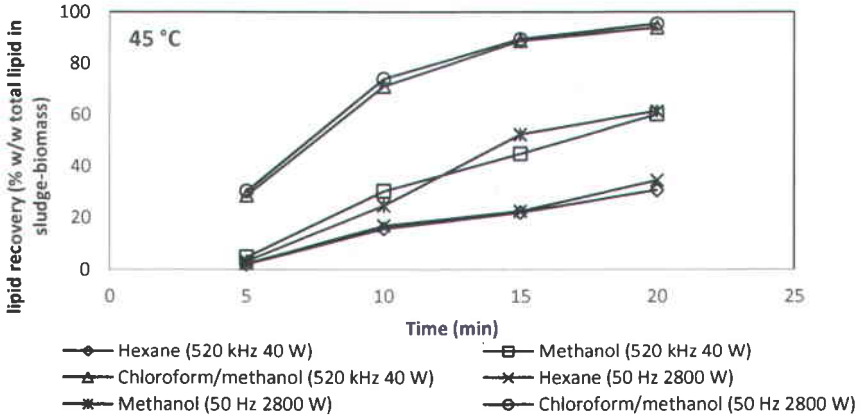


Figure 14.3 **Ultrasonication lipid extraction from sludge-biomass with water as solvent; standard deviation was less than 5%**


25 °C
lipid recovery (% w/w total lipid in sludge-biomass)
100
80
60
40
20
0
0
5
10
15
20
25
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)


35 °C
lipid recovery (% w/w total lipid in sludge-biomass)
100
80
60
40
20
0
0
5
10
15
20
25
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)


45 °C
lipid recovery (% w/w total lipid in sludge-biomass)
100
80
60
40
20
0
0
5
10
15
20
25
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)

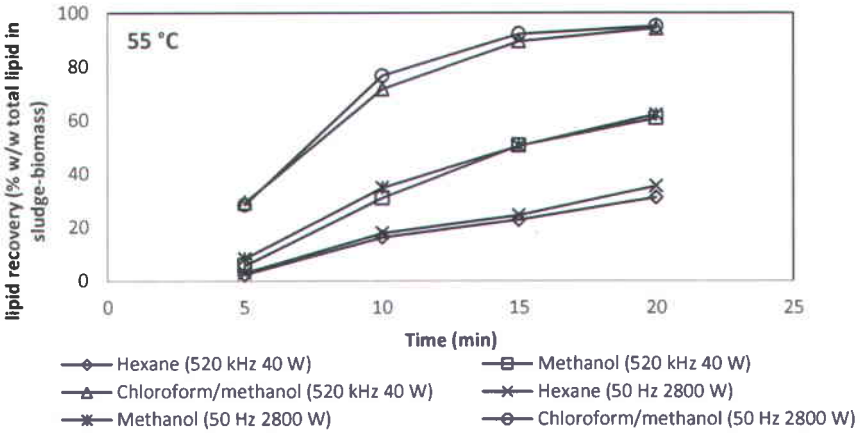
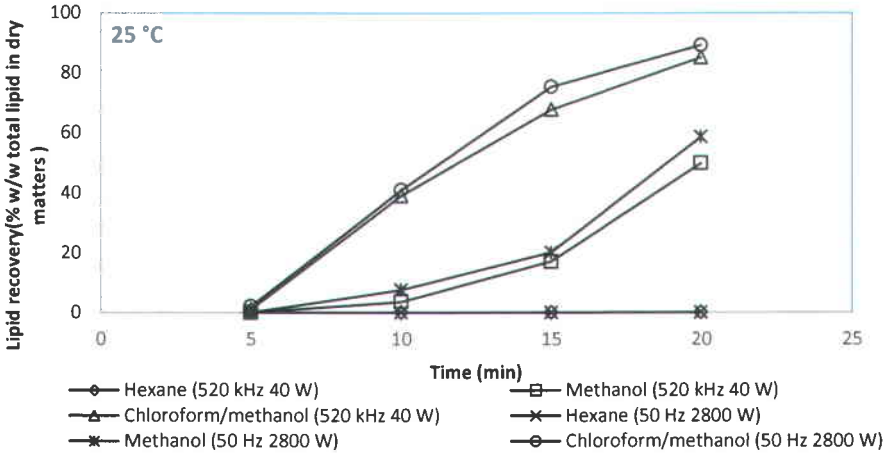
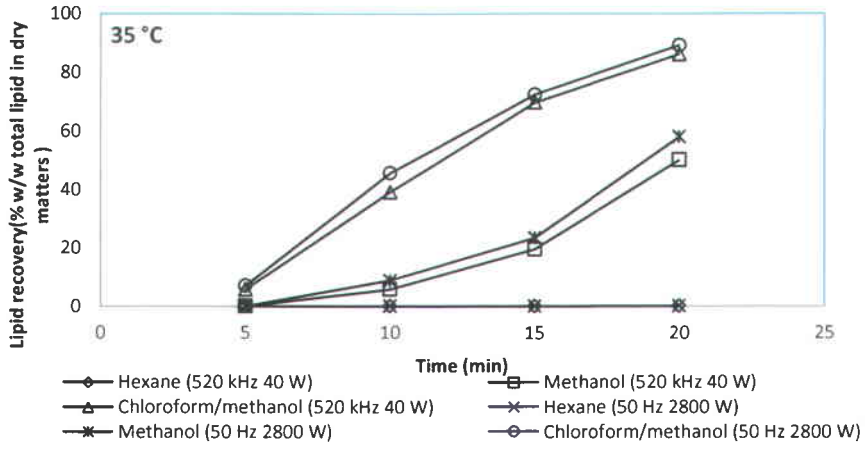
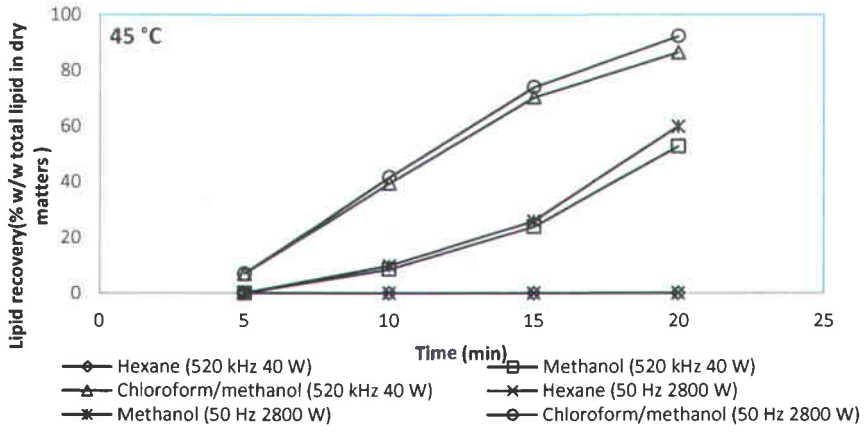


Figure 14.4 **Ultrasonication lipid extraction from sludge-biomass organic solvents; standard deviation was less than 5%**


25 °C
Lipid recovery(% w/w total lipid in dry matters)
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)


35 °C
Lipid recovery(% w/w total lipid in dry matters)
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)


45 °C
Lipid recovery(% w/w total lipid in dry matters)
Time (min)
Hexane (520 kHz 40 W)
Methanol (520 kHz 40 W)
Chloroform/methanol (520 kHz 40 W)
Hexane (50 Hz 2800 W)
Methanol (50 Hz 2800 W)
Chloroform/methanol (50 Hz 2800 W)

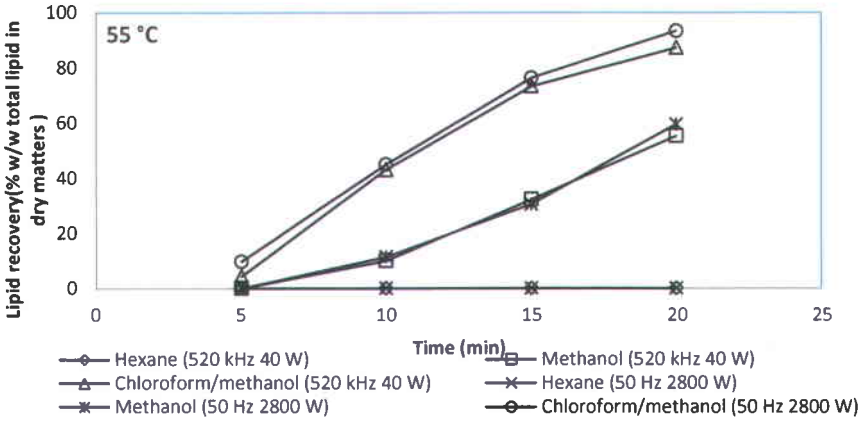
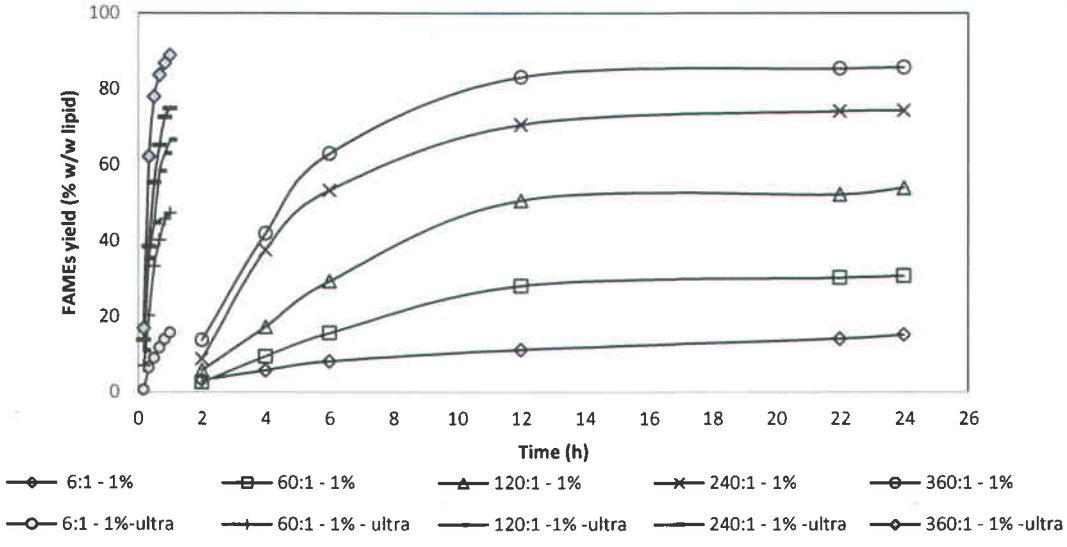
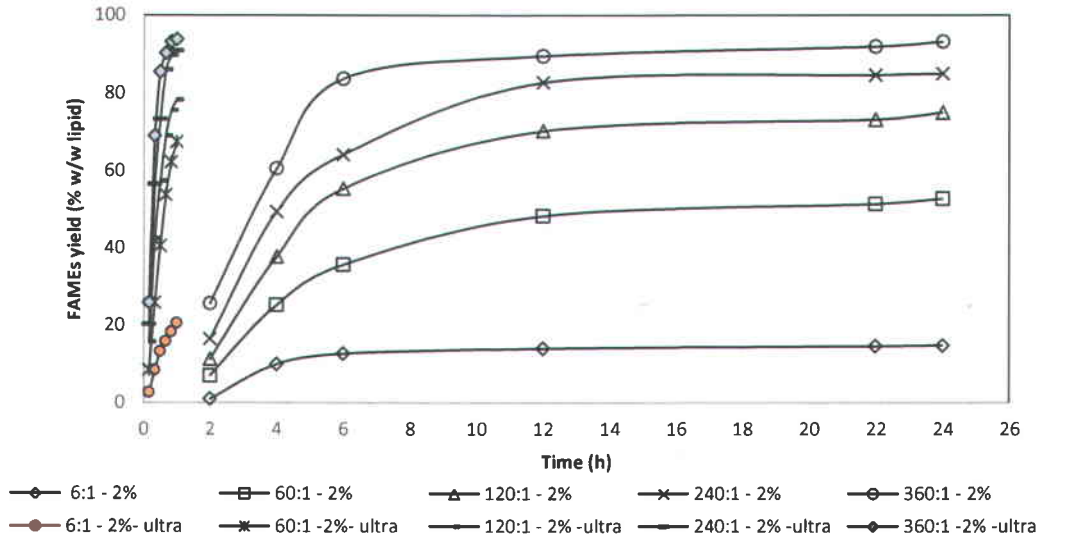


Figure 14.5 **Ultrasonication lipid extraction from original sludge with organic solvents; standard deviation was less than 5%**


