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Production de biodiesel à partir d'huiles microbiennes accumulées dans *Trichosporon oleaginosus ATCC 20905* cultivées dans un mélange de glycérol brut et des boues d'épuration

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RÉSUMÉ

En tant qu'énergie propre et renouvelable, le biodiesel est une alternative prometteuse au diesel. Au cours des dernières décennies, la production de biodiesel augmente rapidement. De nos jours, la production de biodiesel est limitée aux matières premières (huiles végétales et graisses animales). Afin d'augmenter la production de biodiesel, des matières premières alternatives sont nécessaires.

La plupart des micro-organismes peuvent stocker des huiles microbiennes (lipides mono-cellulaires) dans les cellules sous forme d'énergie. Certains micro-organismes ont la capacité d'accumuler une très forte teneur en lipides (masse lipidique/masse sèche) et les lipides sont similaires aux huiles végétales. Les chercheurs accordent une grande attention à la production de biodiesel à partir de lipides. Cependant, la production de biodiesel à partir de lipides n'est pas économiquement viable en raison du coût élevé du substrat, de la faible production de lipides, ce qui limite le processus de production de lipides à grande échelle. De plus, la corrélation entre l'utilisation de substrats, la production de biomasse et la production de lipides n'est toujours pas clairement mise en évidence.

Dans cette étude, des boues d'épuration et du glycérol brut à faible coût ont été étudiés pour la production de lipides à l'aide de la levure oléagineuse *Trichosporon oleaginosus*. Le glycérol brut utilisé dans cette étude contenait une teneur élevée en savon (26,80% p/p) mais une faible teneur en glycérol (15,05% p/p), qui était différente des recherches rapportées dans les littératures. Afin de bien comprendre la possibilité d'utiliser ce glycérol brut pour la production de lipides, les expériences ont d'abord été menées dans un milieu synthétique de glycérol brut. Après l'amélioration et le développement des méthodes de production de lipides dans le glycérol brut, des boues d'épuration ont été utilisées (comme milieu de culture) pour remplacer le milieu synthétique. Des expériences ont été conduites dans des boues d'épuration enrichies avec du glycérol brut. Les résultats obtenus à partir des boues d’épuration enrichies avec du glycérol brut ont ensuite été utilisés pour l'étude de l'équilibre énergétique, l'émission de gaz à effet de serre et l'estimation des coûts pour évaluer la faisabilité de la pratique industrielle de la

production du biodiesel à partir des lipides en utilisant les méthodes développées dans cette étude.

Dans le milieu synthétique de glycérol brut, le rapport C/N optimal, qui était le paramètre le plus important pour la production des teneurs élevées en lipides, a été étudié. Le rapport C/N 45 a été trouvé optimal pour la production de lipides en utilisant *T. Oleaginosus*, cependant, le rapport C/N 30 montrait une production de lipides comparable. Après l'étude du rapport C/N, l'étude cinétique a été réalisée. Contrairement aux recherches précédentes, la concentration en azote a été jugée critique pour la production élevée et rapide de biomasse, une fermentation «Fed-batch», qui était basée sur l'étude cinétique, a ensuite été effectuée. Une production élevée de biomasse de 43,82 g/L et une production élevée de lipides de 21,87 g/L avec une très forte productivité lipidique de 0,42 g/L/h ont été obtenues lors de la fermentation fed-batch.

Le méthanol, qui était le composant principal du glycérol brut, était fatal (à forte concentration) à la croissance cellulaire de la plupart des micro-organismes. La tolérance au méthanol était différente entre les espèces de micro-organismes. *T. oleaginosus* avait une tolérance au méthanol plus élevée que les contaminants (principalement des bactéries). Ainsi, la concentration de méthanol a été ajustée pour la production des lipides en utilisant *T. oleaginosus* dans des conditions non stériles. À la concentration de méthanol de 1,4% p/v, la croissance des contaminants a été inhibée et la croissance de *T. oleaginosus* n'a pas été fortement affectée. La production de lipides à l'aide de *T. oleaginosus* pouvait être réalisée dans des conditions non stériles avec l'aide d'une concentration de méthanol de 1,4% p/v. Une fermentation discontinue dans des conditions non stériles a été réalisée. Une production de biomasse de 43,39 g/L et une production de lipides de 20,42 g/L ont été obtenues, lesquelles concentrations étaient légèrement inférieures à celles obtenues dans la fermentation stérile.

Le savon était le composant secondaire dans le glycérol brut. Il a été constaté que le pH, qui déterminait la concentration de savon dans le milieu, était le paramètre le plus important. Bien que pH 7 ait été optimal pour la croissance de *T. oleaginosus* dans le milieu YPD, la concentration élevée de savon à pH 7 a inhibé significativement la croissance de *T. oleaginosus* lorsque du glycérol brut a été utilisé comme milieu de

fermentation. pH 5 a été trouvé suffisant pour convertir le savon en acide gras libre afin d'éviter l'inhibition du savon. Une fermentation discontinue basée sur le contrôle du pH, qui pourrait être auto-contrôlée, a été réalisée. La production de biomasse et la production de lipides étaient de 65,63 g/L et de 35,79 g/L, respectivement.

Basé sur la loi de la conservation de l'énergie, l'énergie était constante dans un système isolé. Chaque cellule oléagineuse de *T. oleaginosus* peut être considérée comme un système isolé. Par la suite, un nouveau modèle thermodynamique de production de lipides utilisant *T. oleaginosus* a été établi. Une nouvelle équation mathématique a été établie, qui était absolument différente du modèle actuel de Lueding-Piret. La corrélation entre l'utilisation des substrats, la production de biomasse et la production de lipides a été redéfinie.

Les méthodes et les stratégies, qui ont été réalisées dans le milieu synthétique de glycérol brut, ont ensuite été introduites dans un mélange de la boue avec de glycérol brut. Les résultats ont montré que toutes les méthodes développées dans le milieu de glycérol brut pourraient être parfaitement appliquées dans le mélange de la boue avec de glycérol brut.

Le bilan énergétique, les émissions de gaz à effet de serre et l'estimation des coûts ont été étudiés pour évaluer la faisabilité de la production de biodiesel à partir de lipides accumulés par *T. oleaginosus* cultivé dans le milieu composé de boues et de glycérol brut.

L'étude a montré que la production de biodiesel à partir de lipides accumulés par *T. oleaginous* dans le milieu composé de boues et de glycérol brut était faisable et applicable pour des opérations industrielles.

ABSTRACT

As a clean and renewable energy, biodiesel is a promising alternative to diesel. In the last decades, biodiesel production grows rapidly. Nowadays, the biodiesel production is limited to the available feedstocks (vegetable oils and animal fats). In order to increase the biodiesel production, additional and abundant feedstocks are necessary for biodiesel production.

Most of the microorganisms can store microbial oils (also recognized as single cell oils or lipids) in the cells as energy. Some microorganisms have the ability to accumulate very high lipid content (lipid/dry cell mass) and the lipids are found similar to vegetable oils. Researchers pay great attention to produce biodiesel from lipids. However, biodiesel production from lipids is still not economically feasible due to high substrate cost, low lipid production, complicating lipid production and extraction process. In addition, the correlation of substrate utilization, biomass production and lipid production is still unclear.

In this study, free cost sewage sludge and low-cost crude glycerol were investigated for lipid production using oleaginous yeast *Trichosporon oleaginosus*. The crude glycerol used in this study contained high soap content but low glycerol content which was quite different from previous researches done in our lab and reported by others. In order to fully understand the possibility of using this special crude glycerol for lipid production, the experiments were firstly conducted in crude glycerol synthetic medium. After amelioration and development of the lipid production methods in the crude glycerol, sewage sludge was employed to replace the synthetic medium and experiments were conducted in sewage sludge fortified with crude glycerol medium. The results obtained from the culture medium containing sludge and crude glycerol were then used for energy balance, greenhouse gas emission and cost estimation to evaluate the feasibility of industrial practice for the lipid production using the methods developed in this study.

In the crude glycerol synthetic medium, the optimal C/N ratio for lipid production was investigated because C/N ratio was the most important parameter for high lipid production. It was found that the C/N ratio of 45 was optimal for lipid production using *T. oleaginosus*, however, the C/N ratio of 30 showed the comparable lipid production. The results

confirmed that low nitrogen content in the culture media favors the accumulation of lipid in *T. oleaginosus*. Following the C/N ratio investigation, the kinetic study was performed. Different from the previous researches, the nitrogen concentration was found critical for the high and fast biomass production in this study. A fed-batch fermentation was then developed based on the kinetic study, high biomass production of 43.82 g/L and high lipid production of 21.87 g/L with a very high lipid productivity of 0.42 g/L/h were simultaneously achieved in 52 hours of fed-batch fermentation.

Methanol, which was the primary component of crude glycerol, was found fatal to the cell growth of most of the microorganisms at a high concentration. The tolerance toward methanol was different among microorganisms. *T. oleaginosus* had a higher methanol tolerance than the contaminants (mainly bacteria in sludge). Thus, methanol concentration was manipulated to produce lipid using *T. oleaginosus* in non-sterile conditions. At methanol concentration of 1.4% (w/v), the growth of the contaminants was inhibited and the growth of *T. oleaginosus* was not highly affected. It was approved that lipid production using *T. oleaginosus* could be accomplished in non-sterile conditions with the assistance of methanol concentration at 1.4% (w/v). The fed-bath fermentation in non-sterile conditions was performed. Biomass production of 43.39 g/L and lipid production of 20.42 g/L were obtained within 60 hours of the fermentation which were slightly less than those from the sterile fed-batch fermentation.

Soap was the secondary component in the crude glycerol. It was found that pH, which determined the soap concentration in the medium, was the most important parameter for high lipid production. Even though pH 7 was optimal for the growth of *T. oleaginosus* in yeast- peptone -dextrose (YPD) medium; the high soap concentration at pH 7 significantly inhibited the growth of *T. oleaginosus* when crude glycerol was used as fermentation medium. Therefore, pH 5 was found necessary to convert soap to free fatty acid (FFA) in order to avoid the soap inhibition. A fed-batch fermentation based on pH, which could be auto-controlled, was designed. The biomass production and lipid production were 65.63 g/L and 35.79 g/L, respectively, from the pH based fed-batch fermentation.

Based on the law of conservation of energy, the energy was constant, which could only be transformed but neither be generated nor be destroyed, in an isolated system.

Accordingly, each oleaginous cell of *T. oleaginosus* was considered as an isolated system. Thus, the energy in the cell should maintain constantly. Thereafter, a new thermodynamic model of lipid production using *T. oleaginosus* was established. The new mathematic equation was absolutely different from the current Luedking–Piret model. The correlation of substrate utilization, biomass production and lipid production was redefined.

The methods and strategies for high lipid production with the less operational process, which were done in crude glycerol synthetic medium, were then introduced into the sludge fortified with crude glycerol medium. The results showed that all the methods developed in crude glycerol medium had very good applicability in sludge fortified with crude glycerol medium.

Energy balance, greenhouse gas emissions, and cost estimation were studied to evaluate the feasibility of biodiesel production from lipid accumulated by *T. oleaginosus* cultivated in sludge with crude glycerol medium.

The study showed that biodiesel production from lipid accumulated by *T. oleaginosus* in sludge and crude glycerol medium was feasible for industrial practice.

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ABRÉVIATIONS

SCO : Single cell oil

G7 : Group of seven

GHG : Greenhouse Gas

GHE: Greenhouse effect

FAMEs: Fatty Acid Methyl Esters

FFA: Free Fatty Acid

C1: One carbon

GC-MS: Gas Chromatograph –Mass Spectrometer

SS: Total Suspended Solid

TB: Total biomass

LFB: Lipid-free biomass

YPD: Yeast Extract Peptone Dextrose Medium

WWTS: Wastewater treatment sludge

INTRODUCTION

En 2015, le G7 (un groupe de 7 pays comprenant le Canada, la France, l'Allemagne, l'Italie, le Japon, le Royaume-Uni et les États-Unis) a conclu un accord pour éliminer complètement la consommation de combustibles fossiles à la fin de ce siècle. Les tendances mondiales actuelles sont désormais orientées vers le remplacement des combustibles fossiles par de nouvelles énergies.

Les nouvelles énergies comprennent l'énergie solaire, l'énergie géothermique, l'énergie hydraulique, l'énergie éolienne, l'énergie marine, les biocarburants et l'énergie nucléaire. L'abondance et la disponibilité de l'énergie solaire, de l'énergie géothermique, de l'énergie hydroélectrique, de l'énergie éolienne et de l'énergie marine sont déterminées par l'emplacement géographique. Pour transporter ces énergies vers d'autres endroits, il faut mettre en place des installations de base. Les pertes d'énergie pendant le transport et la distribution sont inévitables. Afin d'éviter les grandes constructions et la perte d'énergie, l'énergie nucléaire est considérée comme le premier choix pour le remplacement des combustibles fossiles dans les endroits où d'autres énergies nouvelles sont difficiles à obtenir. Cependant, les deux graves accidents nucléaires (l'explosion de la centrale nucléaire de Tchernobyl et la fuite de la centrale nucléaire de Fukushinia) ont freiné l’interet grandissant de l’utilisation de l'énergie nucléaire. Comparé aux autres énergies nouvelles, le biocarburant a des avantages attrayants inoffensif, abondant, renouvelable et durable (détails au chapitre 2).

Le biocarburant est le carburant qui est produit par des processus biologiques. Le biocarburant contient principalement du bioéthanol, du biodiesel et du biogaz. La production de biogaz par digestion a été développée depuis 1800. En raison de la grande variation des matières premières, il n'y a pas encore de technologie établies et mature pour sa production stable. Ainsi, produire du biogaz à grande échelle reste difficile (détails au chapitre 2). En 2014, la production mondiale de biocarburants s'élevait à 70792 milliers de tonnes (en équivalent pétrole). Parmi ceux-ci, seulement 1% était du biogaz et le reste de 99% était du bioéthanol et du biodiesel (U.S. Department of Energy, 2015). La forte production de bioéthanol et de biodiesel est due à leur caractère additif de carburant. Le

bioéthanol et le biodiesel peuvent être directement utilisés par les moteurs actuels dans certains mélanges. Le mélange d'innocuité du bioéthanol et du biodiesel est de 5% à 25% et de 5% à 20% (v/v), respectivement.

Au Canada, le mélange actuel de bioéthanol à l'essence et de biodiesel au diesel est de 5% (v/v). En fait, le mélange peut aller jusqu'à 20% (v/v), ce qui est toujours sans danger. Cependant, la production de bioéthanol et de biodiesel au Canada est inférieure à la demande. Même s'il existe un grand besoin de production de bioéthanol et de biodiesel, les cultures agricoles devraient d'abord être utilisées pour satisfaire la demande alimentaire pour les humains et les animaux, et le reste d'entre eux peut être utilisé pour produire du biocarburant. À l'heure actuelle, les cultures disponibles au Canada ont été autant utilisées pour la production de bioéthanol que de biodiesel.

Le bioéthanol ou le biodiesel est produit à partir de cultures agricoles. En raison de la faible disponibilité des matières premières, le coût de la production de bioéthanol et de biodiesel augmente progressivement. Il a été rapporté que le coût de production du bioéthanol et du biodiesel était similaire, d'où leur prix de détail est également très similaire (U.S. Department of Energy, 2015). Comparé au bioéthanol, la production de biodiesel présente deux avantages évidents. Lorsque le bioéthanol et le biodiesel sont produits à partir du soja, selon John Manuel (Manuel et al., 2007), le biodiésel fournit un bilan énergétique net positif de 93% par rapport au bioéthanol, ce qui donne un bilan énergétique net positif de seulement 25%. En outre, le biodiesel a réduit les émissions de dioxyde de carbone de 41% par rapport à celles du diesel, mais le bioéthanol ne réduit que de 12% les émissions de dioxyde de carbone par rapport à l'essence. Il suggère que la production de biodiesel peut générer plus d'énergie nette et réduire plus des émissions de gaz à effet de serre que la production du bioéthanol lorsque le même type de matière (ou biomasse) est utilisé (Hill et al., 2006). Comme le gain énergétique net et l'émission de gaz à effet de serre sont les deux facteurs les plus importants pour évaluer la faisabilité d'une nouvelle énergie, le biodiesel est supérieur au bioéthanol en tant que nouvelle énergie. Ainsi, la production de biodiesel prend progressivement le pas sur la production de bioéthanol. En 2011, 80% biocarburant était de l'éthanol et 20% du biodiesel; en 2014, 45% du biocarburant était de l'éthanol et 55% du biodiesel (Shikida et al., 2014).

Le biodiesel, connu sous le nom d'ester méthylique d'acide gras, est utilisé comme additif diesel en raison de sa chaîne carbonée similaire (les chaînes carbonées du biodiesel sont C12 à C22, les chaînes carbonées du diesel sont C6 à C21). Pour les produits chimiques organiques, les caractéristiques chimiques et physiques sont fortement déterminées par les chaînes carbonées, les chaînes carbonées du biodiesel étant dans la gamme des chaînes carbonées du diesel, le biodiesel peut donc être directement utilisé comme additif diesel.

Même si le biodiesel est un bon additif pour le diesel et qu'il y a une forte demande pour produire de plus en plus de biodiesel, la production de biodiesel est limitée en raison de la disponibilité des cultures agricoles en ce moment. Il y a un besoin intense des matières premières alternatives pour la production de biodiesel. Les matériaux devraient être abondants. En outre, le biodiesel produit à partir de nouveaux matériaux devrait gagner de l'énergie nette et réduire l'émission de gaz à effet de serre.

Au Canada, 7% des émissions de gaz à effet de serre sont attribuables aux déchets organiques, comparativement à 25% des combustibles fossiles (Espinosa-Gonzalez et al., 2014). Pendant des siècles, les micro-organismes ont été utilisés pour traiter ou réduire les déchets organiques avant d'être rejetés. Il a été rapporté qu'un groupe de micro-organismes pouvait accumuler une forte concentration d'huiles microbiennes dans leurs cellules lorsqu'ils étaient cultivés dans des déchets organiques. De plus, ces huiles microbiennes sont similaires aux huiles de graines végétales (Cortes et al., 2015). Il indique que ces huiles microbiennes peuvent être utilisées pour produire du biodiesel. Par rapport à la production d'huiles végétales, la production d'huiles microbiennes présente de nombreux avantages : un cycle de vie court, un taux d'accumulation rapide, aucune terre cultivable, facile à contrôler, une large distribution et non affectée par le climat. Il a également été révélé que l'utilisation des huiles microbiennes pour la production du biodiesel pourrait permettre un gain énergétique net et une réduction des émissions de gaz à effet de serre (Zhang et al., 2013). Toutes ces preuves suggèrent que l'utilisation d'huiles microbiennes comme matière première pour la production du biodiesel est un moyen prometteur de résoudre la limitation de la production de biodiesel à partir d'huiles de graines végétales.

Afin de réduire le coût du biodiesel produit à partir d'huiles microbiennes, substrats à faible coût pour la culture de micro-organismes oléagineux, la production d'huile microbienne élevée et le processus moins compliqué étaient favorables. Ainsi, la cinétique de la consommation de substrats, de la production de biomasse et de la production de lipides était nécessaire à la compréhension de leur corrélation et à la recherche des voies permettant d'améliorer la production de lipides. Pour évaluer la faisabilité du procédé, l'émission de gaz à effet de serre, le bilan énergétique et l'estimation des coûts ont été utiles avant l'établissement d'une installation industrielle.

PARTIE I : SYNTHÈSE

1 SYNTHÈSE

1.1 Revue de littérature

1.1.1 Biodiesel

Le biodiesel est l'ester monoalkylique d'acide gras à chaîne longue converti à partir d'huiles végétales ou de graisses animales. Le biodiesel est actuellement utilisé comme additif au diesel. B20 (20% de biodiesel avec 80% de diesel), B5 (5% de biodiesel avec 95% de diesel) et B2 (2% de biodiesel avec 98% de diesel) sont disponibles dans le commerce.

1.1.2 La production actuelle de biodiesel

Le biodiesel est généré par une réaction chimique appelée trans-estérification. Une mole d'huiles ou de graisses réagit avec trois moles d'alcool pour former 3 moles de biodiesel et 1 mole de glycérol en présence d'un catalyseur. Le méthanol est le réactif le plus utilisé dans la pratique en raison de son faible prix. Plusieurs types de catalyseurs tels que la base, l'acide, l'enzyme et les catalyseurs hétérogènes peuvent être utilisés (Lee et al., 1995). Base (NaOH) est le catalyseur le plus couramment utilisé dans le processus de production de biodiesel en raison du faible coût en comparant avec l'enzyme et des catalyseurs hétérogènes, et à haute efficacité par rapport aux acides (Canakci et al., 2008). Cependant, lorsque les acides gras libres dans les huiles de charge sont supérieurs à 0,5% (p/p), l'acide peut être utilisé comme catalyseur pour éliminer la formation de savon (Cvengros et al., 2004; Zhang et al., 2003; Srividya et al., 2011). Le processus de production de biodiesel est illustré à la Figure 1.1.

Tout type d'huile ou de graisse peut être utilisé dans le processus de production de biodiesel. Mais comme les matières premières comptent environ 50-70% du coût de production, les huiles ou graisses ont un impact important sur les coûts de production (Morais et al., 2010; Liang et Jiang, 2013). Les huiles de canola, de maïs, de coton, de lin, de jatropha, d'arachide, de colza, de sésame, de soja, de tournesol ou de graisses de poulet, d'huile de poisson, de saindoux et de suif peuvent être utilisées pour la production

de biodiesel (Meng et al., 2009; Tongprawhan et al., 2014; Converti et al., 2009). Actuellement, le soja, la graine de tournesol et le canola (colza) sont les principales sources d'huiles pour la production de biodiesel en raison de leur disponibilité constante et de leur grande facilité de transformation. Cependant, presque la quantité maximale de cultures (35% de la graine de canola et du soja) après avoir satisfait la demande humaine a été utilisée pour produire du biodiesel au Canada. Néanmoins, il ne peut toujours pas satisfaire toute la demande de biodiesel. Depuis 2013, le Canada a commencé à importer de plus en plus de biodiesel provenant d'autres pays. Il est urgent de trouver d'autres ressources en plus des huiles agricoles pour produire du biodiesel.

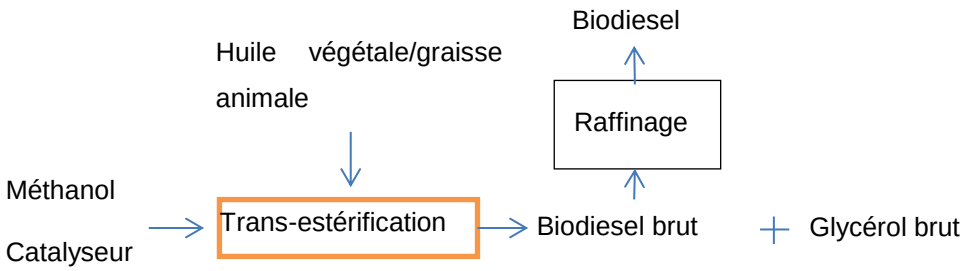

Figure 1. 1 Le processus de la production actuelle de biodiesel

1.1.3 La production de biodiesel à partir d'autres ressources

1.1.3.1 Graisses animales

Les graisses animales sont une autre matière première couramment utilisée pour la production de biodiesel. Similaires aux huiles végétales, ils sont en grande partie nécessaires pour la production des aliments. La production de graisses animales est presque équilibrée avec leur demande alimentaire, ce qui signifie que la source de graisses est limitée après avoir satisfait à la demande de produits alimentaires. De plus, les graisses animales ont généralement un taux de saturation ou une teneur en acides gras libres élevé (> 50% p/p), ce qui entraînerait une viscosité élevée et un faible rendement en biodiesel, respectivement (Lee et al., 1995; Canakci et Sanli, 2008; Sanford et al., 2009).