FAMEs yield (% w/w lipid)
Time (h)
6:1 - 1%
60:1 - 1%
120:1 - 1%
240:1 - 1%
360:1 - 1%
6:1 - 1%-ultra
60:1 - 1% - ultra
120:1 -1% -ultra
240:1 - 1% -ultra
360:1 - 1% -ultra


FAMEs yield (% w/w lipid)
Time (h)
6:1 - 2%
60:1 - 2%
120:1 - 2%
240:1 - 2%
360:1 - 2%
6:1 - 2%- ultra
60:1 -2%- ultra
120:1 - 2% -ultra
240:1 - 2% -ultra
360:1 -2% -ultra

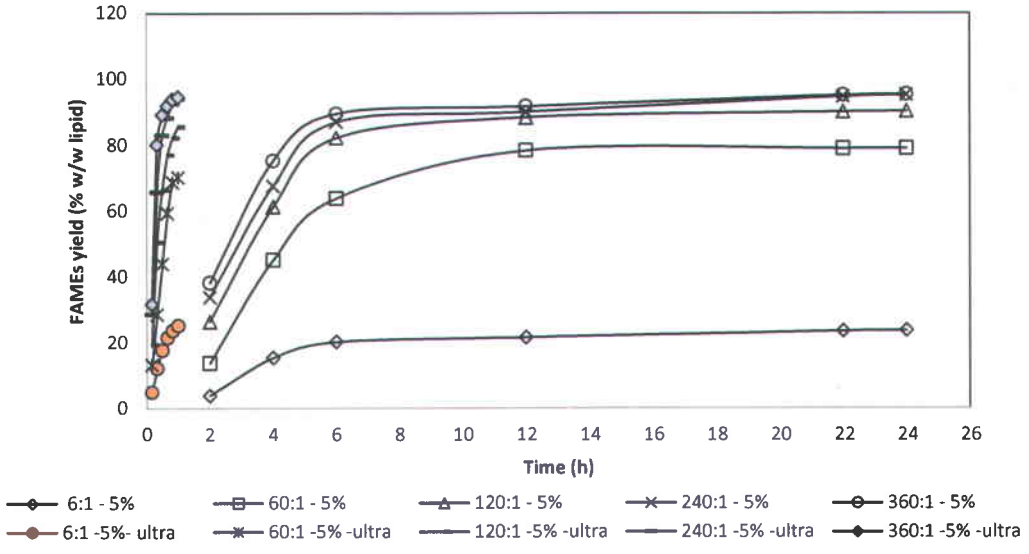
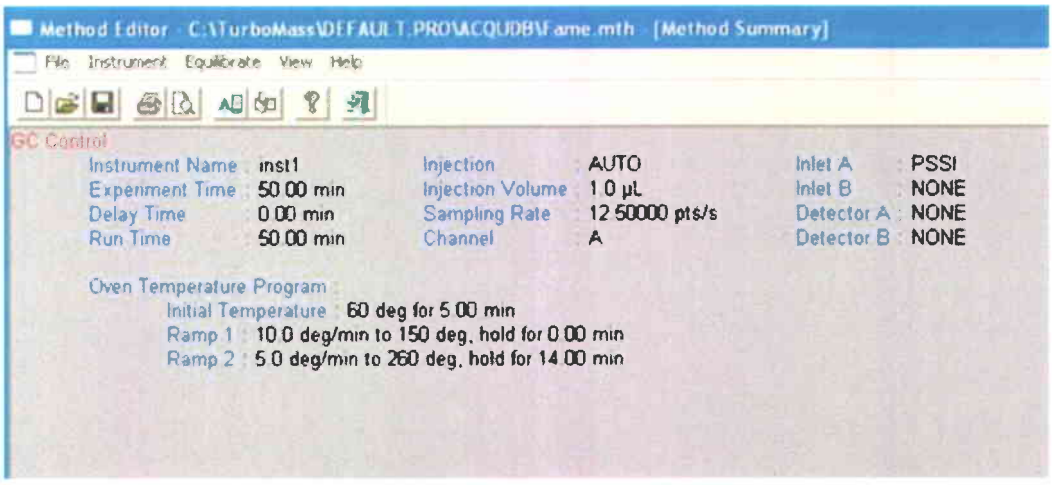


Figure 14.6 **FAME yields from sludge-biomass versus reaction time for the in-situ transesterification with or without ultrasonication; standard deviation is less than 5%**