1.1.3.2 Huiles de cuisson usées

Les huiles de cuisson usées proviennent des cuisines et des activités de transformation des aliments. Comme il s'agit d'un déchet, le biodiesel produit à partir d'huiles usagées réduirait les coûts de production (Zhang et al., 2003; Cvengros et Cvengrosová, 2004; Morais et al., 2010; Srividya et al., 2011). Il peut également atténuer la forte dépendance à l'égard des huiles végétales et des graisses animales pour la production de biodiesel. D'un autre côté, il peut résoudre le problème de l'élimination des huiles de cuisson usagées. L'utilisation d'huiles de cuisson usagées a montré une voie prometteuse pour la production de biodiesel. Cependant, la qualité des huiles de cuisson usées est une préoccupation majeure dans la production de biodiesel car elles contiennent des impuretés telles que de petites particules et certains ingrédients alimentaires. De plus, la quantité d'huiles de cuisson usées est impossible de satisfaire la demande de production de biodiesel.

1.1.3.3 Huiles unicellulaires de microbes oléagineux

Les huiles monocellulaires sont des huiles microbiennes (ou lipides), qui sont accumulés dans les cellules microbiennes en tant que source d'énergie. Les microbes, qui peuvent accumuler plus de 20% de lipides (lipides/les cellules sèches), sont appelés micro-organismes oléagineux (Liang et Jiang, 2013). Les micro-organismes oléagineux comprennent les bactéries, les champignons, les levures et les micro-algues. Certains de ces micro-organismes oléagineux pourraient accumuler une très forte teneur en lipides dans la cellule lorsqu'ils sont cultivés dans des conditions optimales (Meng et al., 2009; He et al., 2015; Singh et al., 2015; Han et al., 2015). Comparé aux graines de plantes telles que le soja, le tournesol, le canola, etc. qui contiennent environ 25 à 35% d'huile, le micro-organisme oléagineux (teneur en lipides jusqu'à 70%) est un remplacement prometteur de la matière première traditionnelle (huiles vegetables). De plus, la composition en acides gras de l'huile microbienne est similaire à celle des huiles végétales (Liu et al., 2015; Zhang et al., 2014; Ryu et al., 2013; Ykema et al., 1988). Une fois que les huiles microbiennes sont extraites des cellules, elles peuvent être directement utilisées pour produire du biodiesel par un procédé similaire à la production de biodiesel à partir d'huiles végétales. La teneur élevée en lipides des micro-organismes

oléagineux et la facilité d'utilisation directe des huiles microbiennes pour produire du biodiesel ont rendu la production de biodiesel à partir d'huiles microbiennes très attrayante.

1.1.4 La Production de biodiesel à partir d'huiles microbiennes

La production de biodiesel à partir d'huiles microbiennes comprend quatre étapes: i) un pré-traitement avant la culture des micro-organismes oléagineux, ii) la culture de micro-organismes oléagineux, iii) la récolte des micro-organismes oléagineux et l'extraction des huiles microbiennes, et iv) la production de biodiesel par trans-estérification (Figure 1.2).

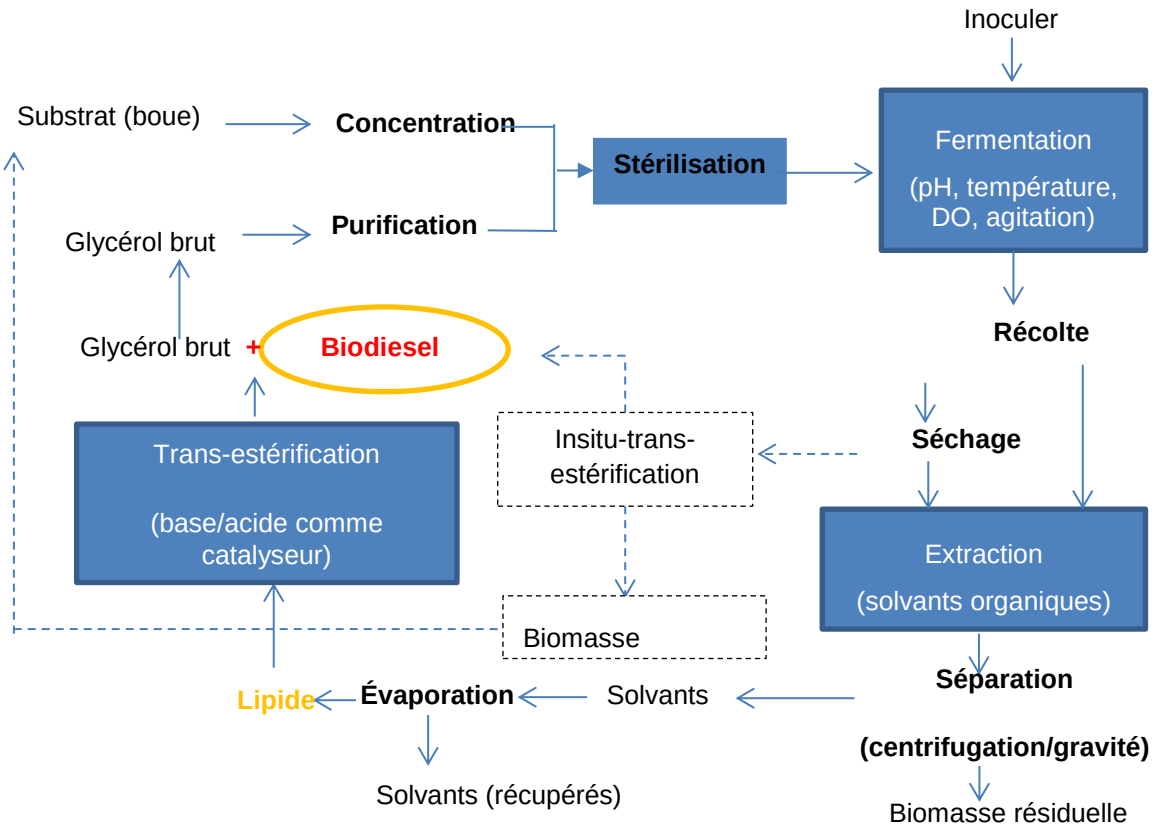

Figure 1. 2 Schéma de production de biodiesel à partir d'huiles microbiennes

Les trois premières étapes du processus de production de biodiesel à partir d'huiles microbiennes visent à produire des huiles microbiennes pour la trans-estérification finale et elles sont essentielles à l'ensemble du processus.

Le pré-traitement du substrat était pour rendre le carbone disponible pour la culture de micro-organismes oléagineux. Selon les matières premières utilisées pour la culture, le pré-traitement peut contenir plusieurs parties. Dans cette étude, les boues d'épuration secondaires et le glycérol brut seront utilisés pour la culture (voir ci-dessous). Les principaux pré-traitements des boues secondaires sont la concentration et la stérilisation, et le pré-traitement principal du glycérol brut est la purification (Hejna et al., 2016; Kong al., 2016).

La culture permet aux micro-organismes oléagineux d'accumuler des huiles microbiennes élevées dans les cellules. Normalement, les paramètres tels que le pH, la température, l'aération et l'agitation doivent être contrôlés dans les conditions optimales pour la croissance rapide du micro-organisme oléagineux (details au chapitre 2).

La récolte de micro-organismes oléagineux et l'extraction d'huiles microbiennes permettent d'obtenir des huiles microbiennes en séparant la biomasse et des lipides. La récolte de micro-organismes oléagineux contient principalement la floculation, la sédimentation, la centrifugation et (ou) le séchage; l'extraction des huiles microbiennes contient l'extraction des lipides et la purification des lipides (Zhang al., 2014). En plus de la méthode traditionnelle, une nouvelle méthode appelée trans-estérification in-situ peut être introduite pour convertir directement la biomasse sèche ou moins humide en biodiesel sans l'extraction d'huiles microbiennes (Go et al., 2016; Zhang et al., 2016).

Le processus de production de biodiesel à partir d'huiles microbiennes est plus compliqué (Figure 1.2) que le procédé de production de biodiesel utilisant des huiles végétales (Figure 1.1), ce qui conduit à un investissement élevé et un coût élevé du biodiesel (Koutinas et al., 2014; Hussain et al., 2016).

En raison du coût élevé, la production de biodiesel à partir d'huiles microbiennes est encore limitée à l'échelle du laboratoire (Benemann et al., 2011; Amanor-Boadu et al., 2014; Santander et al., 2014). Un minimum de 10 US $ est nécessaire pour produire un gallon de biodiesel à partir d'huiles microbiennes accumulées par la levure oléagineuse *Rhodosporidium toruloides* alimentée par le milieu synthétique de glucose. Le coût du biodiesel produit à partir d'huiles végétales n'était que de 3 à 4 US $ par gallon (Davis et al., 2011; Delrue et al., 2012; Ramos et al., 2014). Le coût élevé de biodiesel produit à

partir d'huiles microbiennes est affecté de trois parties: le processus d'opération complexe, le coût élevé du substrat (glucose) et la faible production microbienne d'huile (normalement moins de 20 g/L) (Yang et al., 2015; Béligon et al., 2016; Taskin et al., 2016; Anschau, 2017).

1.1.4.1 Coût élevé du substrat

Les micro-organismes oléagineux autotrophes (micro-algues) utilisent la lumière du soleil pour convertir le dioxyde de carbone en lipide, ce qui nécessite un coût nul pour les besoins en carbone. Cependant, la culture nécessite une grande surface terrestre et l'accumulation d'huile microbienne est lente (Meng et al., 2009; Bellou et al., 2014).

Les micro-organismes hétérotrophes oléagineux sont des promotteurs des huiles microbiennes en raison de leur capacité à accumuler une forte teneur en lipides et un taux de croissance rapide (Ykema et al., 1988; Meng et al., 2009; Kim et al., 2010a; Gao et al., 2013). Cependant, ils ont besoin de sources de carbone (sucres) et d'autres nutriments comme matières premières. La source de carbone et les nutriments de haute qualité sont coûteux, ce qui est la principale cause du coût élevé de la production de biodiesel à partir d'huiles microbiennes (Koutinas et al., 2014). Afin de réduire le coût production de biodiesel, une source alternative de carbone et de nutriments (bon marché ou sans frais supplémentaire) est souhaitable pour l'alimentation de micro-organismes oléagineux hétérotrophes.

Les déchets organiques sont gratuits ou offrent un certain crédit, ce qui peut fortement réduire le coût de la production de biodiesel à partir d'huiles microbiennes. Les déchets organiques sont normalement riches en carbone et/ou en azote et en phosphore, qui sont significativement importants pour la croissance du micro-organisme oléagineux. Par conséquent, les déchets organiques présentent des avantages évidents par rapport à la source de carbone de haute qualité. Actuellement, les déchets organiques sont traités par une série de processus biologique avant d'être rejetés. Cependant, le processus de traitement biologique est coûteux et les sous-produits (boues) peuvent encore causer une pollution grave. Par conséquent, les déchets organiques devraient être directement utilisés pour la culture de micro-organismes oléagineux. L'utilisation directe des déchets organiques pour la culture de micro-organismes oléagineux peut éviter leur rejet et

pollution environnementale. En attendant, le produit (boue) qui contient des huiles microbiennes peut être utilisé pour produire du biodiesel. Déchets organiques incluant les déchets agricoles, industriels et résidentiels (détails au chapitre 2).

Les déchets agricoles sont principalement constitués de résidus de bois et de cultures. Les principales compositions de déchets agricoles sont des fibres (60 à 90%), qui sont difficiles à utiliser par la plupart des microbes.

Les déchets organiques industriels sont généralement hydrolysés et thermolysés au cours du processus de production, ce qui améliore leur biodégradabilité. Cela signifie que les déchets organiques industriels peuvent être directement utilisés pour la culture de micro-organismes oléagineux.

Parmi tous les déchets organiques industriels, le glycérol brut est spécial car il est un sous-produit inévitable de la production de biodiesel par trans-estérification. En 2014, environ 50 millions de tonnes de glycérol brut ont été générées au Canada. Une attention particulière a été accordée à la bonne gestion du glycérol brut, car il n'a presque aucune valeur. Dans une solution de glycérol brut, le savon, le glycerol et le méthanol peuvent être facilement pris par les micro-organismes oléagineux. L'utilisation de glycérol brut comme substrat pour la culture de micro-organismes oléagineux est intéressant car ce procédé gère le sous-produit du processus de production de biodiesel et produit également plus d'huiles microbiennes pour la production de biodiesel. Des chercheurs ont découvert que la levure oléagineuse *Trichosporon oleaginosus* a la capacité d'utiliser du glycérol brut comme substrat pour accumuler une très forte teneur en lipides (lipides/biomasse sèche) (Kitcha et Cheirsilp, 2011; Polburee et al., 2015; Yen et al., 2015; Dobrowolski et al., 2016). Les lipides (huiles microbiennes) accumulés dans les cellules pourraient être utilisés comme matières premières pour produire du biodiesel par trans-estérification. Cela fait du processus de production de biodiesel un cercle vert: la production de biodiesel génère une grande quantité de glycérol brut; le glycérol brut est ensuite utilisé pour alimenter les micro-organismes oléagineux afin d'accumuler les lipides; les lipides sont utilisés pour produire du biodiesel. Dans ce cercle, le glycérol brut peut être éliminé et plus de biodiesel peut être généré. Ce grand avantage attire l'attention sur l'utilisation de glycérol brut comme principale source de carbone pour la culture de

Trichosporon oleaginosus. Néanmoins, le glycérol brut ne contient pas tous les nutriments nécessaires à la croissance du micro-organisme oléagineux. Des nutriments chimiques ou d'autres déchets doivent être ajoutés. Maintenant, les chercheurs se concentrent sur l'utilisation de milieu semi-synthétique de glycérol brut pour la culture de micro-organismes oléagineux (Tableau 1.1). Cependant, la préparation de milieu semi-synthétique de glycérol brut est difficile. Il est préférable de combiner le glycérol brut avec d'autres déchets organiques, qui peuvent fournir des nutriments neccessaires pour la croissance du micro-organisme oléagineux.

Tableau 1. 1 Milieu synthétique de glycérol brut pour la culture de micro-organismes oléagineux

Microorganism	**Milieu synthétique (par litre)**	**Culture**	**C/N**	**Concentration de biomasse (g/L)**	**concentration de Lipide (g/L)**	**Réf.**
Rhodotorula glutinis	30g glycérol 2 g extrait de levure, 2 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g$MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$ et 0.1 g NaCl	pH 5.1 24°C	18.5	8.71	4.61	(Yen et al., 2012)
T. spathulata	40 g glycérol; 10 g extrait de levure 10 g pepthone	pH6.0 24 °C	17	10.40	4.45	(Kitcha, S. et B. Cheirsilp, 2011)
Cryptococcus curvatus (Trichosporon oleaginosus)	20 g glycérol 2.7 g KH_2PO_4; 0.95 g Na_2HPO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g extrait de levure; 4 g $CaCl_2 \cdot 2H_2O$; 0.55 g $FeSO_4 \cdot 7H_2O$; 0.10 g $ZnSO_4 \cdot 7H_2O$; 0.076 g $MnSO_4 \cdot H_2O$; et 100 µl 18 M H_2SO_4.	pH 5.5 28°C	30	31.2	13.8	(Liang et al., 2010)
R. mucilaginosaDiSVA C7.1	20 g glycérol 10 g extrait de levure; 20 g pepthone	25°C	2.7	25	NA	(Taccari et al., 2012)

R. glutinis TISTR 5159	100 g glycérol 10 g glucose, 5 g pepthone , 3 g extrait de levure et 3 g extrait de malt	30°C pH6.0	60	5.70	2.30	(Saenge et al., 2011)
Candida sp	30.0 g/L glycérol, 7.0 g/L KH_2PO_4, 2.5 g/L Na_2HPO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.15 g/L $CaCl_2$, 0.15 g/L $FeCl_3 \cdot 6H_2O$, 0.02 g/L $ZnSO_4 \cdot 7H_2O$, 0.06 g/L $MnSO_4 \cdot H_2O$, 0.5 g/L $(NH_4)_2SO_4$ et 0.5 g/L extrait de levure	pH 6.0 28 °C	75	19.7g/L	9.9g/L	(Duarte et al., 2013)

La boue municipale, qui est largement générée dans la station d'épuration municipale, est l'une des principaux déchets organiques résidentiels. Le principal composant des boues d'épuration municipales est des cellules de micro-organismes, qui sont considérées rationnelles pour la reproduction des micro-organismes.

Dans notre laboratoire, les boues d'épuration ont été utilisées comme matières premières pour cultiver des micro-organismes oléagineux *T. oleaginosus* pour la production de lipides (Zhang et al., 2011; Zhang et al., 2014). Un pré-traitement (hydrolyzation) des boues d'épuration a été réalisé pour améliorer leur biodégradabilité. Il a été découvert que la hydrolyzation alcalino-thermique était le moyen le plus efficace d'améliorer la production de lipides (Zhang et al., 2014). Cependant, la production de lipides était limitée par la limitation de la source de carbone (Zhang et al., 2014). Une tendance similaire a également été observée par d'autres chercheurs lorsque la boue était utilisée comme un nutriment pour la culture de micro-organismes oléagineux (Deeba et al., 2016; Liu et al., 2016). Le glycérol purifié a été ajouté au milieu de boue pour augmenter la source de carbone (Xu et al., 2012; Yang al., 2014). La combinaison des boues d'épuration et du glycérol a considérablement augmenté la production de biomasse et l'accumulation de

lipides (Angerbauer et al., 2008; Zhang et al., 2014; Ma et al., 2016). Ainsi, la combinaison de boues et de glycérol brut sera utilisée pour la culture de la levure oléagineuse *Trichosporon oleaginosus* dans cette étude.

La combinaison des boues et de glycérol purifiée a été bien étudiée pour la culture de levure oléagineuse *T. oleaginosus*. La cinétique de l'utilisation du glycérol et sa relation avec la production de biomasse et l'accumulation de lipides ont été bien déterminées par les chercheurs précédents (Papanikolaou et Aggelis, 2002). Puisque chaque composant du substrat a sa cinétique d'utilisation unique, et le glycérol brut est un mélange de trois substrats (savon ou acide gras libre, méthanol, glycérol) qui est différent du glycérol pur. Comme présenté dans le Tableau 1.1, il a montré que la levure oléagineuse, cultivée avec du milieu synthétique de glycérol brut, produit normalement une faible production de biomasse et une faible accumulation de lipides. Par conséquent, les études, qui visent à augmenter la production de biomasse et l'accumulation de lipides, doivent être effectuées en utilisant le glycerol brut comme la culture.

1.1.4.2 Processus d'opération compliqué

La production des huiles microbiennes (pré-traitement des substrats, culture de micro-organismes, récolte de micro-organismes et extraction des lipides) est très compliquée et nécessite une haute comsuption d'énergie et un investissement coûteux en équipement.

Dans cette étude, la levure oléagineuse *Trichosporon oleaginosus* sera cultivée dans des boues melangé avec du glycérol brut, les boues d'épuration serviront d'éléments neccessaires pour la croissance de la souche et le glycérol brut sera de fournir du carbone supplémentaire pour l'accumulation d'huile microbienne. Cependant, il y a un pré-traitement inévitable avant la culture de la souche, qui est la stérilisation. La stérilisation a généralement lieu à 121 ° C pendant 15 minutes. La stérilisation nécessite un apport énergétique élevé et un équipement de haute qualité. En comparaison, il a été rapporté que certaines algues oléagineuses peuvent accumuler une forte teneur en lipides dans la cellule sans aucun pré-traitement, ce grand avantage est l'un des facteurs importants qui ont fait de la production de biodiesel à partir d'huiles microbiennes produites par les algues dans la pratique industrielle.

Afin de rattraper les recherches sur la production d'huile microbienne produites par algues oléagineuses, il faut étudier des méthodes qui peuvent produire de l'huile microbienne en utilisant de la levure oléagineuse sans stérilisation. Des études ont révélé que le méthanol peut favoriser ou inhiber la croissance des micro-organismes en faisant varier sa concentration (Chen et al., 1976). De plus, chaque souche a sa tolérance différente de la concentration en méthanol (Dewez et al., 2003; Salakkam et Webb, 2015). La détermination de la plage de concentration en methanol pour inhiber la croissance de contaminants et favoriser concomitamment la croissance de la souche productrice de lipides, peut fournir un moyen de produire des lipides dans les conditions non stérilisées.

Le glycérol brut, qui sera utilisé comme source de carbone dans cette étude, est un mélange de glycérol, méthanol, savon, eau, etc. Le méthanol dans le glycérol brut peut être utilisé pour éviter l'étape de stérilisation de la levure oléagineuse.

1.1.4.3 Faible production de lipides

En plus d'appliquer des déchets organiques pour réduire le coût, l'approche qui concerne principalement l'augmentation de l'accumulation de lipides (huiles microbiennes) peut également réduire les coûts de production des lipides.

Les lipides accumulés dans les cellules oléagineuses sont affectés par le nombre de cellules (ou la concentration de biomasse) et la teneur en lipides des cellules. La condition idéale est d'obtenir plus de cellules qui accumulent une forte teneur en lipides. Ainsi, l'augmentation de l'accumulation de lipides peut être effectuée à partir de deux approches: i) soit en augmentant le nombre de cellules (concentration) ou par ii) en augmentant la teneur en lipides des cellules. Tout stress environnemental, qui a un impact sur l'un d'eux ou les deux, affecterait l'accumulation de lipides. Les paramètres pH, température, oxygène dissous (DO), rapport carbone/azote (C/N), oligoéléments et mode de fermentation ont été considérés comme les paramètres les plus importants pour l'accumulation de lipides (Peng et al., 2015; San Pedro et al., 2016; Wang et al., 2016). D'après le Tableau 1.1, la concentration de la biomasse, l'accumulation de lipides et la productivité lipidique sont différentes lorsque la levure oléagineuse était cultivée par un milieu synthétique de glycérol brut ou de glycérol brut combiné avec des boues. De plus, le rapport C/N était également différent. L'étude cinétique, qui peut révéler l'utilisation du

substrat, la production de biomasse et l'accumulation de lipides, devrait être étudiée pour mieux comprendre l'accumulation de lipides et améliorer la production de lipides. En outre, le stress environnemental devrait être étudié, car les conditions environnementalles de culture peuvent grandement influencer la production de lipides.

Normalement, les microbes oléagineux pourraient produire des lipides dans une large gamme de pH. *Candida Curvata* accumule une quantité similaire de lipide (40-50% p/p) de pH 3,5 à 5,7 (Hall et Ratledge, 1977). Cependant, la tendance n'était pas adaptée à tous les microbes. Certains chercheurs ont mentionné que la plus forte production de lipides par *Rhodotorula glutin* est survenue à pH 4 et que le pH plus bas ou plus élevé a entraîné une diminution significative de l'accumulation de lipides (Johnson et al., 2014). Afin d'accumuler des lipides élevées, les micro-organismes oléagineux doivent être cultivés dans leur condition optimale de pH.