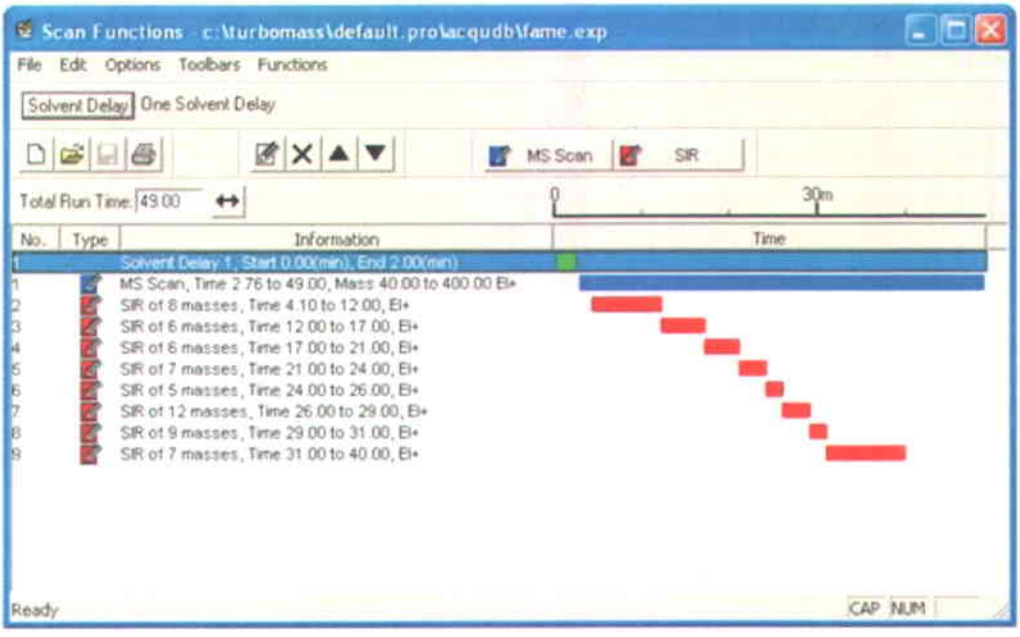
ANNEXES

ANNEXE 1: FAMEs analysis with GC-MS

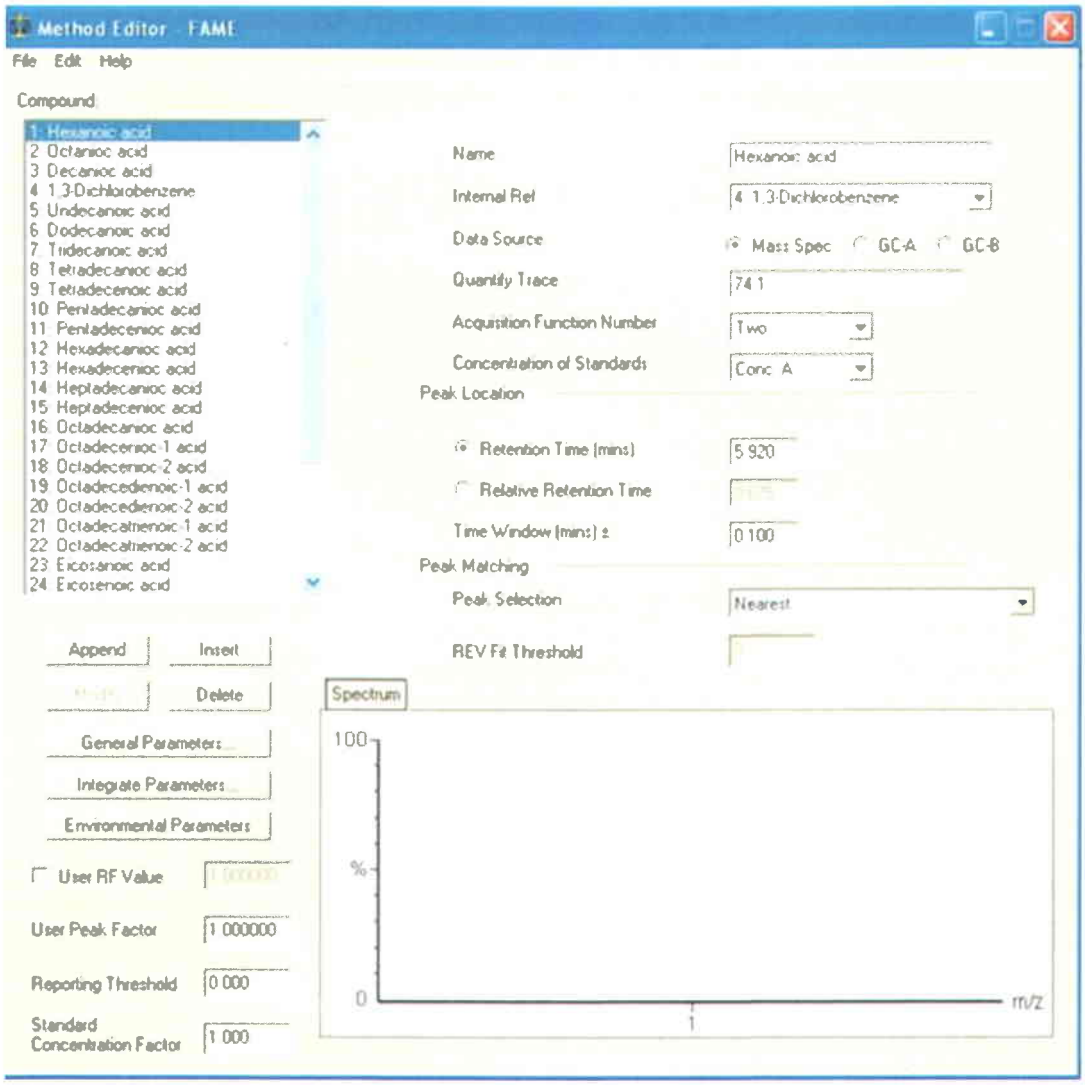
ANNEXE 1.1. GC method



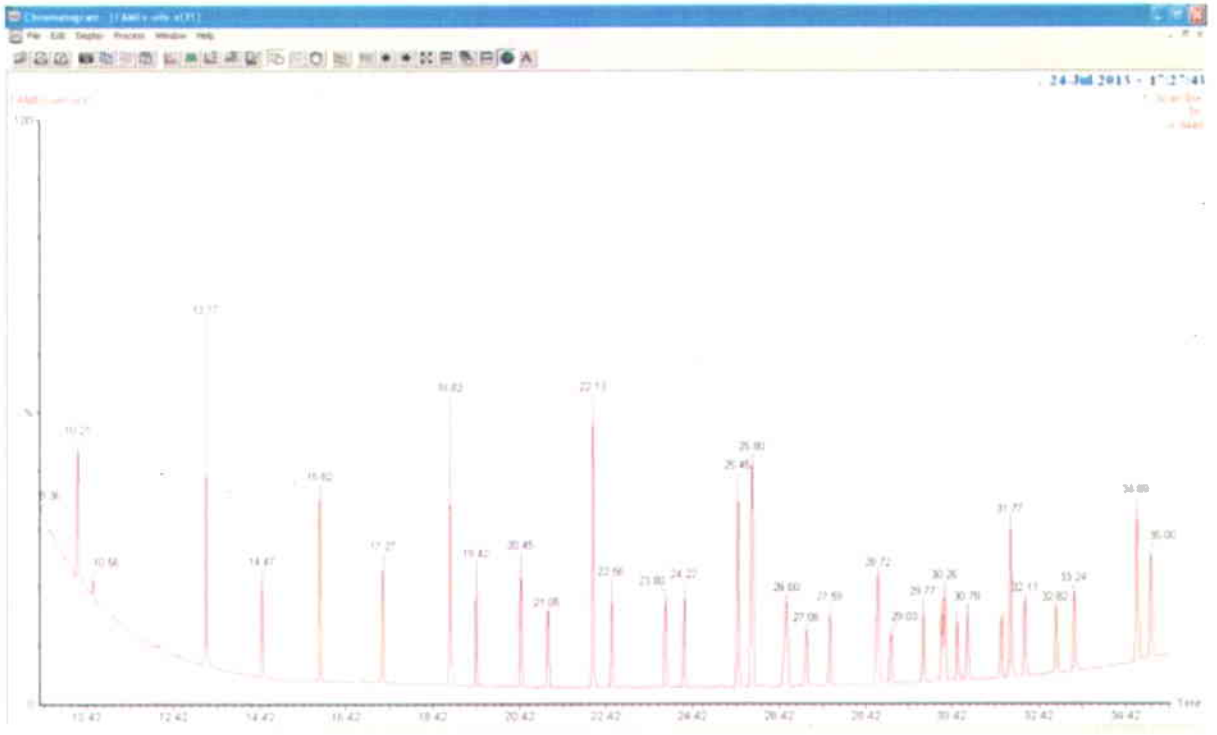
ANNEXE 1.2. MS method



ANNEXE 1.3. Quantification method



ANNEXE 1.4 Chromatogram of the 37 FAMEs



ANNEXE 1.5 FAMEs produced from sludge derived oils

Fatty acid	pH 2	pH 2 struvite formation	pH nature	pH nature struvite formation	pH 12	pH 12 struvite formation
C6:0	107.2	111.37	70.64	81.92	58.45	66.64
C8:0	310.78	239.81	210.23	249.31	139.37	184.44
C10:0	807.87	484.89	397.23	562.59	199.16	355.15
C11:0	253.67	196.83	161.79	227.31	97.44	135.44
C12:0	2655.1	1524.67	1063.47	1671.16	749.17	974.3
C13:0	1183.2	872.21	712.38	1040.33	547.86	647.79
C14:0	9703.18	6269.33	3052.08	5378.54	2836.17	2832.39
C14:1	535.98	391.56	257.21	403.96	224.66	235.35
C15:0	5630.83	4018.34	2153.19	3758.26	2258.86	1953.75
C15:1	438.48	171.5	210.9	153.93	128.01	124.14
C16:0	45250.87	34796.59	14914.25	30620.49	20321.86	15086.1
C16:1	36994.26	22551.33	9564.51	17966.57	8804.46	8066.27
C17:0	4425.94	3383.79	1851.35	3198.54	2341.09	1718.25
C18:0	57752.43	42746.93	21641.68	36869.78	31072.71	18380.04
C18:1	75676.69	58825.51	16494.59	51194.35	23949.76	15784.85
C18:2	42830.34	30089.22	13960.23	26837.17	18134.63	14557.97
C18:3	13083.97	12201.31	25637.19	8626.74	33943.28	26234.23
C20:0	1835.35	1427.62	899.82	1270.95	1288.94	691.6
C20:1	2021.85	1581.13	1008.29	1461.59	1425.9	811.09
C20:2	476.72	429.39	365.48	439.62	425.74	390.03
C21:0	488.64	408.38	262.83	348.52	294.15	239.03
C22:0	1384.09	1148.04	716.53	950.35	1050.5	549.62
C22:1	1288.14	1097.07	687.1	908.46	974.64	527.34
C22:2	0	505.87	0	295.37	0	0
C23:0	613.06	540.67	368.45	453.73	513.08	309.05
C24:0	1037.49	933.87	562.22	754.19	854.94	461.43
C24:1	3041.62	3831.01	1418.13	2042.98	2467.43	1179.37
Total PPM	309827.75	230778.24	118641.77	197766.71	155102.26	112495.66

fatty acid SLY		fatty acid SOF		fatty acid T.O.			fatty acid secondary sludge		
C6:0	0.22	C6:0	0.15	C6:0	0.36	41.72	C6:0	0.23	61
C8:0	0.45	C8:0	0.32	C8:0	0.09	34.58	C8:0	0.92	34
C10:0	0.41	C10:0	0.49	C10:0	0.16		C10:0	0.75	5
C11:0	0.23	C11:0	0.18	C11:0	1.04		C11:0	0.61	
C12:0	1.43	C12:0	1.04	C12:0	0.82		C12:0	0	
C13:0	0.77	C13:0	0.66	C13:0	0.05		C13:0	0	
C14:0	3.48	C14:0	3.51	C14:0	2.56		C14:0	1.9	
C14:1	0.52	C14:1	0.21	C14:1	2.26		C14:1	3.68	
C15:0	2.34	C15:0	2.14	C15:0	1.27		C15:0	3.17	
C15:1	0.48	C15:1	0.11	C15:1	0.31		C15:1	1.11	
C16:0	14.92	C16:0	13.36	C16:0	11.82		C16:0	34.82	
C16:1	11.07	C16:1	12.54	C16:1	12.17		C16:1	18.23	
C17:0	2.29	C17:0	1.62	C17:0	4.28		C17:0	2.39	
C18:0	17.68	C18:0	17.73	C18:0	17.24		C18:0	12.18	
C18:1	15.49	C18:1	24.36	C18:1	18.09		C18:1	9.17	
C18:2	12.71	C18:2	13.03	C18:2	16.12		C18:2	2.57	
C18:3	5.64	C18:3	4.64	C18:3	5.09		C18:3	1.09	
C20:0	0.93	C20:0	0.56	C20:0	0.19		C20:0	1.82	
C20:1	1.04	C20:1	0.85	C20:1	0.56		C20:1	1.12	
C20:2	1.24	C20:2	0.16	C20:2	2.25		C20:2	1.25	
C21:0	0.72	C21:0	0.17	C21:0	1.19		C21:0	0.07	
C22:0	0.91	C22:0	0.42	C22:0	0.38		C22:0	0.38	
C22:1	0.83	C22:1	0.39	C22:1	0.68		C22:1	0.68	
C22:2	1.03	C22:2	0.03	C22:2	0.24		C22:2	0.09	

ANNEXE 2: Lipid content of microbes cultivated with sludge

	Primary				
SLY	1	2	3	average	Standard deviation
0	6.14	6.47	6.26	6.29	0.17
12	7.55	7.81	7.44	7.6	0.19
24	8.15	8.09	7.46	7.9	0.38
36	8.59	8.93	8.61	8.71	0.19
48	9.02	8.74	8.85	8.87	0.14
60	9.15	9.26	8.68	9.03	0.31
72	9.19	9.16	8.74	9.03	0.25

	secondary				
SLY	1	2	3	average	Standard deviation
0	5.12	5.47	4.89	5.16	0.29
12	8.74	8.92	9.43	9.03	0.36
24	14.62	14.37	14.57	14.52	0.13
36	27.23	27.09	26.98	27.1	0.13
48	32.45	32.27	31.79	32.17	0.34
60	32.18	32.37	31.75	32.1	0.32
72	32.36	32.11	31.83	32.1	0.27

	mixed				
SLY	M-1	M-2	M-3	average	Standard deviation
0	7.14	6.83	6.85	6.94	0.17
12	7.65	7.83	7.74	7.74	0.09
24	9.37	9.73	9.94	9.68	0.29
36	14.17	14.43	13.97	14.19	0.23
48	16.56	16.58	17.17	16.77	0.35
60	26	26.35	26.04	26.13	0.19
72	26.47	26.79	26.57	26.61	0.16

pulp and paper

SLY	1	2	3	average	Standard deviation
0	9.76	10.13	10.26	10.05	0.26
12	16.04	16.37	15.98	16.13	0.21
24	20.13	20.64	20.67	20.48	0.30
36	24.27	24.54	25.23	24.68	0.50
48	30.35	30.62	30.68	30.55	0.18
60	32.22	32.57	32.47	32.42	0.18
72	32.75	33.1	32.85	32.9	0.18

Primary

SOF	1	2	3	average	Standard deviation
0	7.25	7.53	7.48	7.42	0.15
12	7.43	7.38	7.99	7.6	0.34
24	12.64	13.01	12.57	12.74	0.24
36	15.49	14.93	15.06	15.16	0.29
48	15.64	15.17	16.14	15.65	0.49
60	15.32	15.71	15.41	15.48	0.20
72	15.69	15.38	15.37	15.48	0.18

secondary

SOF	1	2	3	average	Standard deviation
0	6.07	6.38	5.94	6.13	0.23
12	8.86	8.97	9.26	9.03	0.21
24	14.79	15.02	14.71	14.84	0.16
36	24.01	24.29	24.27	24.19	0.16
48	31.44	31.74	31.65	31.61	0.15
60	32.36	32.54	32.36	32.42	0.10
72	32.27	32.05	31.98	32.1	0.15

mixed

SOF	1	2	3	average	Standard deviation
0	6.82	6.83	6.66	6.77	0.10
12	8.69	8.32	8.64	8.55	0.20
24	14.15	14.35	14.07	14.19	0.14
36	20.8	20.71	20.44	20.65	0.19
48	25.82	25.71	25.42	25.65	0.21
60	27.13	26.79	26.9	26.94	0.17
72	26.53	26.77	26.53	26.61	0.14

pulp and paper

SOF	1	2	3	average	Standard deviation
0	10.86	11.21	10.84	10.97	0.21
12	13.54	13.21	13.42	13.39	0.17
24	19.05	19.32	19.2	19.19	0.14
36	26.33	26.51	26.51	26.45	0.10
48	34.04	34.23	34.3	34.19	0.13
60	34.11	34.18	33.8	34.03	0.20
72	33.85	33.64	33.64	33.71	0.12

secondary sludge (g/L) sly	Ave-bio10	Ave-Lipid10	Ave-bio15	Ave-Lipid15	Ave-bio20	Ave-Lipid20	Ave-bio25	Ave-Lipid25	Ave-bio30	Ave-Lipid30
0	3.81	6.15	3.79	6.11	3.76	6.06	3.79	6.11	3.8	6.13
12	4.23	6.82	4.62	7.45	4.83	7.79	5.42	8.74	5.6	9.03
24	6.31	10.18	6.99	11.27	7.54	12.16	8.97	14.47	9.2	14.84
36	8.23	13.27	8.43	13.60	12.46	20.10	15.13	24.40	15	24.19
48	10.14	16.35	11	17.74	15.17	24.47	18.65	30.08	19.6	31.61
60	10.24	16.52	11.27	18.18	15.21	24.53	19.03	30.69	20.1	32.42
72	10.18	16.42	11.32	18.26	15.28	24.65	18.96	30.58	19.9	32.10

secondary sludge (g/L) sof	Ave-bio10	Ave-Lipid10	Ave-bio15	Ave-Lipid15	Ave-bio20	Ave-Lipid20	Ave-bio25	Ave-Lipid25	Ave-bio30	Ave-Lipid30
0	3.85	6.21	3.82	6.16	3.8	6.13	3.85	6.21	3.81	6.15
12	4.67	7.53	4.73	7.63	4.91	7.92	5.52	8.90	6.01	9.69
24	6.24	10.06	7.05	11.37	7.64	12.32	9.04	14.58	8.93	14.40
36	7.69	12.40	8.69	14.02	12.53	20.21	15.37	24.79	16.74	27.00
48	10.17	16.40	11.2	18.06	16.14	26.03	18.83	30.37	19.79	31.92
60	10.22	16.48	11.37	18.34	16.21	26.15	19.01	30.66	20.4	32.90
72	10.24	16.52	11.42	18.42	16.28	26.26	18.92	30.52	20.51	33.08

pp sludge (g/L) sof	Ave-bio10	Ave-Lipid10	Ave-bio15	Ave-Lipid15	Ave-bio20	Ave-Lipid20	Ave-bio25	Ave-Lipid25	Ave-bio30	Ave-Lipid30
0	6.81	10.98	6.79	10.95	6.76	10.90	6.79	10.95	6.8	10.97
12	8.23	13.27	8.62	13.90	8.83	14.24	8.42	13.58	8.6	13.87
24	10.31	16.63	9.99	16.11	10.54	17.00	9.97	16.08	10.03	16.18
36	11.26	18.16	11.76	18.97	15.23	24.56	15.13	24.40	15	24.19
48	12.49	20.15	13	20.97	18.17	29.31	18.03	29.08	18.6	30.00
60	12.24	19.74	14.27	23.02	21.79	35.15	20.14	32.48	20.73	33.44
72	12.18	19.65	14.32	23.10	21.77	35.11	20.16	32.52	20.69	33.37

pp sludge (g/L) sly	Ave-bio10	Ave-Lipid10	Ave-bio15	Ave-Lipid15	Ave-bio20	Ave-Lipid20	Ave-bio25	Ave-Lipid25	Ave-bio30	Ave-Lipid30
0	6.79	10.95	6.76	10.90	6.82	11.00	6.82	11.00	6.78	10.94
12	8.26	13.32	8.59	13.85	8.91	14.37	8.53	13.76	8.61	13.89
24	10.29	16.60	9.98	16.10	10.64	17.16	10.94	17.65	10.67	17.21
36	11.33	18.27	11.67	18.82	15.73	25.37	16.17	26.08	16.08	25.94
48	12.56	20.26	13.23	21.34	18.27	29.47	20.17	32.53	19.97	32.21
60	12.64	20.39	14.53	23.44	20.13	32.47	20.86	33.65	20.46	33.00
72	12.68	20.45	14.52	23.42	20.18	32.55	20.91	33.73	20.39	32.89