Cependant, le pH optimal rapporté était différent, même lorsque la même souche était utilisée pour produire des lipides (Tableau 1.2). Par exemple, lorsque la micro-algue *Neochloris oleoabundans* a été utilisée pour produire des lipides, le pH optimal rapporté pourrait être de 8,0-9,5 (Santos et al., 2014; Peng et al., 2015). Les chercheurs ont également rapporté un pH optimal différent pour la levure oléagineuse *Cryptococcus curvatus* (*Trichosporon oleaginosus*) (Cui et al., 2012; Yu et al., 2014). De plus, même dans des conditions de pH optimal, la production de biomasse et l'accumulation de lipides étaient différentes selon les chercheurs (Santos et al., 2014; Peng et al., 2015). Il doit y avoir des raisons inexpliquées qui doivent être découvertes pour le pH optimal de chaque microbe oleagineau.

Tableau 1. 2 pH optimal pour certains microbes oléagineux

Micro-organism	Nom	PH optimal	Concentration de biomasse (g/L)	Teneur en lipides (p/p)	Référence
Microalgue	*Chlorococcum sp.*	pH 8.0 à 8.5	1.75	56%	(Harwati et al., 2012)
Microalgue	*Neochloris oleoabundans*	pH 8.5-9.5	1.32	24.7%	(Peng et al., 2015)
Microalgue	*Neochloris oleoabundans*	pH 8.0	5	29.9%	(Santos et al., 2014)

Microalgue	*Scenedesmus obliquus*	pH 7.0	6-9	40%	(Breuer et al., 2013)
Levure	*Rhodosporidium toruloides*	pH 4.0	97.18	27.57%	(Dias et al., 2015)
Levure	*Trichosporon fermentans*	pH 6.5	28.1 g/l	62.4%	(Zhu et al., 2008)
Levure	*Cryptococcus curvatus*	pH 6.0	7.11	38.53 %	(Cui et al., 2012)
Levure	*Cryptococcus curvatus*	pH 5.5	10.7	30.8%	(Yu et al., 2014)
Levure	*Yarrowia lipolytica*	pH 7.0	-	55%	(Liu et al., 2016b)

Lorsque la levure oléagineuse *Cryptococcus curvatus* (*Trichosporon oleaginosus*) a été utilisée pour produire des huiles microbiennes, la température optimale rapportée par les chercheurs était presque la même (Tableau 1.3) qui était de 30,0 ° C. La même souche que *Trichosporon oleaginosus* sera utilisée dans cette recherche, donc la température optimale sera considérée à 30,0 ° C.

Tableau 1. 3 Température optimale pour *Cryptococcus curvatus* (*Trichosporon oleaginosus*)

Température optimale (°C)	Concentration de biomasse (g/L)	Teneur en lipides (% p/p)	Référence
30.0	8.73	24.7	(Espinosa-Gonzalez et al., 2014b)
30.0	80.0	20.0	(Béligon et al., 2015)
28.0	50.4	37.7	(Ryu et al., 2013b)
30.0	51.8	61.8	(Zhang et al., 2011a)

Il est bien connu que les micro-organismes oléagineux accumulent une teneur élevée en lipides dans les cellules dans des conditions riches en carbone et en azote limité. Par conséquent, le rapport C/N était considéré comme le paramètre le plus important, ce qui affecte l'accumulation de lipides (Wiebe et al., 2012; Braunwald et al., 2013; Ma et al., 2016). L'étape critique de la culture de micro-organismes oléagineux est de trouver le rapport C/N optimal pour l'accumulation de lipides dans le milieu. Voies métaboliques sont différentes avec le changement du rapport C/N dans les cellules oléagineuses. Avec un faible rapport C N, les lipides accumulés dans les cellules peuvent être digérés pour générer de l'énergie, qui est utilisée pour soutenir les activités cellulaires. Avec un rapport

C/N équilibré, le micro-organisme oléagineux peut rapidement générer des cellules (augmentation du nombre de cellules) plutôt que d'accumuler des lipides (huiles microbiennes). Avec un rapport C/N optimal pour l'accumulation des lipides, une partie du carbone est directement utilisée pour l'accumulation des lipides, ce qui est idéal pour l'accumulation de lipides par les micro-organismes oléagineux. Avec un rapport C/N élevé, l'inhibition se produit et le surdosage en carbone peut être utilisé par les microorganismes oléagineux pour produire une quantité élevée d'acide organique (acide citrique) (Azim et al., 2008; Abu et al., 2015). Le rapport C/N idéal de chaque micro-organisme oléagineux dépend de la souche et de la composition du milieu. Normalement, lorsque les sources de carbone dans le milieu sont faciles à utiliser par les micro-organismes oléagineux, le rapport C/N optimal est faible. Par exemple, le glycérol est plus facile à utiliser par les micro-organismes oléagineux comparé aux déchets lignocellulosiques. Ainsi, le rapport C/N optimal rapporté, quand le glycérol a été utilisé comme source de carbone, était de 30 à 60, soit 120 ou plus lorsque le matériau lignocellulosique était une source de carbone (Liang et al., 2010; Ruan et al., 2012; Duarte et al., 2013; Cheirsilp et Kitcha, 2015; Patel et al., 2015).

Lorsque plus d'une source de carbone est présente dans le milieu, le rapport C/N optimal peut être très différent de celui d'un milieu source de carbone solo. Kraisintu et al. ont observé que le rapport C/N optimal était de 35 si le glucose était la seule source de carbone, qui était de 180 lorsque le glucose était combiné avec du glycérol (le glycérol solitaire était d'environ 60) (Kraisintu et al., 2010).

Dans cette étude, la solution de glycérol brut (contenant du méthanol, du savon, du glycérol et d'AGL) sera utilisée comme principale source de carbone pour la culture de *Trichosporon oleaginosus*. La solution de glycérol brut est un mélange de méthanol, de savon, de glycérol et de catalyseur. Le rapport C/N optimal peut être différent de celui obtenu dans le milieu glycérol purifié ou avec celui obtenu dans une solution de glycérol brut de composition différente (Ryu et al., 2013; Gao et al., 2014; Saenge et al., 2011; Matsakas et al., 2014; Uçkun Kiran et al., 2012; Hassan et al., 1996). Par conséquent, il est nécessaire d'étudier le rapport C/N optimal pour *Trichosporon oleaginosus* avec le glycérol brut dans cette recherche.

Les oligo-éléments sont des nutriments essentiels pour les micro-organismes pour leurs fonctions normales en quantité infime. La constitution de chaque élément trace est généralement inférieure à 0,01% (p/v) de la masse corporelle. Ils sont principalement: chrome, cobalt, cuivre, fluor, iode, fer, manganèse, molybdène, sélénium et zinc (Strachan, 2010).

Comme discuté ci-dessus, les boues seront utilisées pour fournir des éléments de base pour la croissance cellulaire dans cette étude. Il est supposé que les oligo-éléments contenus dans les boues d'épuration sont suffisants pour la croissance de *Trichosporon oleaginosus*.

Dans le mode discontinu conventionnel (mode batch), tous les nutriments sont introduits dans le réacteur au début suivi de l'inoculation du micro-organisme. Rien d'autre que de l'oxygène, un agent antimousse et un acide ou une base (employés pour contrôler le pH) sont ajoutés aux réacteurs pendant la fermentation. Le mode batch est facile à préparer et à réaliser, mais normalement le produit désiré est généré à faible concentration. Pour produire un niveau élevé de produit désiré, le mode fed-batch est largement effectué. Le mode fed-batch est le processus biotechnologique dans lequel les substrats nutritifs sont introduits dans le réacteur à travers de multiples étapes au cours de la culture. Normalement, un milieu basique est ajouté au réacteur au début pour appuyer la croissance cellulaire. Au fur et à mesure de la fermentation, les nutriments sont ajoutés par plusieurs étapes.

Le mode fed-batch est supérieur au mode batch conventionnel lorsqu'une concentration élevée en nutriments a un effet négatif sur la production du produit désiré. Pour l'accumulation de lipides, il nécessite un rapport C/N élevé (concentration élevée en carbone). Une concentration élevée en carbone peut inhiber la croissance des microbes. Afin d'améliorer l'accumulation de lipides mais sans affecter la division cellulaire, le mode fed-batch doit être appliqué. Li et al. ont rapporté qu'une concentration élevée de biomasse (151,5 g/L) et une teneur en lipides (48% p/p) étaient atteintes en alimentant la levure oléagineuse *Rhorosporium toruloides Y4* avec du glucose en mode fed-batch (Li et al., 2007). Jusqu'à présent, la concentration de biomasse la plus élevée (185 g/L), la teneur en lipides la plus élevée (76% p/p) et la productivité lipidique la plus élevée (1g/L/h)

ont été obtenues par la fermentation fed-batch (Koutinas et al., 2014). Par conséquent, le mode fed-batch doit être utilisé pour obtenir une production élevée d'huile microbienne. Pour utiliser le mode fed-batch, les stratégies d'alimentation sont essentielles. De nos jours, la stratégie d'alimentation est basée soit sur le pic de la concentration de CFU, soit sur l'épuisement des nutriments. Cela signifie que lorsque le pic de la concentration de CFU s'est produit ou que certains éléments nutritifs ont été épuisés à un moment, ce point de temps a été utilisé pour l'alimentation de nutriments supplémentaires. Cependant, le point temporel peut ne pas être optimal. De plus, il n'y avait pas d'ajustement fiable pour la quantité de nutriments supplémentaires introduits dans le réacteur.

En conclusion, le pH et le rapport C/N sont considérés comme les paramètres les plus importants. Le mode fed-batch pour améliorer la production d'huiles microbiennes sera utilisé. Une nouvelle stratégie d'alimentation en contrôlant le pH serat découverte dans cette étude.

Le bilan énergétique, l'émission de gaz à effet de serre et l'estimation des coûts sont importants pour évaluer la faisabilité de la production de biodiesel à partir du procédé proposé dans cette étude (détails au chapitre 2).

1.2 Problématique

1.2.1 Pénurie d'huiles pour la production de biodiesel

Les huiles végétales et les graisses animales sont actuellement utilisées comme matières premières pour la production de biodiesel. Cependant, les quantités disponibles d'huiles sont limitées en considérant les besoins pour la consommation humaine. Il y a un grand besoin de trouver des matières premières alternatives pour satisfaire la production de biodiesel.

1.2.2 Coût élevé de biodiesel produit à partir d'huiles microbiennes

Les huiles microbiennes comme matière première pour produire du biodiesel présentent un grand avantage par rapport aux huiles végétales et aux graisses animales, car les microbes oléagineux se développent plus vite et ont une grande capacité à accumuler

des huiles microbiennes dans les cellules. Cependant, le coût élevé est un gros obstacle à la production de biodiesel à partir d'huiles microbiennes.

1.2.3 Processus complexes de production de biodiesel à partir d'huiles microbiennes

Le procédé de production de biodiesel à partir d'huiles microbiennes contient un pré-traitement de substrats, la culture de micro-organismes, la récolte de micro-organismes et l'extraction de lipides, ce qui est très complexe.

1.2.4 Manque de corrélation suffisante entre la consommation du substrat, la production de biomasse et la production de lipides.

Une étude cinétique est généralement réalisée pour étudier la corrélation entre l'utilisation du substrat, la production de biomasse et la production de lipides. Cependant, les méthodes ou les équations utilisées pour l'étude cinétique peuvent ne pas convenir aux micro-organismes oléagineux. Par conséquent, la corrélation entre l'utilisation du substrat, la production de biomasse et la production de lipides n'est pas claire.

1.2.5 Faible production d'huiles microbiennes

De nombreux microbes oléagineux ont la capacité d'utiliser des déchets organiques pour produire des huiles microbiennes. Cependant, la production d'huiles microbiennes à partir de déchets organiques est la plupart du temps inférieure à celle provenant de sources de carbone (glucose) de haute qualité.

1.2.6 Pas de stratégies d'alimentation fiables pour le mode fed-batch

Des chercheurs ont essayé d'augmenter la production d'huiles microbiennes en utilisant le mode fed-batch, les stratégies d'alimentation utilisées dans les recherches précédentes n'étaient pas fiables.

1.3 Hypothèses et objectifs de recherche

1.3.1 Hypothèses

Pour rendre la production de biodiesel à partir d'huiles microbiennes réalisable pour la pratique industrielle, les hypothèses suivantes doivent être vérifiées:

- La fermentation sans stérilisation doit être effectuée pour simplifier le processus et réduire la consommation d'énergie.
- La culture de micro-organismes oléagineux par les déchets organiques peut fortement réduire le coût de la production de biodiesel.
- L'étude cinétique est importante pour la compréhension de corrélation entre l'utilisation du glycérol brut, la production de biomasse et l'accumulation de lipides.
- Le rapport C/N optimal doit être étudié lorsque la solution de glycérol brut est utilisée comme source de carbone.
- Le mode fed-batch peut améliorer la concentration de biomasse et de lipides. Des nouvelles stratégies d'alimentation devraient être testées.
- Le processus de production de biodiesel à partir d'huiles microbiennes devrait être étudié par le bilan énergétique et l'analyse techno-économique afin d'évaluer sa faisabilité industrielle.

1.3.2 Objectifs de recherche

L'objectif principal est de contribuer au développement d'une filière industrielle de production de biodiesel à partir d'huiles microbiennes. Pour ce faire, les substrats (glycérol brut et boues d'épuration) seront utilisés comme milieu de culture de *Trichosporon oleaginosus*.

1. Étudier et déterminer le rapport C/N optimal en utilisant un milieu de culture synthétique à base de glycérol brut en présence de *Trichosporon oleaginosus*.
2. Étudier la corrélation ou cinétique de la consommation de substrat, de la production de biomasse et de l'accumulation de lipides.
3. Évaluer la possibilité d'éliminer le processus de stérilisation en appliquant la solution de glycérol brut à la culture de *Trichosporon oleaginosus*.

4. Étudier la relation entre le pH et la concentration de savon, qui peut être utilisé comme stratégie d'alimentation.
5. Appliquer le processus de fermentation discontinue (fed-batch) en vue d'améliorer la concentration de biomasse et d'accumuler davantage de lipides dans *Trichosporon oleaginosus* pour la production de biodiesel.
6. Optimiser le rapport C/N lors de la croissance de la culture de *Trichosporon oleaginosus* dans des boues enrichies de glycérol brut.
7. Étudier le bilan énergétique, les émissions de gaz à effet de serre et le coût du procédé pour évaluer la faisabilité industrielle de cette étude.

1.3.3 Originalité du travail

Les boues d'eaux usées enrichies en glycérol brut (contenant du savon élevé, du méthanol et des concentrations relativement faibles en glycérol) n'ont pas été utilisées pour cultiver *T. oleaginosus*.

La cinétique de consommation du glycérol brut (contenant des concentrations élevées en savon , du méthanol et des concentrations relativement faibles en glycérol) et leur relation avec la production de biomasse et l'accumulation de lipides n'ont pas été étudiées.

La stérilisation est nécessaire pour la culture de la levure oléagineuse, qui consomme beaucoup d'énergie. L'application directe de méthanol dans le milieu de glycérol brut pour éviter le processus de stérilisation n'a pas été étudiée.

Le pH qui peut refléter la concentration de savon n'a pas été étudié en tant que paramètre pour guider l'addition de substrats dans le mode discontinue.

Le mode fed-batch a été étudié pour augmenter la production de biomasse et de lipides, mais les stratégies d'alimentation n'étaient pas basées sur la concentration précise en nutriments. Dans cette étude, le pH sera utilisé comme la stratégie d'alimentation, qui peut être contrôlée automatiquement en mode discontinue.

L'analyse techno-économique de la production de biodiesel à partir d'huiles microbiennes produites par *Trichosporon oleaginosus* dans les boues d'épuration et le milieu glycérol brut n'a pas été explorée en mode discontinue.

1.4 Méthodologies

1.4.1 Le plan expérimental global

Le but de l'étude était de développer un procédé industriel réalisable pour la production de biodiesel à partir d'huiles microbiennes accumulées par la levure oléagineuse *Trichosporon oleaginosus* cultivée dans un mélange de glycérol brut et de des boues d'épuration. Tout d'abord, une solution de glycérol brut a été recueillie et caractérisée. Le rapport C/N, qui était le paramètre le plus important pour la production de lipides, a été varié et étudié pour la culture de *T. oleaginosus* dans le milieu synthétique de glycérol brut. Suite à l'étude du rapport C/N, les résultats expérimentaux ont été utilisés pour l'étude cinétique afin de révéler la corrélation entre la consommation de substrat, la croissance cellulaire, la production de biomasse et l'accumulation de lipides. Selon l'étude cinétique et la loi de la conservation de l'énergie, un nouveau modèle d'accumulation de lipides a été proposé. Une fermentation fed-batch a été conçue et exploitée pour augmenter simultanément la production de lipides et la productivité des lipides. . La stérilisation du milieu de fermentation, qui devait assurer une production élevée de lipides, nécessitait une consommation d'énergie élevée. Le méthanol, composant principal de la solution de glycérol brut, a été étudié pour aider *T. oleaginosus* à produire des lipides dans des conditions non stériles afin d'éviter le processus de stérilisation. Le savon, le deuxième composant du glycérol brut, était un inhibiteur de la souche productrice de lipides. Le pH a été étudié pour déterminer sa relation avec la forme du savon (savon ou acide gras libre) et son impact sur la croissance cellulaire de *T. oleaginosus*. De plus, une fermentation de type fed-batch basé sur le contrôle du pH a été conçu et utilisé pour simplifier le processus de production de lipides et augmenter la production de lipides. Après l'amélioration de production de lipides et la simplification du processus dans le milieu synthétique de glycérol brut, des boues d'épuration ont été introduites pour remplacer les produits chimiques du milieu synthétique de glycérol brut. Les boues d'épuration ont été recueillies, caractérisées et combinées avec du glycérol brut pour la culture de *T. oleaginosus*. L'abondance de l'azote dans les boues d'épuration a été étudiée. Les résultats obtenus dans le mélange de glycérol brut et des boue d'épuration ont été utilisés pour l'étude du bilan énergétique, des émissions de gaz à effet de serre

et le coût du processus afin d'évaluer la faisabilité industrielle de la production de biodiesel à partir d'huiles microbiennes accumulées par *T. oleaginosus* dans le mélange de glycérol brut et des boue d'épuration.

1.4.2 Matériaux, fermentation et les méthodes analytiques

1.4.2.1 La souche

La souche productrice de lipides était la levure oléagineuse *Trichosporon oleaginosus* (ATCC 20905).

1.4.2.2 Milieux de la fermentation

Le milieu synthétique de glycérol brut contenait 0.95 g/L de Na_2HPO_4, 1.616 g/L de NH_4Cl, 0.2 g/L de $MgSO_4 \cdot 7H_2O$, 0.4 g/L de peptone, 1 g/L de $CaCl_2 \cdot 2H_2O$, 0.55 g/L de $FeSO_4 \cdot 7H_2O$, 0.1 g/L de $ZnSO_4 \cdot 7H_2O$, and 0.076 g/L de $MnSO_4 \cdot H_2O$ et la quantité souhaitée de glycérol brut.

Le mélange de glycérol brut et des boues d'épuration contenait 30 g/L de boues et 45 mL de glycérol brut.

1.4.2.3 Les méthodes analytiques

Le glycérol brut a été caractérisé selon les méthodes présentées au chapitre 4.

Les boues d'épuration ont été caractérisées selon les méthodes présentées dans un article publié (Zhang et al., 2014).

Les méthodes employées pour déterminer la concentration de biomasse, lipide, glycérol, savon, méthanol et la teneur en lipides sont présentées au chapitre 4.

1.4.2.4 Fermentation

La fermentation a été réalisée dans un fermenteur de 15 L (volume utile: 10 L, Biogenie, Qc, Canada) équipé d'accessoires et d'un système de contrôle logique programmable pour oxygène dissous (DO), pH, anti-mousse, et température. Le logiciel (iFix 3.5, Intellution, USA) a permis le contrôle automatique et l'intégration de tous les paramètres via l'API.

1.4.3 Méthodologie de l'objectif spécifique

1.4.3.1 Méthodologie de l'objectif 1: Étudier et déterminer le rapport C/N optimal en utilisant un milieu de culture synthétique à base de glycérol brut en présence de *Trichosporon oleaginosus*

Basé sur des résultats antérieurs et ceux de notre laboratoire, le rapport C / N de 20, 30, 45 et 60 a été étudié pour révéler son impact sur la production de lipides et le rapport C / N optimal pour *T. oleaginosus*.

Pour préparer un milieu de fermentation de 10 litres, 290, 430, 650 et 860 mL de glycérol brut ont étéutilisés pour obtenir un rapport C / N de 20, 30, 45 et 60, respectivement. Après stérilisation et inoculation, la fermentation a été réalisée à pH 5, à une température de 30 ° C et une concentration d'oxygène dissous supérieure à 35%. Pendant la fermentation, 150 mL d'échantillon a été prélevé à un intervalle de 6 heures et stocké à 4 ° C (détails au chapitre 4).

1.4.3.2 Méthodologie de l'objectif 2: Étudier la corrélation ou cinétique de la consommation de substrat, de la production de biomasse et de l'accumulation de lipides

Les résultats obtenus à partir de l'étude du rapport C/N (objectif 1) ont été utilisés pour déterminer la cinétique de consommation de glycérol brut par rapport à la production de biomasse et l'accumulation de lipides par les équations suivantes:

Taux de consommation de glycérol ou de savon = dS/dt

Productivité de la biomasse = dX/dt

Productivité lipidique = dP/dt

Où dt est un intervalle de temps; dS est la concentration de substrat (glycérol ou savon) consommée dans l'intervalle de temps dt; dX est la biomasse produite dans l'intervalle de temps dt et dP est le lipide accumulé dans l'intervalle de temps dt.

Pour déterminer si le substrat (glycérol ou savon) était destiné à la croissance de la biomasse ou à l'accumulation de lipides, le rendement en biomasse et le rendement en lipides ont été calculés selon les équations suivantes:

Rendement de la biomasse = $(X_{t2}-X_{t1})/(S_{t1}-S_{t2})$

Rendement en lipides = $(P_{t2}-P_{t1})/(S_{t1}-S_{t2})$

Où X_{t2} et X_{t1} représentent les concentrations de biomasse sans lipide aux temps t2 et t1; P_{t2} et P_{t1} représentent les concentrations de lipides aux temps t2 et t1; S_{t2} et S_{t1} représentent les concentrations de substrat (glycérol ou savon) aux temps t2 et t1.

La cinétique de la consommation de substrat, la production de biomasse et l'accumulation de lipides ont révélé quel substrat (glycérol ou savon) était utilisé plus rapidement et révélaient si l'utilisation du substrat correspondait à la production de biomasse ou à l'accumulation de lipides. Ces résultats ont été utilisés pour la conception de la fermentation fed-batch. Dans la fermentation fed-batch, la concentration initiale en azote a été doublée pour la production de biomasse rapide. Des multiples charges de glycérol brut ont été ajoutées pendant la période de production rapide de biomasse (16 h à 32 h de la fermentation) (détails au chapitre 4).

Selon l'étude cinétique et la loi de la conservation de l'énergie, chaque cellule oléagineuse était considérée comme un système isolé. L'énergie à l'intérieur de la cellule oléagineuse a été considérée comme constante et une équation thermodynamique a été proposée (détails au chapitre 7).