T.O	Ave-bio	Ave-lipid	sd	Ave-bio	Ave-lipid	sd
0	9.07	6.46	0.31	14.17	6.66	0.32
12	11.23	8.26	0.14	16.23	9.81	0.3
24	9.41	11.44	0.52	13.11	13.11	0.47
36	7.09	16.23	0.63	12.08	16.09	0.61
48	5.83	18.09	0.44	10.96	22.38	0.51
60	5.41	17.69	0.18	10.36	22.59	0.33
72	5	17.04	0.18	9.79	22.18	0.29

Ave-bio	Ave-lipid	sd	Ave-bio	Ave-lipid	sd	Ave-bio	Ave-lipid	sd
19.24	5.92	0.38	24.77	7.08	0.19	29.33	7.22	0.12
22.05	11.89	0.46	27.31	13.26	0.36	33.12	15	0.42
18.46	15.11	0.52	23.98	18.37	0.18	28.92	21.03	0.53
16.67	23.17	0.41	21.39	28.54	0.34	26.04	30.76	0.24
15.38	30.11	0.39	20.22	36.82	0.15	19.76	37.69	0.52
14.76	29.45	0.48	18.96	36.66	0.34	19.05	36.55	0.53
14.22	29.07	0.11	18.66	33.91	0.52	18.72	35.02	0.24

SS=10	AVE-pH 2	AVE-pH2 struvite	AVE-pH nature	AVE-ph n struvite	AVE-pH12	AVE-ph 12 struvite
0	6.09	5.88	6.34	6.17	5.19	5.33
12	10.05	9.46	8.43	8.29	9.16	9.24
18	11.76	12.09	11.23	10.66	12.43	12.69
24	13.91	14.23	12.76	11.83	14.17	14.88
36	16.28	17.24	15.23	15.42	18.23	18.1
42	18.21	17.83	16.14	16.35	19.15	19.01
48	18.21	18.02	17.09	16.85	19.09	18.92
60	18.05	17.64	16.83	16.43	18.67	18.47
66	17.73	17.32	16.55	16.15	18.03	18.34
72	17.44	17.11	16.24	16.12	17.64	17.92

SS=15	AVE-pH 2	AVE-pH2 struvite	AVE-pH nature	AVE-ph n struvite	AVE-pH12	AVE-ph 12 struvite
0	6.29	6.01	6.66	6.83	6.21	6.02
12	12.08	10.22	9.81	9.47	13.22	13.17
18	14.11	12.69	11.16	11.32	15.29	15.03
24	15.95	14.17	13.11	13	16.74	18.43
36	20.12	19.03	17.09	17.74	21.7	21.42
42	21.06	21.13	19.69	19.48	22.83	22.63
48	21.11	21.33	20.78	21	22.64	22.41
60	21.03	21.08	20.59	20.73	22.21	22.08
66	20.95	20.92	20.46	20.39	21.82	21.83
72	20.74	21.14	20.12	20.28	21.64	21.8

SS=20	AVE-pH 2	AVE-pH2 struvite	AVE-pH nature	AVE-ph n struvite	AVE-pH12	AVE-ph 12 struvite
0	6.12	6.02	5.92	6.13	5.89	6.09
12	13.18	12.67	11.89	12.28	15.92	14.87
18	18.25	17.55	16.24	17.16	22.14	18.73
24	21.35	22.79	20.11	19.34	26.19	24.89
36	27.31	27.94	25.34	25.01	31.47	29.84
42	29.73	29.58	28.38	28.56	32.64	32.17
48	30.7	30.47	30.11	30.01	32.57	32.09
60	30.64	30.32	29.45	29.87	32.43	31.84
66	30.32	30.19	29.11	29.76	32.19	31.73
72	30.12	30.04	29.07	29.43	32.22	31.62

SS=25	AVE-pH 2	AVE-pH2 struvite	AVE-pH nature	AVE-ph n struvite	AVE-pH12	AVE-ph 12 struvite
0	6.58	6.32	7.08	6.67	6.18	6.3
12	14.23	14.11	13.26	12.86	18.27	17.39
18	19.26	18.54	15.36	16.74	23.64	22.97
24	26.18	24.52	19.27	18.45	30.15	27.58
36	33.46	34.19	27.54	27.63	34.28	36.03
42	36.13	35.79	33.17	32.19	37.12	36.86
48	36.09	36.34	35.86	35.15	37.02	36.91
60	35.88	36.02	35.66	35.07	36.73	36.43
66	35.67	35.85	35.24	34.75	36.59	36.2
72	34.12	34.63	33.91	34.56	36.07	35.19

SS=30	AVE-pH 2	AVE-pH2 struvite	AVE-pH nature	AVE-ph n struvite	AVE-pH12	AVE-ph 12 struvite
0	6.6	6.65	7.22	6.83	6.57	6.21
12	17.32	16.49	15	14.29	21.09	16.39
18	22.17	21.58	19.46	17.64	26.53	25.47
24	29.32	27.39	24.18	20.52	30.42	31.16
36	35.12	34.29	31.76	28.49	36.59	36.01
42	37.07	36.93	34.18	33.78	38.76	37.21
48	37.07	36.93	36.29	35.23	38.8	37.19
60	36.81	36.51	36.55	36.05	38.64	36.87
66	36.47	36.07	36.09	35.82	38.43	36.1
72	36.12	36.02	35.13	35.46	37.25	35.76

ANNEXE 3: Cost estimation of sludge for biodiesel production

ANNEXE 3.1 Biodiesel from lipid from original sludge

EXECUTIVE SUMMARY (2011 prices)

==========

TOTAL CAPITAL INVESTMENT 7356000 $

CAPITAL INV. CHARGED TO THIS PROJECT 7356000 $

OPERATING COST 5285000 $/year

PRODUCTION RATE 9379990 kg/year of Biodiesel

UNIT PRODUCTION COST 0.530 $/kg of Biodiesel

TOTAL REVENUES 14223000 $/year

GROSS MARGIN 62.84 %

RETURN ON INVESTMENT 93.67 %

PAYBACK TIME 1.07 years

IRR AFTER TAXES 75.39 %

NPV (at 7.0 % interest) 48723000 $

==========

MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2011 prices)

==========

Quantity/ Description Unit Cost Cost

Stand-by ($) ($)

1/0 RDR-101 Rotary Dryer 100000 100000

Drum Diameter = 2.81 m

Drum Length = 14.04 m

1/0 gR-101 grinder 50000 50000

Rated Throughput = 10822.71 kg/h

1/0 MSX-101 Mixer-Settler Extractor 170000 170000

Rated Throughput = 66.9 m^3/h

Number of Stages = 1.0

1/0 EV-101 Evaporator 200000 200000

Number of Effects = 1

Area per Effect = 320.00 m^2

1/0 V-101 Receiver Tank 10000 10000

Volume = 250.39 L

Diameter = 0.47 m

3/0 V-102 Stirred Jacket Vessel 100000 300000

Volume = 35128.25 L

Diameter = 2.62 m

1/0 V-106 Stirred Jacket Vessel 100000 100000

Volume = 8202.30 L

Diameter = 1.61 m

1/0 V-107 Decanter Tank 8000 8000

Volume = 0.89 L

Diameter = 0.06 m

3/0 BC-103 Bowl Centrifuge 50000 150000

Rated Throughput = 0.00 L/min

1/0 EV-102 Evaporator 70000 70000

Number of Effects = 1

Area per Effect = 2.27 m^2

1/0 V-103 Decanter Tank 8000 8000

Volume = 37.67 L

Diameter = 0.21 m

1/0 C-101 Distillation Column 62000 62000

Number of Stages = 52

1/0 C-102 Distillation Column 57000 57000

Number of Stages = 49

Cost of Unlisted Equipment 143000

==========

TOTAL EQUIPMENT PURCHASE COST 1428000

==========

FIXED CAPITAL ESTIMATE SUMMARY (2011 prices)

==========

A. TOTAL PLANT DIRECT COST (TPDC) (physical cost)

1. Equipment Purchase Cost $ 1428000
2. Installation 826000
3. Process Piping 500000
4. Instrumentation 571000
5. Insulation 43000
6. Electricals 143000

7. Buildings 143000

8. Yard Improvement 71000

9. Auxiliary Facilities 143000

TPDC = 3867000

B. TOTAL PLANT INDIRECT COST (TPIC)

10. Engineering 773000

11. Construction 1160000

TPIC = 1933000

C. TOTAL PLANT COST (TPDC+TPIC) TPC = 5800000

12. Contractor's fee 290000

13. Contingency 580000

(12+13) = 870000

==========

D. DIRECT FIXED CAPITAL (DFC) TPC+12+13 = 6670000

==========

LABOR REQUIREMENT AND COST SUMMARY

==========

Section Labor Hours Labor Cost

Name Per Year $/year %

Main Section 52444 779000 100.00

TOTAL 52444 779000 100.00

==========

RAW MATERIALS COST SUMMARY

==========

Raw Unit Cost Annual Amount Cost

Material ($/kg) (kg) ($/yr) %

Methanol 0.004 83452646.30 350501 23.46

Hexane 0.005 158355258.24 791776 52.99

Acetone 0.004 81871967.68 294739 19.73

Sulfuric Acid 0.160 130765.23 20922 1.40

Sodium Hydroxid 0.340 106552.11 36228 2.42

Sludge 0.000 2152148907 0 0.00

TOTAL 2476066096.94 1494000 100.00

==========

UTILITY REQUIREMENTS (2011 prices)

==========

ELECTRICITY

Procedure Equipment Annual Amount Cost

Name Name (kWh) ($/yr)

P-4 gR-101 85716 5143

P-11 V-102 2574 154

P-15 V-106 2 0

P-7 BC-103 198000 11880

Unlisted Equipment 17893 1074

General Load 53680 3221

SUBTOTAL 21472

HEAT TRANSFER AGENT : Steam (4.2000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-6 MSX-101 5997564 25190

P-8 EV-101 328523806 1379800

P-12 EV-102 769841 3233

P-1 C-101 847929 3561

P-10 C-102 1293320 5432

SUBTOTAL 1417216

HEAT TRANSFER AGENT : Cooling Water (0.1000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-1 C-101 50798841 5080

P-10 C-102 61070729 6107

SUBTOTAL 11187

HEAT TRANSFER AGENT : Chilled Water (0.4000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-11 V-102 339345969 135738

P-7 BC-103 33922592 13569

SUBTOTAL 149307

==========

TOTAL 1599183

==========

ANNUAL OPERATING COST - SUMMARY (2011 prices)

==========

Cost Item $/Year %

Raw Materials 1 494 000 28.27

Labor-Dependent 779 000 14.74

Equipment-Dependent 1 296 000 24.52

Laboratory/QC/QA 117 000 2.21

Utilities 1 599 000 30.26

==========

TOTAL 5 285 000 100.00

==========

PROFITABILITY ANALYSIS (2011 prices)

==========

A. DIRECT FIXED CAPITAL $ 6670000

B. WORKING CAPITAL 352000

C. STARTUP COST 334000

D. TOTAL INVESTMENT (A+B+C) 7356000

E. INVESTMENT CHARGED TO THIS PROJECT 7356000

F. REVENUE STREAM FLOWRATES

kg/year of total flow (in solvent revover) 321 544 465

kg/year of total flow (in S2) 2 066 234 354

kg/year of total flow (in Na2SO4) 288 389

kg/year of total flow (in methanol rec.) 784 238

kg/year of total flow (in Biodiesel) 9 379 990

kg/year of total flow (in Glycerol) 985 919

G. PRODUCTION (UNIT) COST

$/kg of Biodiesel 0.530

H. SELLING/PROCESSING PRICE

$/MT of total flow (in solvent revover) 0.000

$/MT of total flow (in S2) 0.000

$/kg of total flow (in Na2SO4) 0.150

$/kg of total flow (in methanol rec.) 0.430

$/kg of total flow (in Biodiesel) 1.460

$/kg of total flow (in Glycerol) 0.150

I. REVENUES ($/year)

solvent revover 0

S2 0

Na2SO4 43000

methanol rec. 337000

Biodiesel 13695000

Glycerol 148000

---------- ----------

Total Revenues 14223000

J. ANNUAL OPERATING COST 5285000

K. GROSS PROFIT (I-J) 8938000

L. TAXES (30 %) 2682000

M. NET PROFIT (K-L + Depreciation) 6891000

==========

GROSS MARGIN 62.84 %

RETURN ON INVESTMENT 93.67 %

PAYBACK TIME (years) 1.07

==========

MT = Metric Ton = 1.000 kg

ANNEXE 3.2 Biodiesel from lipid from microorganism cultivated with sludge

EXECUTIVE SUMMARY (2011 prices)

==========

TOTAL CAPITAL INVESTMENT 32652000 $

CAPITAL INV. CHARGED TO THIS PROJECT 32652000 $

OPERATING COST 19946000 $/year

PRODUCTION RATE 31755817 kg/year of Biodiesel

UNIT PRODUCTION COST 0.628 $/kg of Biodiesel

TOTAL REVENUES 46478000 $/year

GROSS MARGIN 57.08 %

RETURN ON INVESTMENT 57.45 %

PAYBACK TIME 1.74 years

IRR AFTER TAXES 52.11 %

NPV (at 7.0 % interest) 124608000 $

==========

MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2011 prices)

==========

Quantity/ Description Unit Cost Cost

Stand-by ($) ($)

4/0 ST-101 Heat Sterilizer 50000 200000

Diameter = 10.00 m

Length = 0.10 m

30/0 V-101 Seed Fermentor 50000 1500000

Volume = 830000.00 L

Diameter = 7.06 m

35/0 BC-102 Bowl Centrifuge 50000 1750000

Sigma Factor = 109901.82 m^2

2/0 RDR-101 Rotary Dryer 50000 100000

Drum Diameter = 2.93 m

Drum Length = 14.63 m

1/0 MSX-101 Mixer-Settler Extractor 100000 100000

Rated Throughput = 144.4 m^3/h

Number of Stages = 1.0

2/0 EV-101 Evaporator 200000 400000

Number of Effects = 1

Area per Effect = 799.05 m^2

9/0 V-102 Stirred Jacket Vessel 100000 900000

Volume = 37761.33 L

Diameter = 2.68 m

1/0 EV-102 Evaporator 70000 70000

Number of Effects = 1

Area per Effect = 0.91 m^2

1/0 V-103 Decanter Tank 10000 10000

Volume = 547.99 L

Diameter = 0.52 m

3/0 V-104 Stirred Jacket Vessel 100000 300000

Volume = 32113.88 L

Diameter = 2.54 m

1/0 V-105 Decanter Tank 34000 34000

Volume = 641.55 L

Diameter = 0.55 m

1/0 C-101 Distillation Column 64000 64000

Number of Stages = 52

1/0 C-102 Distillation Column 94000 94000

Number of Stages = 52

Cost of Unlisted Equipment 614000

==========

TOTAL EQUIPMENT PURCHASE COST 6136000

==========

FIXED CAPITAL ESTIMATE SUMMARY (2011 prices)

==========

A. TOTAL PLANT DIRECT COST (TPDC) (physical cost)

1. Equipment Purchase Cost $ 6136000
2. Installation 3174000
3. Process Piping 2148000
4. Instrumentation 2454000
5. Insulation 184000
6. Electricals 614000
7. Buildings 614000
8. Yard Improvement 307000
9. Auxiliary Facilities 614000

TPDC = 16243000

B. TOTAL PLANT INDIRECT COST (TPIC)

10. Engineering 4061000

11. Construction 5685000

TPIC = 9746000

C. TOTAL PLANT COST (TPDC+TPIC) TPC = 25989000

12. Contractor's fee 1299000

13. Contingency 2599000

(12+13) = 3898000

==========

D. DIRECT FIXED CAPITAL (DFC) TPC+12+13 = 29887000

==========

LABOR REQUIREMENT AND COST SUMMARY

==========

Section Labor Hours Labor Cost

Name Per Year $/year %

Main Section 49651 1256000 100.00

TOTAL 49651 1256000 100.00

==========

RAW MATERIALS COST SUMMARY

==========

Raw Unit Cost Annual Amount Cost

Material ($/kg) (kg) ($/yr) %

Air 0.000 4514460023.39 0 0.00

Water 0.000 4929015062.51 0 0.00

Methanol 0.040 25569486.28 1022779 16.97

Chloroform 0.003 1575849091.73 4727547 78.45

Sodium Hydroxid 0.340 638965.67 217248 3.61

HCl 0.100 583056.18 58306 0.97

Dry sludge 0.000 79870709.16 0 0.00

TOTAL 11125986394.92 6026000 100.00

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UTILITY REQUIREMENTS (2011 prices)

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ELECTRICITY

Procedure Equipment Annual Amount Cost

Name Name (kWh) ($/yr)

P-4 BC-102 198000 11880

P-9 BC-103 198000 11880

P-11 V-102 8300 498

P-15 V-104 28 2

Unlisted Equipment 25270 1516

General Load 75811 4549

SUBTOTAL 30325

HEAT TRANSFER AGENT : steam (2.0000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-1 ST-101 42256362 84513

P-7 EV-101 1640650664 3281301

P-12 EV-102 932197 1864

P-3 C-101 5869490 11739

P-10 C-102 44150614 88301

SUBTOTAL 3467719

HEAT TRANSFER AGENT : Steam (4.2000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-5 MSX-101 19126194 80330

SUBTOTAL 80330

HEAT TRANSFER AGENT : Cooling Water (0.1000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-1 ST-101 2791451773 279145

P-3 C-101 229895232 22990

P-10 C-102 2086289815 208629

SUBTOTAL 510764

HEAT TRANSFER AGENT : Chilled Water (0.4000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-8 V-101 5290566383 2116227

P-4 BC-102 33922592 13569

P-9 BC-103 33922592 13569

P-11 V-102 1152404065 460962

SUBTOTAL 2604326

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TOTAL 6693463

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ANNUAL OPERATING COST - SUMMARY (2011 prices)

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Cost Item $/Year %

Raw Materials 6026000 30.21

Labor-Dependent 1256000 6.30

Equipment-Dependent 5782000 28.99

Laboratory/QC/QA 188000 0.94

Utilities 6693000 33.56

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TOTAL 19946000 100.00

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PROFITABILITY ANALYSIS (2011 prices)

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A. DIRECT FIXED CAPITAL $ 29887000

B. WORKING CAPITAL 1271000

C. STARTUP COST 1494000

D. TOTAL INVESTMENT (A+B+C) 32652000

E. INVESTMENT CHARGED TO THIS PROJECT 32652000

F. REVENUE STREAM FLOWRATES

kg/year of total flow (in recov. solvents) 1596908233

kg/year of total flow (in Recov. methanol) 1026200

kg/year of total flow (in Biodiesel) 31755817

kg/year of total flow (in Glycerol) 3338810

G. PRODUCTION (UNIT) COST

$/kg of Biodiesel 0.628

H. SELLING/PROCESSING PRICE

$/MT of total flow (in Recov. methanol) 40.000

$/kg of total flow (in Biodiesel) 1.460

$/MT of total flow (in Glycerol) 22.000

I. REVENUES ($/year)

Recov. methanol 41000

Biodiesel 46363000

Glycerol 73000

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Total Revenues 46478000

J. ANNUAL OPERATING COST 19946000

K. GROSS PROFIT (I-J) 26532000

L. TAXES (40 %) 10613000

M. NET PROFIT (L-K + Depreciation) 18758000

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GROSS MARGIN 57.08 %

RETURN ON INVESTMENT 57.45 %

PAYBACK TIME (years) 1.74

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MT = Metric Ton = 1.000 kg

ANNEXE 4: Lipid production from crude glycerol

time	Biomass growth 2.5% pure glycerc	Biomass growth 2.5	Biomass growth 2.5	average	sd
0	0.36	0.37	0.39	0.37	0.02
8	0.89	0.92	0.87	0.89	0.03
16	1.63	1.54	1.6	1.59	0.05
24	3.52	3.46	3.7	3.56	0.12
32	4.96	4.89	4.92	4.92	0.04
40	6.27	5.79	6.59	6.22	0.40
48	8.73	8.77	8.62	8.71	0.08
66	9.1	9.94	10.14	9.73	0.55
72	10.96	10.82	10.91	10.90	0.07
96	10.63	10.55	10.37	10.52	0.13
120	10.02	10.05	10	10.02	0.03

time	pure glycerol concentration (g/L)	pure glycerol conce	pure glycerol conce	Average	sd
0	24.46	23.87	23.42	23.92	0.52
8	23.87	22.18	23.02	23.02	0.85
16	20.45	20.38	20.46	20.43	0.04
24	16.76	16.77	16.76	16.76	0.01
32	13.46	13.52	13.55	13.51	0.05
40	10.47	10.49	10.41	10.46	0.04
48	4.19	5.22	6.13	5.18	0.97
66	3.02	3.02	3.06	3.03	0.02
72	0.17	0.23	0.25	0.22	0.04
96	0	0	0	0.00	0.00
120	0	0	0	0.00	0.00

time	pure glycerol Lipid content			average	sd
0	12.07	12.11	12.14	12.11	0.04
8					
16					
24	28.65	28.59	28.63	28.62	0.03
32					
40					
48	48.14	48.11	48.14	48.13	0.02
66	48.23	49.31	50.27	49.27	1.02
72	49.17	49.17	49.22	49.19	0.03
96	49.03	50	48.02	49.02	0.99
120	48.67	46.69	46.31	47.22	1.27

time	Biomass growth at 2.5% c	Biomass growth at 2.!	Biomass growth a average		sd
0	0.37	0.35	0.37	0.36	0.01
8	0.54	0.55	0.58	0.56	0.02
16	0.99	1.04	1.02	1.02	0.03
24	1.28	1.28	1.22	1.26	0.03
32	2.59	2.57	2.63	2.60	0.03
40	3.62	3.91	4.08	3.87	0.23
48	5.53	5.64	5.69	5.62	0.08
66	6.49	6.55	6.6	6.56	0.06
72	7.24	7.02	7.51	7.26	0.25
96	7.33	7.34	7.46	7.38	0.07
120	7.05	7.92	7.75	7.58	0.44

time	crude glycerol concentration (g/L)		average	st	
0	24.23	24.15	24.21	24.20	0.04
8	23.76	23.76	23.69	23.74	0.04
16	22.79	22.84	22.82	22.82	0.03
24	21.93	21.91	22.01	21.95	0.05
32	19.76	19.68	19.71	19.72	0.04
40	15.52	16.94	16.49	16.98	1.49
48	13.23	13.26	13.23	13.24	0.02
66	8.77	8.79	8.73	8.76	0.03
72	6.88	6.84	6.86	6.86	0.02
96	6.35	6.32	6.33	6.33	0.02
120	4.66	6.14	6.29	5.70	0.90

time	crude glycerol Lipid content		average	sd	
0	12.07	12.11	12.14	12.11	0.04
8					
16					
24	22.4	22.33	22.39	22.37	0.04
32					
40					
48	35.22	35.23	35.27	35.24	0.03
66	37.14	37.09	37.11	37.11	0.03
72	36.69	36.66	40.63	38.66	1.97
96	38.72	38.69	38.76	38.72	0.04
120	39.88	36.88	38.92	38.56	1.53

time	Biomass growth at 2	Biomass growth	Biomass growth at 2.5	average	sd
0	0.36	0.36	0.35	0.36	0.02
8	0.84	0.87	0.88	0.86	0.02
16	1.62	1.3	1.48	1.47	0.16
24	3.04	3.33	3.56	3.31	0.26
32	4.86	4.39	4.68	4.64	0.24
40	6.01	5.95	6.05	6.01	0.04
48	8.54	8.52	8.49	8.52	0.03
66	9.75	9.57	9.88	9.73	0.16
72	10.38	10.28	10.3	10.32	0.05
96	9.93	9.84	9.92	9.90	0.05
120	9.55	9.61	9.18	9.45	0.23

time	purified glycerol 25 concentration (g/L)			average	st
0	23.7	23.69	23.71	23.70	0.01
8	22.73	22.69	22.63	22.68	0.05
16	21.55	21.53	21.5	21.53	0.03
24	15.43	18.5	18.5	17.48	1.77
32	14.19	14.22	14.23	14.21	0.02
40	11.26	11.25	11.23	11.26	0.03
48	5.29	5.33	5.27	5.30	0.03
66	2.72	2.77	2.75	2.75	0.03
72	0.32	0.35	0.34	0.34	0.02
96	0	0	0	0.00	0.00
120	0	0	0	0.00	0.00

time	purified glycerol 25g/L Lipid content			average	sd
0	12.07	12.11	12.14	12.11	0.04
8					
16					
24	24.54	27.53	27.49	26.52	1.71
32					
40					
48	39.83	39.86	39.89	39.86	0.03
66	40.14	40.13	40.1	40.12	0.02
72	43.33	46.34	43.3	44.32	1.75
96	43.63	43.65	43.62	43.63	0.02
120	41.25	43.28	45.31	43.28	2.03