1.4.3.3 Méthodologie de l'objectif 3: Évaluer la possibilité d'éliminer le processus de stérilisation en appliquant la solution de glycérol brut à la culture de *Trichosporon oleaginosus*.

Une concentration optimale de méthanol pourrait inhiber la croissance des contaminants et favoriser concomitamment la croissance de la souche productrice de lipides qui pourrait fournir un moyen de produire des lipides sans la stérilisation du milieu.

Le glycérol brut contenant une teneur élevée en méthanol a été directement utilisé pour la culture de *Trichosporon oleaginosus*. La concentration de méthanol de 0%, 1,4%, 2,2%, 3,3% et 4,4% (p/v) a été étudiée. En conséquence, la solution de glycérol brut désirante a été calculée et utilisée pour préparer le milieu de fermentation. Les fermentations ont été réalisées dans des conditions similaires à l'objectif 1 (détails au chapitre 5).

1.4.3.4 Méthodologie de l'objectif 4: Étudier la relation entre le pH et la concentration de savon, qui peut être utilisé comme stratégie d'alimentation.

Le savon ($C_{17}H_{35}COONa$) était un produit chimique d'alcali fort et d'acide faible. Lorsque le savon était présent dans le milieu, il pouvait être considéré comme un tampon. Les fermentations sous rapport C/N optimal (déterminé dans l'objectif 1) ont été réalisées à différents pH pour étudier l'effet du pH sur la forme du savon (savon à pH élevé et acide gras libre à pH bas) et l'effet la production de lipides (détails au chapitre 7).

1.4.3.5 Méthodologie de l'objectif 5: Appliquer le processus de fermentation discontinue (fed-batch) en vue d'améliorer la concentration de biomasse et d'accumuler davantage de lipides dans *Trichosporon oleaginosus* pour la production de biodiesel.

La fermentation dans du milieu synthétique de glycerol brut a été effectuée sans contrôle du pH pour vérifier la variation du pH lors de la fermentation. Il a été constaté que le pH diminuait continuellement.

Un litre de solution de glycérol brut sans méthanol a été ajusté à pH 11 et ensuite utilisé pour contrôler le pH de fermentation. Lorsque le pH de fermentation a diminué, la solution de glycérol brute à pH 11 a été pompée automatiquement dans le fermenteur contrôlé par la sonde de pH. Le contrôle du pH a été combiné avec l'alimentation du glycérol brut (détails au chapitre 7).

1.4.3.6 Méthodologie de l'objectif 6: Optimiser le rapport C/N lors de la croissance de la culture de *Trichosporon oleaginosus* dans des boues enrichies de glycérol brut.

Les boues générées dans l'usine de traitement des eaux usées, ont été introduites pour remplacer les produits chimiques dans le milieu synthétique de glycérol brut.

Après la caractérisation des boues, il a été constaté que la source d'azote dans les boues pourrait ne pas être suffisante pour assurer une production de lipides élevée et rapide. Ainsi, NH_4Cl a été ajouté dans le mélange de glycérol brut et des boues pour optimiser le rapport C/N et comparé à l'expérience sans l'addition de NH_4Cl (détails au chapitre 8).

1.4.3.7 Méthodologie de l'objectif 7: Étudier le bilan énergétique, les émissions de gaz à effet de serre et le coût du procédé pour évaluer la faisabilité industrielle de cette étude.

L'étude de faisabilité industrielle de production de biodiesel à partir d'huiles microbiennes accumulées par *Trichosporon oleaginosus* dans le mélange de glycérol brut et des boues a permis d'étudier le bilan énergétique, les émissions de gaz à effet de serre et l'estimation des coûts.

Dans l'approche du bilan énergétique, des émissions de gaz à effet de serre et de l'estimation des coûts, la première étape consistait à concevoir le processus de production de biodiesel en fonction des matières premières sélectionnées, des voies de réaction et de la capacité de production. La production de biodiesel à partir d'huiles microbiennes comprenait la production de lipides, l'extraction de lipides, la formation de biodiesel et la purification de biodiesel. Le calcul commencé à la production de matières premières (huiles microbiennes ou lipides) a pris fin jusqu'à l'obtention de biodiesel pur.

Les teneurs énergétiques de l'électricité, des combustibles fossiles, des vapeurs et du méthanol, du glycérol et du savon étaient considérées comme énergie directe et utilisées dans le calcul de l'énergie, tandis que l'énergie consommée pour produire d'autres produits chimiques était considérée comme énergie indirecte. Les éléments énergétiques connexes, l'apport énergétique total, l'apport énergétique net, le gain énergétique et le rapport énergétique ont été calculés (détails au chapitre 8).

L'étude a pris en compte les émissions de CO_2, de CH_4 et de N_2O provenant de sources spécifiques d'énergie et de matériaux consommés, de l'utilisation de combustibles, de l'électricité et de produits chimiques. Les potentiels de réchauffement planétaire du Groupe d'experts intergouvernemental sur l'évolution du climat (GIEC) ont été appliqués aux émissions de CH_4 (21 éqCO_2) et de N_2O (310 éqCO_2) pour calculer les émissions de CO_2 équivalent (détails au chapitre 8).

Dans cette étude, Superpro Designer a été utilisé pour estimer le coût de la production de biodiesel. Les prix des produits chimiques provenaient d'ICIS Pricing, qui était le principal service de production de rapports sur les prix des produits pétrochimiques et énergétiques pour l'industrie chimique mondiale. Les prix des équipements provenaient

des guides officiels des solutions IRON, qui indiquaient les valeurs et les tendances de l'équipement en Amérique du Nord (détails au chapitre 8).

1.5 Résultats et discussion

Comme mentionné ci-dessus, le principal problème de la production de biodiesel à partir d'huiles microbiennes est le coût élevé qui a contribué au coût élevé du substrat; processus compliquant; faible production de lipides. En outre, il était nécessaire de comprendre la base de la production de lipides qui était la corrélation entre l'utilisation du substrat, la production de biomasse et la production de lipides. En conséquence, les résultats de cette étude ont été discutés à partir de quatre parties: 1) améliorer la production de lipides à partir de déchets organiques constitués de boues et de glycérol brut dans cette étude; 2) simplifier le processus de production de biodiesel à partir de lipides; 3) étudier la cinétique ou la corrélation de l'utilisation du substrat, la production de biomasse et la production de lipides; 4) évaluer la faisabilité de la production de biodiesel à partir de lipides accumulés par la levure oléagineuse *Trichosporon oleaginosus*.

1.5.1 Amélioration de la production de lipides à partir de déchets organiques

1.5.1.1 Effet du rapport C/N sur la production de lipides dans le milieu synthétique de glycérol brut

Dans cette étude, la solution de glycérol brut, contenant peu de glycérol mais de fortes impuretés (méthanol, savon et catalyseur), a été utilisée pour la production de lipides à l'aide de *T. oleaginosus*. Comme indiqué, une concentration élevée de méthanol pourrait inhiber la croissance de la souche (Chen et al., 1976), ainsi le méthanol a été évaporé du glycérol brut avant d'être utilisé. Le savon et le glycérol ont servi de sources de carbone. Les rapports C/N (p/p) de 20, 30, 45 et 60 ont été étudiés pour évaluer son impact sur la production de lipides de *T. oleaginosus*.

La concentration de biomasse augmentait avec le rapport C/N de 20 à 45 et diminuait avec l'augmentation du rapport C/N à 60. La concentration maximale de biomasse au

rapport C/N de 45 (24,80 g/L) était légèrement supérieure que le rapport C/N de 30 (23,70 g/L). Cette augmentation est principalement due à l'augmentation de la concentration lipidique de 11,26 g/L (rapport C/N de 30) à 12,14 g/L (rapport C/N de 45). Le rapport C/N de 30 était suffisant pour la production de biomasse et le rapport C/N de 45 était un peu favorable à la production de lipides.

La teneur en lipides était de 22,98%, 47,50%, 48,95% et 52,02% (p/p) au rapport C/N de 20, 30, 45 et 60, respectivement. Ceci a indiqué qu'un rapport C/N plus élevé était favorable pour que *T. oleaginosus* accumule une plus grande teneur en lipides.

Au rapport C/N de 30, la déplétion de glycérol et d'AGL a conduit à une diminution rapide de la concentration en lipides à la fin de la fermentation. Il a été rapporté qu'une réduction de la concentration en lipides et de la teneur en lipides était due à l'utilisation de lipides comme source d'énergie après l'épuisement de la source de carbone dans le milieu (Taskin et al., 2016). Au rapport C/N de 45 et 60, malgré la présence de source de carbone, la concentration en lipides a encore légèrement diminué à la fin de la fermentation. Cela pourrait être dû à une limitation de l'azote, ce qui est critique pour le métabolisme cellulaire (Rakicka et al., 2015a).

1.5.1.2 Importance de l'azote pour la production de lipides élevée et rapide

Il a été constaté que l'azote pourrait limiter la production de biomasse et de lipides dans la fermentation discontinue. Ainsi, la concentration de nitrogène a été doublée de 0,51 g/L à 1,02 g/L dans la fermentation fed-batch. La concentration de la biomasse (43,82 g/L) et la concentration en lipides (21,87 g/L) ont été réalisées dans la fermentation fed-batch.

1.5.1.3 Effet du pH sur la production de lipides

Dans cette étude, le pH optimal de la levure oléagineuse *T. oleaginousus* a été étudié et discuté dans différents milieux. La variation du pH pendant la fermentation a été étudiée.

Il a été trouvé que les conditions de pH acide faible ou neutre (pH 5,5, 6,5 et 7) étaient avantageuses pour la croissance cellulaire de *T. oleaginosus* dans le milieu de extrait de levure peptone dextrose et de la boue. Cependant, la production de cellules a été significativement affectée par l'ajout de glycérol brut dans les boues à pH 6 et 7 qui n'était

pas fortement affecté à pH 5. Il est prédit que certains composants de la solution de glycérol brut affectaient fortement la production cellulaire de *T. oleaginosus* à pH 6 et 7, mais très légèrement à pH 5.

En investissant les composants de la solution de glycérol brut, les deux principaux composants contribuant comme sources de carbone étaient le savon et le glycérol car le méthanol était déjà évaporé du glycérol brut avant d'être utilisé. Le glycérol était stable avec la variation du pH, ce qui signifiait que la variation du pH n'affectait pas la fonction du glycérol. Comparativement, le savon pourrait être fortement affecté par la variation de pH. Dans des conditions de pH élevé, il s'est formé sous forme de savon, qui était très soluble dans le milieu. Dans des conditions de faible pH, le savon a été converti en acide gras libre (AGL), moins soluble dans le milieu (0,34 g/L à 25 ° C) (Khuwijitjaru et al., 2002).

Savon (ou détergent) était un inhibiteur important des micro-organismes dans le système d'eau naturel. Le savon pourrait fortement affecter la croissance des micro-organismes en diminuant leurs paramètres de motilité, en altérant leur orientation et en transformant leur morphologie (Azizullah et al., 2011). Une fois que le savon a été converti en AGL, il a été trouvé que l'inhibition de l'acide oléique était mineure (Angelidaki & Ahring, 1992). Ainsi, un pH bas, qui convertissait le savon en AGL, était favorable lorsque du glycérol brut contenant une forte teneur en savon était utilisé pour la culture de *T. oleaginosus*. Dans cette étude, le pH 5 était considéré optimal pour la croissance de *T. oleaginosus* en utilisant du glycérol brut comme milieu de fermentation.

1.5.1.4 Amélioration de la production de lipides par fermentations fed-batch

Dans cette étude, deux types de stratégies d'alimentation ont été utilisés dans les fermentations fed-batch.