time	Biomass growt	Biomass growth	Biomass growt average		sd
0	0.35	0.37	0.36	0.36	0.01
8	0.93	0.97	0.91	0.94	0.03
16	1.63	1.65	1.59	1.62	0.03
24	3.46	3.51	3.49	3.49	0.03
32	4.87	4.91	4.83	4.87	0.04
40	6.31	6.45	6.01	6.26	0.22
48	8.67	8.64	8.66	8.66	0.02
66	9.83	9.93	10.28	10.01	0.24
72	9.74	10.69	11.81	10.75	1.04
96	10.93	10.99	10.94	10.95	0.03
120	11.1	11.07	11.07	11.08	0.02

time	purified glycerol 50 concentration (g/L)		average	st	
0	48.25	48.31	48.26	48.27	0.03
8	47.03	47.04	46.98	47.02	0.03
16	45.55	45.53	45.47	45.52	0.04
24	43.29	43.25	43.31	43.28	0.03
32	38.46	41.53	41.39	40.46	1.73
40	37.13	37.06	37.11	37.10	0.04
48	31.24	31.29	31.27	31.27	0.03
66	26.69	26.68	26.64	26.67	0.03
72	23.43	23.44	23.43	23.43	0.01
96	18.54	23.11	23.09	21.58	2.63
120	19.21	19.31	19.25	19.26	0.05

time	purified glycerol 50 g/l Lipid content		average	sd	
0	12.07	12.11	12.14	12.11	0.04
8					
16					
24	28.74	28.77	28.72	28.74	0.03
32					
40					
48	43.26	43.25	43.22	43.24	0.02
66	44.17	46.19	45.16	45.17	1.01
72	47.3	47.32	47.33	47.32	0.02
96	45.05	49.09	47.1	47.08	2.02
120	47.13	47.17	47.15	47.15	0.02

time	Biomass growth at 7.5	Biomass gro	Biomass	average	sd
0	0.36	0.34	0.37	0.36	0.02
8	0.75	0.83	0.59	0.72	0.12
16	1.05	1.1	1.08	1.08	0.03
24	1.81	1.89	1.92	1.87	0.06
32	2.9	2.94	2.87	2.90	0.04
40	4.11	4.15	4.13	4.13	0.02
48	5.06	5.21	4.99	5.09	0.11
66	6.44	6.86	6.59	6.63	0.21
72	8.51	8.82	8.38	8.57	0.23
96	9.43	9.43	9.45	9.44	0.01
120	9.59	9.6	9.64	9.61	0.03

time	purified glycerol 75 co	purified glyce	purified	average	st
0	75.22	75.29	75.26	75.26	0.04
8	74.26	74.35	74.29	74.30	0.05
16	73.49	73.45	73.42	73.45	0.04
24	71.55	71.49	71.46	71.50	0.05
32	69.04	69.01	68.98	69.01	0.03
40	67.17	67.13	67.15	67.15	0.02
48	64.03	63.96	63.99	63.99	0.04
66	60.17	60.15	60.19	60.17	0.02
72	56.21	53.26	53.23	54.23	1.71
96	49.46	50.42	50.41	50.10	0.55
120	48.29	47.33	49.31	48.31	0.99

time	purified glycerol 75 g/L	Lipid content		average	sd
0	12.07	12.11	12.14	12.11	0.04
8					
16					
24	29.74	29.69	29.77	29.73	0.04
32					
40					
48	40.33	45.32	44.29	43.31	2.63
66	44.11	47.17	47.14	46.14	1.76
72	48.06	48.09	48.13	48.09	0.04
96	48.12	48.2	48.17	48.16	0.04
120	48.26	48.27	48.26	48.26	0.01

time	Biomass growth at 10	Biomass growtl	Biomass gro	average	sd
0	0.37	0.35	0.36	0.36	0.01
8	0.62	0.66	0.65	0.64	0.02
16	0.88	0.89	0.89	0.89	0.01
24	1.31	1.35	1.29	1.32	0.03
32	2.27	2.31	2.25	2.28	0.03
40	3.15	3.22	3.27	3.21	0.06
48	3.73	4.09	4.36	4.06	0.32
66	5.19	4.93	4.62	4.91	0.29
72	5.67	5.69	5.63	5.66	0.03
96	6.36	6.38	6.42	6.39	0.03
120	5.96	6.97	6.51	6.48	0.51

time	purified glycerol 100 (	purified glycerc	purified glyc	average	st
0	91.46	91.52	91.5	91.49	0.03
8	90.89	90.81	90.82	90.84	0.04
16	89.97	89.92	89.88	89.92	0.05
24	88.67	88.59	88.62	88.63	0.04
32	86.39	86.35	86.39	86.38	0.02
40	84.13	84.05	84.12	84.10	0.04
48	82.92	82.97	82.96	82.95	0.03
66	80.79	80.74	80.77	80.77	0.03
72	78.84	78.82	78.79	78.82	0.03
96	77.25	77.19	77.23	77.22	0.03
120	76.53	76.62	76.62	76.59	0.05

time	purified glycerol 100g/L Lipid content			average	sd
0	12.07	12.11	12.14	12.11	0.04
8					
16					
24	29.05	29.03	29.03	29.04	0.01
32					
40					
48	42.17	42.17	42.23	42.19	0.03
66	45.46	45.44	45.39	45.43	0.04
72	44.66	49.14	49.18	47.66	2.60
96	47.82	47.82	47.88	47.84	0.03
120	47.91	47.93	47.87	47.90	0.03

ANNEXE 5 : Energy balance of biofuel production from crude glycerol

ANNEXE 5.1 Biodiesel production from crude glycerol

Items					
biomass concentration g biomass/g glyce	0.63				
lipid content	60%				
lipid extraction efficiency	0.95			11138.95851	
lipid yield (kg)	1000000				
80% crude glycerol demand (L)	3480924.534				
Total fermentation volume (m3)	35087.7193				
Biodiesel energy content (MJ/KG)	37.8				
fermentation	Amount (kg)	unit energy content (MJ/L)	power (KWH)	Energy (GJ)	
NH4-SO4	24561.40351	8.98			220.5614035
MgSO4	17543.85965	12.12			212.6315789
KH2PO4	35087.7193	10.3			361.4035088
HCl	11138.95851	1			11.13895551
crude glycerol 80% (100 g/L)	3480924.534	10.19			28376.4968
agitation				12280.70175	44.21052632
aeration				5044.889218	18.16160119
pumping				317.7357914	1.143859649
					29245.74823
Cell disruption	time (h)	flow rate (m3/h)	power (KWH)	Energy (GJ)	
bead milling (three pass)	1	6	526315.7895		1894.736842
					1894.736842
transesterification	Amount (ton)	unit energy content (MJ/kg)	power (KWH)	Energy (GJ)	
NaOH (2% w/w lipid)	20	18.5			370
Methanol	88.8624	22			1954.9728
mixing			27769.5		99.9702
heating					222.156
					2647.099
Purification	Amount (ton)	unit consumption (kJ/kg)	power (KWH)	Energy (GJ)	
drying		313.5			290.191275
Total energy					34077.77535
	amount (kg)	unit energy content (MJ/kg)			290.191275
Biomass	754385.9649	7.95			5997.368421
glycerol	129591	11.74			1521.39834
Biodiesel yield	925650	37.8			34989.57
					42508.33676
credites					7518.766761
net nergy input					26559.00859
energy output					34989.57
energy balance					8430.56141
energy ratio					1.317427564

ANNEXE 5.2 Hydrogen production from crude glycerol

		unit energy (MJ/kg)	power (KWH)	Energy (GJ)
crude glycerol amount (L)	3480924.534	10.19		28376.4968
glycerol content	2784739.627			
glycerol concentration (kg/m3	25			
fermentation volume (m3)	111389.5851			
yeast extract (1kg/m3) kg	111389.5851	6.46		719.5767197
K2HPO4 (4.6KG/M3) kg	512392.0914	10.3		5277.638541
water (m3)	111389.5851	0.05		5.569479254
HCL (3.2 mL/L 1MHCl)	11138958.51	1		11.13895851
agitation (0.35 kWh/m3)			38986.35478	140.3508772
NaOH (1.89 g /g CO2) kg	2807894.737	18.5		51946.05264
pumping (32.6 Kj/m3)				3.631300474
centrifuge (1 kWh/m3)			111389.5851	401.0025063
Total energy input				86881.45782
Biomass (13.8 g/L) kg	1537176.274	7.95		12220.55138
H2 (24.25 mol/kg glycerol) mo	67529935.96	122		16477.30437
CO2 (0.5 mol/mol H2) mol	33764967.98	0		0
total energy output				16477.30437
H2 1mol=22.41 L	1513345.865			
CO2 1 mol=22.4 L				
H2 1 mol=2 g	135.0598719			
	1485658.591			
Net energy input				74660.90644
energy balance				-58183.60207
Energy ratio				0.220695209

ANNEXE 5.3 Biogas production from crude glycerol

		unit energy (M power (KWH)		Energy (GJ)
crude glycerol amount (L)	3480924.534	10.19		28376.4968
glycerol content	2784739.627			
glycerol concentration (kg/m3)	10			
fermentation volume (m3)	278473.9627			
SRT (15d)	11603.08178			
fermenter volume (m3)	1000			
number of fermenter	11.60308178			
HCl (3.2 L/m3 1MHCl)	11138958.51	1		11.13895851
Water (1m3/m3 methane)	556947.9254	0.05		27.84739627
Agitation (0.4kWh/m3)			111389.5851	401.0025063
heating (1.16 kWh/m3/C)			3230297.968	11629.07268
pumping (0.2 kWh/m3)			55694.79254	200.5012532
others (0.05 kWh/m3)			13923.69814	50.12531329
sludge (50 g/L)	274993.0382			
dry sludge (tonne)	13923.69814			
Total energy input				43867.05654
		UNIT ENERGY (MJ/m3)		
Biogas yeild (200 m3/tonne glycero	556947.9254	21.43		11935.39404
biogas upgrading				1312.893345
sludge as fertilizer	222.7791702	8.34		1857.978279
residual sludge as fertilizer	44.55583404	8.34		371.5956559
net energy input				43495.46088
energy output				11935.39404
energy balance				-31560.06684
Energy ratio				0.274405508

ANNEXE 5.4 Ethanol production from crude glycerol

	kg	unit energy (MJ/kg)	power (KWH)	Energy (GJ)
crude glycerol amount (L)	3480924.534	10.19		28376.4968
glycerol content	2784739.627			
glycerol concentration (kg/m3)	10			
fermentation volume (m3)	278473.9627			
SRT (48 h)	1489.165576			
fermenter volume (m3)	1000			
number of fermenter	1.489165576			
HCl (3.2 L/m3 1MHCl)	11138958.51	1		11.13895851
Water	278473.9627	0.05		13.92369814
yeast (5g/L)	1392369.814	6.46		8994.708996
K2HPO4 (5 g/L)	1392369.814	10.3		14341.40908
agitation (0.35 kWh/m3)			97465.88695	350.877193
distillation (10.62 MJ/kg ethanol)				10942.35589
NaOH (1.89 g /g CO2) kg	257052.6316	18.5		4755.473685
Centrifuge (1 kWh/m3)			278473.9627	1002.506266
Total energy input				68788.89057
Biomass (4 g/L)	1113895.851	7.95		8855.472014
ethanol (0.37 g/g glycerol)	1030353.662	30		30910.60986
hydrogen (1.11 mmol/g glycerol)	3091060.986	122		377.1094403
CO2 (1.11 mmol/g glycerol)	3091060.986	0		0
Net energy input				59556.30911
Energy balance				-28645.69925
Energy ratio				0.519014867

ANNEXE 6: COST ESTIMATION OF BIODIESEL PRODUCTION FROM CRUDE GLYCEROL FERMENTATION

ANNEXE 6.1 Biodiesel production from crude glycerol (80% glycerol; <2% methanol)

EXECUTIVE SUMMARY (2013 prices)

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TOTAL CAPITAL INVESTMENT 1845000 $

CAPITAL INV. CHARGED TO THIS PROJECT 1845000 $

OPERATING COST 540000 $/year

PRODUCTION RATE 998709 kg/year of lipid (in S-110)

UNIT PRODUCTION COST 0.541 $/kg of lipid (in S-110)

TOTAL REVENUES 849000 $/year

GROSS MARGIN 36.33 %

RETURN ON INVESTMENT 25.65 %

PAYBACK TIME 3.90 years

IRR AFTER TAXES 16.02 %

NPV (at 7.0 % interest) 1126000 $

==========

MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2013 prices)

==========

Quantity/ Description Unit Cost Cost

Stand-by ($) ($)

2/0 V-102 Fermentor 165000 330000

Volume = 119214.72 L

Diameter = 3.70 m

1/0 V-107 Fermentor 44000 44000

Volume = 11072.34 L

Diameter = 1.67 m

2/0 BM-101 Bead Mill 49000 98000

Grinding Volume = 0.45 m^3

1/0 OS-101 Flotation Tank 9000 9000

Horizontal Area = 1.64 m^2

3/0 V-101 Receiver Tank 68000 204000

Volume = 71905.79 L

Diameter = 3.13 m

Cost of Unlisted Equipment 36000

==========

TOTAL EQUIPMENT PURCHASE COST 722000

==========

FIXED CAPITAL ESTIMATE SUMMARY (2013 prices)

==========

A. TOTAL PLANT DIRECT COST (TPDC) (physical cost)

1. Equipment Purchase Cost $ 722000

2. Installation 306000

3. Process Piping 144000

4. Instrumentation 130000

5. Insulation 22000

6. Electricals 72000

TPDC = 1397000

B. TOTAL PLANT INDIRECT COST (TPIC)

7. Engineering 140000

TPIC = 140000

C. TOTAL PLANT COST (TPDC+TPIC) TPC = 1536000

8. Contractor's fee 77000

9. Contingency 123000

(8+9) = 200000

==========

D. DIRECT FIXED CAPITAL (DFC) TPC+8+9 = 1736000

==========

LABOR REQUIREMENT AND COST SUMMARY

==========

Section Labor Hours Labor Cost

Name Per Year $/year %

Main Section 7920 158000 100.00

TOTAL 7920 158000 100.00

==========

RAW MATERIALS COST SUMMARY

==========

Raw Unit Cost Annual Amount Cost

Material ($/kg) (kg) ($/yr) %

---

wastewatersludg 0.000 57185.38 0 0.00

KH2PO4 1.200 1429.64 1716 68.56

Water 0.000 25479900.84 0 0.00

Yeast 2.000 285.93 572 22.85

glycerol (80% w 0.000 3668615.25 0 0.00

Magne Sulfate 0.350 348.83 122 4.88

Amm. Sulfate 0.130 714.82 93 3.71

TOTAL 29208480.68 3000 100.00

==========

UTILITY REQUIREMENTS (2013 prices)

==========

ELECTRICITY

Procedure Equipment Annual Amount Cost

Name Name (kWh) ($/yr)

P-2 V-102 755344 45321

P-1 V-107 16037 962

P-4 BM-101 72707 4362

Unlisted Equipment 52756 3165

General Load 158267 9496

SUBTOTAL 63307

HEAT TRANSFER AGENT : Cooling Water (0.1000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-2 V-102 113138750 11314

P-1 V-107 1988423 199

SUBTOTAL 11513

HEAT TRANSFER AGENT : Chilled Water (0.4000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-4 BM-101 29845392 11938

SUBTOTAL 11938

==========

TOTAL 86757

==========

ANNUAL OPERATING COST - SUMMARY (2013 prices)

==========

Cost Item	$/Year	%
Raw Materials	3000	0.46
Labor-Dependent	158000	29.31
Equipment-Dependent	269000	49.78
Laboratory/QC/QA	24000	4.40
Utilities	87000	16.05
TOTAL	540000	100.00

PROFITABILITY ANALYSIS (2013 prices)

A. DIRECT FIXED CAPITAL $ 1736000

B. WORKING CAPITAL 23000

C. STARTUP COST 87000

D. TOTAL INVESTMENT (A+B+C) 1845000

E. INVESTMENT CHARGED TO THIS PROJECT 1845000

F. REVENUE STREAM FLOWRATES

kg/year of lipid (in S-110) 998709

G. PRODUCTION (UNIT) COST

$/kg of lipid (in S-110) 0.541

H. SELLING/PROCESSING PRICE

$/kg of lipid (in S-110) 0.850

I. REVENUES ($/year)

S-110 849000

J. ANNUAL OPERATING COST 540000

K. GROSS PROFIT (I-J) 308000

O. NET PROFIT (K + Depreciation) 473000

==========

GROSS MARGIN 36.33 %

RETURN ON INVESTMENT 25.65 %

PAYBACK TIME (years) 3.90

==========

ANNEXE 6.2 Biodiesel production from crude glycerol (80% glycerol; 15% methanol)

EXECUTIVE SUMMARY (2013 prices)

==========

TOTAL CAPITAL INVESTMENT 1 118 000 $

CAPITAL INV. CHARGED TO THIS PROJECT 1 118 000 $

OPERATING COST 406 000 $/year

PRODUCTION RATE 1 005 896 kg/year of lipid (in S-110)

UNIT PRODUCTION COST 0.404 $/kg of lipid (in S-110)

TOTAL REVENUES 855 000 $/year

GROSS MARGIN 52.49 %

RETURN ON INVESTMENT 49.02 %

PAYBACK TIME 2.04 years

IRR AFTER TAXES 36.02 %

NPV (at 7.0 % interest) 2 658 000 $

==========

MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2013 prices)

==========

Quantity/ Description Unit Cost Cost

Stand-by ($) ($)

2/0 V-102 Fermentor 129 000 258 000

Volume = 80519.09 L

Diameter = 3.25 m

1/0 V-107 Fermentor 34 000 34 000

Volume = 7463.94 L

Diameter = 1.47 m

2/0 BM-101 Bead Mill 39 000 78 000

Grinding Volume = 0.30 m^3

1/0 OS-101 Flotation Tank 7 000 7 000

Horizontal Area = 1.10 m^2

2/0 V-101 Receiver Tank 20 000 40 000

Volume = 72405.05 L

Diameter = 3.13 m

Cost of Unlisted Equipment 22 000

==========

TOTAL EQUIPMENT PURCHASE COST 440 000

==========

FIXED CAPITAL ESTIMATE SUMMARY (2013 prices)

==========

A. TOTAL PLANT DIRECT COST (TPDC) (physical cost)

1. Equipment Purchase Cost $ 440 000
2. Installation 178 000
3. Process Piping 88 000
4. Instrumentation 79 000
5. Insulation 13 000
6. Electricals 44 000

TPDC = 841 000

B. TOTAL PLANT INDIRECT COST (TPIC)

7. Engineering 84 000

TPIC = 84 000

C. TOTAL PLANT COST (TPDC+TPIC) TPC = 926 000

8. Contractor's fee 46 000

9. Contingency 74 000

(8+9) = 120 000

==========

D. DIRECT FIXED CAPITAL (DFC) TPC+8+9 = 1 046 000

==========

LABOR REQUIREMENT AND COST SUMMARY

==========

Section Labor Hours Labor Cost

Name Per Year $/year %

Main Section 7 920 158 000 100.00

TOTAL 7 920 158 000 100.00

==========

RAW MATERIALS COST SUMMARY

==========

Raw Unit Cost Annual Amount Cost

Material ($/kg) (kg) ($/yr) %

---

wastewatersludg 0.000 204 000.00 0 0.00

Water 0.000 16 986 600.00 0 0.00

glycerol (80% w 0.000 2 445 750.00 0 100.00

TOTAL 19 636 350.00 0 100.00

==========

UTILITY REQUIREMENTS (2013 prices)

==========

ELECTRICITY

Procedure Equipment Annual Amount Cost

Name Name (kWh) ($/yr)

P-2 V-102 510 169 30 610

P-1 V-107 10 811 649

P-4 BM-101 72 706 4 362

Unlisted Equipment 37 105 2 226

General Load 111 316 6 679

SUBTOTAL 44 526

HEAT TRANSFER AGENT : Cooling Water (0.1000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-2 V-102 76 548 866 7 655

P-1 V-107 1 343 166 134

SUBTOTAL 7 789

HEAT TRANSFER AGENT : Chilled Water (0.4000 $/1000 kg)

Procedure Equipment Annual Amount Cost

Name Name (kg) ($/yr)

P-4 BM-101 24 148 346 9 659

SUBTOTAL 9 659

==========

TOTAL 61 975

==========

ANNUAL OPERATING COST - SUMMARY (2013 prices)

==========

Cost Item $/Year %

Raw Materials 0 0.00

Labor-Dependent 158 000 38.99

Equipment-Dependent 162 000 39.90

Laboratory/QC/QA 24 000 5.85

Utilities 62 000 15.26

==========

TOTAL 406 000 100.00

==========

PROFITABILITY ANALYSIS (2013 prices)

==========

A. DIRECT FIXED CAPITAL $ 1 046 000

B. WORKING CAPITAL 20 000

C. STARTUP COST 52 000

D. TOTAL INVESTMENT (A+B+C) 1 118 000

E. INVESTMENT CHARGED TO THIS PROJECT 1 118 000

F. REVENUE STREAM FLOWRATES

kg/year of lipid (in S-110) 1 005 896

G. PRODUCTION (UNIT) COST

$/kg of lipid (in S-110) 0.404

H. SELLING/PROCESSING PRICE

$/kg of lipid (in S-110) 0.850

I. REVENUES ($/year)

S-110 855 000

J ANNUAL OPERATING COST 406 000

K. GROSS PROFIT (I-J) 449 000

L. NET PROFIT (K + Depreciation) 548 000

==========

GROSS MARGIN 52.49 %

RETURN ON INVESTMENT 49.02 %

PAYBACK TIME (years) 2.04

ANNEXE 7: Utrasonication aided lipid extraction

Strain	*Trichosporon oleaginosus*								
kHz	**W**	**Solvent**	**Time**	**Temp C**	**Lipid cont.1**	**Lipid cont.2**	**Lipid cont.3**	**Average**	**Std Dev**
520	40	hexane	5	25	2.32	2.26	2.24	2.27	0.04
520	40	hexane	10	25	13.09	13.18	13.14	13.14	0.05
520	40	hexane	15	25	15.09	14.97	14.99	15.02	0.06
520	40	hexane	20	25	16.43	16.48	16.42	16.44	0.03
520	40	methanol	5	25	3.55	3.61	3.58	3.58	0.03
520	40	methanol	10	25	18.23	18.26	18.26	18.25	0.02
520	40	methanol	15	25	36.11	36.09	36.18	36.13	0.05
520	40	methanol	20	25	37.84	37.93	37.92	37.90	0.05
520	40	chlor/meth	5	25	18.86	18.86	18.81	18.84	0.03
520	40	chlor/meth	10	25	54.22	54.26	54.22	54.23	0.02
520	40	chlor/meth	15	25	60.37	60.4	60.4	60.39	0.02
520	40	chlor/meth	20	25	60.76	60.81	60.71	60.76	0.05
520	40	hexane	5	35	2.99	2.97	2.97	2.98	0.01
520	40	hexane	10	35	9.31	9.42	9.38	9.37	0.06
520	40	hexane	15	35	16.27	16.22	16.23	16.24	0.03
520	40	hexane	20	35	16.17	16.14	16.17	16.16	0.02
520	40	methanol	5	35	3.85	3.89	3.9	3.88	0.03
520	40	methanol	10	35	25.19	25.24	25.23	25.22	0.03
520	40	methanol	15	35	37.49	37.54	37.45	37.49	0.05
520	40	methanol	20	35	39.19	39.2	39.12	39.17	0.04
520	40	chlor/meth	5	35	21.54	21.52	21.59	21.55	0.04
520	40	chlor/meth	10	35	53.86	53.89	53.92	53.89	0.03
520	40	chlor/meth	15	35	60.38	60.42	60.46	60.42	0.04
520	40	chlor/meth	20	35	60.5	60.43	60.43	60.45	0.04
520	40	hexane	5	45	3.05	3.04	2.99	3.03	0.03
520	40	hexane	10	45	14.24	14.29	14.3	14.28	0.03
520	40	hexane	15	45	16.31	16.35	16.31	16.32	0.02
520	40	hexane	20	45	16.25	16.26	16.26	16.26	0.01
520	40	methanol	5	45	3.72	3.77	3.73	3.74	0.03
520	40	methanol	10	45	23.33	23.33	23.28	23.31	0.03
520	40	methanol	15	45	37.44	37.42	37.49	37.45	0.04
520	40	methanol	20	45	38.24	38.23	38.19	38.22	0.03
520	40	chlor/meth	5	45	18.75	18.74	18.73	18.74	0.01
520	40	chlor/meth	10	45	54.11	54.07	54.11	54.10	0.02
520	40	chlor/meth	15	45	58.76	58.79	58.73	58.76	0.03

520	40	chlor/meth	20	45	60.88	60.91	60.91	60.90	0.02
520	40	hexane	5	55	2.84	2.8	2.85	2.83	0.03
520	40	hexane	10	55	14.18	14.22	14.18	14.19	0.02
520	40	hexane	15	55	16.22	16.23	16.22	16.22	0.01
520	40	hexane	20	55	17.62	17.6	17.57	17.60	0.03
520	40	methanol	5	55	4.01	4.03	4.1	4.05	0.05
520	40	methanol	10	55	19.31	19.33	19.35	19.33	0.02
520	40	methanol	15	55	36.44	36.47	36.48	36.46	0.02
520	40	methanol	20	55	39.11	39.06	39.09	39.09	0.03
520	40	chlor/meth	5	55	22.45	22.48	22.53	22.49	0.04
520	40	chlor/meth	10	55	54.19	54.17	54.17	54.18	0.01
520	40	chlor/meth	15	55	60.55	60.5	60.54	60.53	0.03
520	40	chlor/meth	20	55	60.91	60.95	60.96	60.94	0.03
50	2800	hexane	5	25	5.22	5.32	5.25	5.26	0.05
50	2800	hexane	10	25	23.19	23.15	23.17	23.17	0.02
50	2800	hexane	15	25	25.12	25.12	25.13	25.12	0.01
50	2800	hexane	20	25	26.36	26.33	26.34	26.34	0.02
50	2800	methanol	5	25	6.17	6.13	6.13	6.14	0.02
50	2800	methanol	10	25	28.14	28.17	28.15	28.15	0.02
50	2800	methanol	15	25	43.19	43.17	43.14	43.17	0.03
50	2800	methanol	20	25	45.3	45.28	45.35	45.31	0.04
50	2800	chlor/meth	5	25	20.15	20.17	20.14	20.15	0.02
50	2800	chlor/meth	10	25	52.24	52.25	52.21	52.23	0.02
50	2800	chlor/meth	15	25	59.96	59.92	59.93	59.94	0.02
50	2800	chlor/meth	20	25	60.78	60.83	60.85	60.82	0.04
50	2800	hexane	5	35	7.45	7.48	7.53	7.49	0.04
50	2800	hexane	10	35	15.38	15.33	15.34	15.35	0.03
50	2800	hexane	15	35	25.76	25.79	25.79	25.78	0.02
50	2800	hexane	20	35	26.06	26.09	26.11	26.09	0.03
50	2800	methanol	5	35	13.57	13.58	13.51	13.55	0.04
50	2800	methanol	10	35	29.25	29.26	29.17	29.23	0.05
50	2800	methanol	15	35	44.41	44.42	44.49	44.44	0.04
50	2800	methanol	20	35	45.51	45.52	45.48	45.50	0.02
50	2800	chlor/meth	5	35	22.19	22.14	22.12	22.15	0.04
50	2800	chlor/meth	10	35	53.16	53.15	53.09	53.13	0.04
50	2800	chlor/meth	15	35	59.48	59.51	59.53	59.51	0.03
50	2800	chlor/meth	20	35	60.92	60.92	60.89	60.91	0.02
50	2800	hexane	5	45	7.62	7.61	7.65	7.63	0.02
50	2800	hexane	10	45	20.45	20.49	20.49	20.48	0.02
50	2800	hexane	15	45	25.85	25.81	25.76	25.81	0.05
50	2800	hexane	20	45	26.17	26.15	26.14	26.15	0.02

50	2800	methanol	5	45	13.61	13.62	13.62	13.62	0.01
50	2800	methanol	10	45	31.31	31.3	31.37	31.33	0.04
50	2800	methanol	15	45	43.91	43.92	43.96	43.93	0.03
50	2800	methanol	20	45	45.47	45.49	45.43	45.46	0.03
50	2800	chlor/meth	5	45	21.48	21.46	21.45	21.46	0.02
50	2800	chlor/meth	10	45	54.29	54.24	54.33	54.29	0.05
50	2800	chlor/meth	15	45	60.66	60.72	60.71	60.70	0.03
50	2800	chlor/meth	20	45	60.99	61.02	60.93	60.98	0.05
50	2800	hexane	5	55	7.24	7.23	7.25	7.24	0.01
50	2800	hexane	10	55	24.55	24.53	24.49	24.52	0.03
50	2800	hexane	15	55	26.14	26.21	26.16	26.17	0.04
50	2800	hexane	20	55	26.93	26.91	26.93	26.92	0.01
50	2800	methanol	5	55	14.18	14.17	14.14	14.16	0.02
50	2800	methanol	10	55	28.38	28.36	28.45	28.40	0.05
50	2800	methanol	15	55	45.22	45.26	45.21	45.23	0.03
50	2800	methanol	20	55	46.19	46.15	46.22	46.19	0.04
50	2800	chlor/meth	5	55	24.24	24.21	24.24	24.23	0.02
50	2800	chlor/meth	10	55	54.72	54.68	54.67	54.69	0.03
50	2800	chlor/meth	15	55	60.52	60.55	60.55	60.54	0.02
50	2800	chlor/meth	20	55	60.91	60.9	60.94	60.92	0.02

Strain	SKF-5								
kHz	W	Solvent	Time	Temp C	Lipid cont.1	Lipid cont.2	Lipid cont.3	Average	Std Dev
520	40	hexane	5	25	1.59	1.67	1.66	1.64	0.04
520	40	hexane	10	25	6.77	6.68	6.7	6.72	0.05
520	40	hexane	15	25	7.05	7.03	6.98	7.02	0.04
520	40	hexane	20	25	7.45	7.48	7.53	7.49	0.04
520	40	methanol	5	25	2.54	2.61	2.58	2.58	0.04
520	40	methanol	10	25	12.43	12.45	12.49	12.46	0.03
520	40	methanol	15	25	13.17	13.17	13.12	13.15	0.03
520	40	methanol	20	25	13.08	13.09	13.11	13.09	0.02
520	40	chlor/meth	5	25	12.01	12.08	12.09	12.06	0.04
520	40	chlor/meth	10	25	19.81	19.82	19.84	19.82	0.02
520	40	chlor/meth	15	25	31.19	31.18	31.15	31.17	0.02
520	40	chlor/meth	20	25	32.51	32.55	32.51	32.52	0.02
520	40	hexane	5	35	1.71	1.69	1.74	1.71	0.03
520	40	hexane	10	35	6.76	6.83	6.82	6.80	0.04
520	40	hexane	15	35	6.92	6.89	6.91	6.91	0.02
520	40	hexane	20	35	7.56	7.58	7.52	7.55	0.03
520	40	methanol	5	35	2.63	2.65	2.65	2.64	0.01
520	40	methanol	10	35	16.71	16.68	16.74	16.71	0.03
520	40	methanol	15	35	19.19	19.17	19.25	19.20	0.04
520	40	methanol	20	35	21.26	21.23	21.21	21.23	0.03
520	40	chlor/meth	5	35	9.03	9.05	9.1	9.06	0.04
520	40	chlor/meth	10	35	23.19	23.21	23.16	23.19	0.03
520	40	chlor/meth	15	35	32.37	32.35	32.33	32.35	0.02
520	40	chlor/meth	20	35	33.91	33.87	33.95	33.91	0.04
520	40	hexane	5	45	1.53	1.52	1.57	1.54	0.03
520	40	hexane	10	45	6.84	6.89	6.92	6.88	0.04
520	40	hexane	15	45	7.21	7.26	7.19	7.22	0.04
520	40	hexane	20	45	7.63	7.66	7.61	7.63	0.03
520	40	methanol	5	45	2.54	2.51	2.57	2.54	0.03
520	40	methanol	10	45	12.82	12.85	12.78	12.82	0.04
520	40	methanol	15	45	18.16	18.14	18.19	18.16	0.03
520	40	methanol	20	45	20.31	20.37	20.31	20.33	0.03
520	40	chlor/meth	5	45	9.44	9.47	9.53	9.48	0.05
520	40	chlor/meth	10	45	25.54	25.58	25.57	25.56	0.02
520	40	chlor/meth	15	45	32.48	32.51	32.47	32.49	0.02
520	40	chlor/meth	20	45	33.19	33.16	33.16	33.17	0.02
520	40	hexane	5	55	1.51	1.6	1.56	1.56	0.05
520	40	hexane	10	55	6.94	6.92	6.97	6.94	0.03
520	40	hexane	15	55	7.22	7.27	7.17	7.22	0.05

520	40	hexane	20	55	7.46	7.44	7.51	7.47	0.04
520	40	methanol	5	55	2.77	2.71	2.72	2.73	0.03
520	40	methanol	10	55	14.58	14.57	14.62	14.59	0.03
520	40	methanol	15	55	18.33	18.32	18.28	18.31	0.03
520	40	methanol	20	55	20.33	20.36	20.36	20.35	0.02
520	40	chlor/meth	5	55	10.33	10.35	10.31	10.33	0.02
520	40	chlor/meth	10	55	27.27	27.26	27.3	27.28	0.02
520	40	chlor/meth	15	55	33.26	33.27	33.31	33.28	0.03
520	40	chlor/meth	20	55	34.21	34.19	34.26	34.22	0.04
50	2800	hexane	5	25	2.16	2.17	2.19	2.17	0.02
50	2800	hexane	10	25	9.44	9.41	9.45	9.43	0.02
50	2800	hexane	15	25	11.09	11.06	11.14	11.10	0.04
50	2800	hexane	20	25	11.23	11.27	11.22	11.24	0.03
50	2800	methanol	5	25	3.31	3.36	3.31	3.33	0.03
50	2800	methanol	10	25	17.64	17.63	17.68	17.65	0.03
50	2800	methanol	15	25	22.09	22.15	22.05	22.10	0.05
50	2800	methanol	20	25	22.31	22.32	22.37	22.33	0.03
50	2800	chlor/meth	5	25	12.76	12.73	12.71	12.73	0.03
50	2800	chlor/meth	10	25	25.66	25.61	25.71	25.66	0.05
50	2800	chlor/meth	15	25	32.48	32.48	32.51	32.49	0.02
50	2800	chlor/meth	20	25	33.19	33.16	33.15	33.17	0.02
50	2800	hexane	5	35	1.21	1.2	1.24	1.22	0.02
50	2800	hexane	10	35	9.55	9.62	9.57	9.58	0.04
50	2800	hexane	15	35	10.88	10.86	10.86	10.87	0.01
50	2800	hexane	20	35	11.26	11.28	11.24	11.26	0.02
50	2800	methanol	5	35	3.42	3.43	3.46	3.44	0.02
50	2800	methanol	10	35	17.75	17.73	17.78	17.75	0.03
50	2800	methanol	15	35	22.22	22.25	22.19	22.22	0.03
50	2800	methanol	20	35	22.29	22.31	22.27	22.29	0.02
50	2800	chlor/meth	5	35	14.27	14.26	14.25	14.26	0.01
50	2800	chlor/meth	10	35	26.72	26.76	26.71	26.73	0.03
50	2800	chlor/meth	15	35	31.38	31.36	31.38	31.37	0.01
50	2800	chlor/meth	20	35	34.09	34.07	34.14	34.10	0.04
50	2800	hexane	5	45	1.32	1.38	1.3	1.33	0.04
50	2800	hexane	10	45	9.71	9.73	9.73	9.72	0.01
50	2800	hexane	15	45	11.01	11.05	10.98	11.01	0.04
50	2800	hexane	20	45	11.31	11.37	11.34	11.34	0.03
50	2800	methanol	5	45	3.64	3.65	3.61	3.63	0.02
50	2800	methanol	10	45	18.03	17.97	17.94	17.98	0.05
50	2800	methanol	15	45	22.19	22.21	22.17	22.19	0.02
50	2800	methanol	20	45	22.37	22.29	22.31	22.32	0.04

50	2800	chlor/meth	5	45	15.3	15.31	15.27	15.29	0.02
50	2800	chlor/meth	10	45	28.16	28.16	28.19	28.17	0.02
50	2800	chlor/meth	15	45	32.87	32.84	32.85	32.85	0.02
50	2800	chlor/meth	20	45	34.27	34.29	34.31	34.29	0.02
50	2800	hexane	5	55	1.26	1.23	1.21	1.23	0.03
50	2800	hexane	10	55	9.66	9.64	9.67	9.66	0.02
50	2800	hexane	15	55	10.96	10.92	10.91	10.93	0.03
50	2800	hexane	20	55	11.38	11.42	11.37	11.39	0.03
50	2800	methanol	5	55	3.89	3.84	3.91	3.88	0.04
50	2800	methanol	10	55	18.19	18.15	18.17	18.17	0.02
50	2800	methanol	15	55	22.21	22.24	22.26	22.24	0.03
50	2800	methanol	20	55	22.38	22.34	22.31	22.34	0.04
50	2800	chlor/meth	5	55	14.99	14.96	14.95	14.97	0.02
50	2800	chlor/meth	10	55	28.46	28.51	28.49	28.49	0.03
50	2800	chlor/meth	15	55	34.11	34.19	34.17	34.16	0.04
50	2800	chlor/meth	20	55	34.28	34.23	34.22	34.24	0.03