Premièrement, la stratégie d'alimentation était basée sur le point de temps. Il a été observé qu'une productivité élevée de la biomasse conduit à un taux élevé de consommation de carbone. Ainsi, la source de carbone devrait être suffisante lorsque la productivité élevée de la biomasse s'est produite, qui a duré de 16 h à 32 h. Il a indiqué que la source de carbone devrait être alimentée pendant 16 h à 32 h. Pour minimiser l'inhibition de la source de carbone en excès, l'alimentation de la source de carbone a été divisée en trois étapes. À 16 h, 10,28 g/L de source de carbone (à partir de glycérol et

d’AGL) ont été ajoutés au milieu. À 24 h, 10,28 g/L de carbone (à partir de glycérol et AGL) ont été introduits dans le fermenteur pour la deuxième fois. À 32 h, 15,42 g/L de carbone (à partir de glycérol et AGL) ont été introduits dans le milieu pour la troisième fois. La concentration maximale de biomasse (43,82 g/L) et de lipides (21,87 g/L) avec une productivité lipidique de 0,42 g/L/h ont été obtenues.

Deuxièmement, la stratégie d'alimentation était basée sur le pH. Il a été précédemment observé que le pH diminuait continuellement. Ainsi, une solution alcaline de glycérol brut, sans solution acide, a été utilisée pour contrôler le pH pendant la fermentation discontinue. Dans cette étude, la fermentation fed-batch, auto-contrôlée avec une solution de glycérol brut (pH = 10,98), a atteint une production élevée de biomasse (65,63 g/L), une production élevée de lipides (35,79 g/L) et une teneur élevée en lipides (54,53% p/p). La concentration de glycérol et d'AGL sont maintenues à un niveau élevé (concentration de glycérol entre 6,22 g/L et 17,75 g/L, concentration d'AGL entre 2,3 g/L et 10,08 g/L) mais pas trop élevée pour provoquer l'inhibition du substrat pendant la fermentation.

1.5.2 Simplifier le processus de production de biodiesel à partir de lipides

1.5.2.1 Élimination de la stérilisation et la production de lipides dans des conditions non stériles

Lorsque la concentration de méthanol est dans la plage de croissance de la levure oléagineuse mais inhibe celle des contaminants (principalement les bactéries), la production de lipides à partir de levure oléagineuse peut être réalisée dans des conditions non stérilisées en manipulant la concentration de méthanol dans cette gamme.

Dans cette étude, un glycérol brut, contenant une forte teneur en méthanol, a été utilisé pour la production de lipides à partir de la levure oléagineuse *Trichosporon oleaginosus* dans des conditions non stériles. Dans cette étude, une concentration de méthanol de 1,4% (p/v), 2,2% (p/v), 3,3% (p/v) et 4,4% (p/v) ont été utilisées pour étudier la concentration optimale de méthanol sur la production de lipides à partir de *T. oleaginosus* dans la fermentation non stérilisée.

Dans la fermentation avec une concentration de méthanol de 1,4% p/v, le nombre d'UFC de *T. oléagineux* était beaucoup plus élevé que celui des contaminants pendant la

fermentation. À une concentration de methanol de 2,2% (p/v), il a été observé que l'inhibition de la croissance de *T. oleaginosus* et de contaminants se produisait sévèrement. À une concentration de méthanol de 3,3% (p/v), la croissance de *T. oleaginosus* et des contaminants s'est presque complètement arrêtée. A une concentration en methanol de 4,4% (p/v), la croissance de *T. oleaginosus* et des contaminants a été inhibée dans une plus grande mesure. Par conséquent, la concentration de méthanol de 1,4% (p/v) était considérée optimale pour la culture de *T. oleaginosus* dans des conditions non stériles. Ainsi, le processus de stérilisation pourrait être évité.

1.5.2.2 Stratégie d'alimentation auto-contrôlée dans la fermentation discontinue

Il est bien connu que la fermentation fed-batch peut améliorer la production de lipides. Cependant, les stratégies d'alimentation, qui étaient basées sur le point de temps ou le taux de dilution, ne sont ni précises ni contrôlées automatiquement. Dans cette étude, il a été constaté que le pH déterminait la concentration de savon. Ainsi, une stratégie d'alimentation autocontrôlée, basée sur le pH, a été conçue.

La fermentation fed-batch, basée sur le pH et contrôlée automatiquement, a maintenu la concentration des substrats à un niveau élevé (glycérol entre 6,22 g/L et 17,75 g/L; savon entre 2,5 g/L et 6,5 g/L). Une concentration très élevée de biomasse et de lipides, qui était de 65,63 g /L et de 35,79 g/L, respectivement, a été atteinte.

1.5.3 La cinétique ou la corrélation de l'utilisation du substrat, la production de biomasse et la production de lipides

1.5.3.1 L'étude cinétique utilisant les modèles actuels pour révéler la croissance cellulaire, l'utilisation du substrat, la production de biomasse et la production de lipides

Le taux de croissance cellulaire spécifique, la productivité de la biomasse, la productivité lipidique et le taux de consommation de substrat (glycérol et acide gras libre) ont été calculés pour révéler la corrélation entre l'utilisation du substrat, la croissance cellulaire, la production de biomasse et la production de lipides.

D'après l'étude cinétique, il a été constaté que: 1). Le taux de croissance spécifique était légèrement affecté par la variation de la concentration de glycérol brut lorsque la source de carbone était suffisante dans le milieu. En dehors de la source de carbone, les nutriments, notamment l'azote et le phosphore, étaient importants pour la croissance des micro-organismes, et la croissance cellulaire serait limitée lorsque ces nutriments sont déficients. 2). La productivité de la biomasse était fortement associée au taux de consommation de substrat, ce qui indiquait qu'une productivité élevée de la biomasse signifiait un taux élevé de consommation de substrat. 3). La productivité élevée des lipides est normalement apparue après la forte productivité de la biomasse.

1.5.3.2 Une nouvelle modélisation thermodynamique de l'accumulation de lipides *Trichosporon oleaginosus*

Dans cette étude, chaque cellule oléagineuse était considérée comme un système isolé. Selon la loi de conservation de l'énergie, l'énergie serait constante dans un système isolé. Ainsi, l'énergie générée par l'utilisation du substrat serait égale à l'énergie consommée pour tous les produits. Par conséquent, un modèle thermodynamique de l'accumulation de lipides dans le micro-organisme oléagineux a été établie. A partir du modèle de Lueding-Piret utilisé couramment, la production de lipides était associée à la croissance ou non associée à la croissance, ce qui signifiait que la production de lipides était déterminée par la production de biomasse. D'après le modèle établi dans cette étude, la relation entre la production de biomasse et de lipides était la compétition pour l'énergie provenant de l'utilisation du substrat.

1.5.4 La faisabilité de la production de biodiesel à partir de lipides accumulés par la levure oléagineuse *Trichosporon oleaginosus*

1.5.4.1 Production de lipides à partir d'un mélange de boues et glycérol brut

Les boues, largement générées par les usines de traitement des eaux usées, étaient riches en tous les éléments nécessaires à la croissance cellulaire. Les méthodes de fermentation développées dans le milieu synthétique de glycérol brut ont été utilisées dans le mélange de boues et glycérol brut.

Nos recherches antérieures ont montré que l'azote était important pour la croissance cellulaire et la production de lipides. La source d'azote dans les boues pourrait ne pas être suffisante pour la production élevée de lipides. Ainsi, de l'azote supplémentaire a été ajouté dans le mélange de boues et glycérol brut. Les résultats expérimentaux de la fermentation avec addition d'azote ont été comparés à la fermentation sans addition d'azote.

La plus forte concentration de biomasse provenant de la fermentation avec addition d'azote était de 55,33 g/L, soit 42,66 g/L provenant de la fermentation sans addition d'azote. La fermentation avec addition d'azote a également montré une meilleure production de lipides et une meilleure teneur en lipides. La concentration lipidique la plus élevée dans la fermentation avec addition d'azote était de 26,98 g/L avec une teneur en lipides de 48,77% (poids/poids) qui était de 17,88 g/L et 41,91% (poids/poids), respectivement, dans la fermentation sans addition d'azote.

1.5.4.2 L'évaluation de la faisabilité par le bilan énergétique, l'émission de gaz à effet de serre, l'estimation du coût

Les résultats expérimentaux obtenus par fermentation avec l'addition d'azote a été introduite pour l'évaluation de la faisabilité. Il a trouvé que le biodiesel produit à partir de lipides a obtenu un gain énergétique net de 2395,89 GJ/lot avec un rapport d'énergie (sortie d'énergie/entrée d'énergie) de 1,78. Le biodiesel a réduit 3355,30 tonnes d'émission de CO_2/lot. L'estimation des coûts a montré que le coût unitaire du biodiesel était de 0,63 (prix de détail 0,90 $/kg). Par conséquent, la production de biodiesel à partir de lipides accumulés par *T. oleaginosus* dans le mélange de boues et glycérol brut avec addition d'azote pourraient être mis en pratique industrielle.

1.6 Conclusions et recommandations

1.6.1 Conclusions

- Dans un milieu synthétique de glycérol brut, le rapport C/N de 30 était optimal pour *T. oleaginosus* afin de convertir efficacement le glycérol brut en biomasse et en lipide. Le

rapport C/N de 45 était optimal pour que *T. oleaginosus* produise une production de biomasse et de lipides plus élevée.

- Sur la base de l'étude cinétique, la production de biomasse était fortement associée à l'utilisation du substrat et l'azote était important pour la production de biomasse élevée et rapide.

- Dans la fermentation fed-batch dirigée par l'étude cinétique des fermentations discontinues, une production élevée de biomasse de 43,82 g/L et une production élevée de lipides de 21,87 g/L ont été atteintes.

- Le méthanol a été capable d'aider *T. oleaginosus* à surmonter l'inhibition des contaminants (principalement des bactéries) à une concentration de 1,4% p/v. Ainsi, la production de lipides à l'aide de *T. oleaginosus* pourrait être accomplie dans des conditions non stériles et la stérilisation pourrait être évitée.

- En raison de l'absence d'enzyme C1 (carbone un), le méthanol ne peut être utilisé par *T. oleaginosus*. La perte de méthanol était due à l'évaporation.

- Le savon était un inhibiteur de la croissance cellulaire de *T. oleaginosus*. Par conséquent, le pH était le paramètre le plus important lorsque du glycérol brut contenant une teneur élevée en savon était utilisé comme milieu de fermentation parce que le pH déterminait la concentration de savon dans le milieu.

- Bien que pH 7 ait été optimal pour *T. oleaginosus*, dans le milieu de l'extrait de levure peptone dextrose, pH 5 a été considéré comme optimal dans cette étude sur la considération de convertir le savon en acide gras libre et d'éviter l'inhibition du savon.

- La fermentation fed-batch, basée sur le pH, pourrait contrôler automatiquement l'alimentation et atteindre une production de biomasse (65,63 g/L) et de lipides (35.79 g/L) très élevée.

- Basés sur la loi de l'énergie de conservation, les cellules oléagineuses étaient considérées comme des systèmes isolés et l'énergie dans les cellules était constante.

- Le substrat à haute teneur en énergie était favorable pour un rendement élevé de lipide en substrat.

- Les méthodes et les stratégies développées dans le milieu synthétique de glycérol brut pourraient être parfaitement employées dans le mélange de boue et de glycérol brut.
- L'étude du bilan, l'estimation des émissions de gaz à effet de serre, l'estimation des coûts a montré que la production de biodiesel à partir de lipides accumulés par *T. oleaginosus* dans le mélange de boue et de glycérol brut a une faisabilité industrielle.

1.6.2 Recommandations

À partir des études, les recommandations suivantes peuvent être envisagées:

- Les méthodes pour prolonger simultanément la croissance de la biomasse et des lipides devraient être découvertes.
- Un fermenteur, dans lequel la biomasse peut être collectée automatiquement, devrait être disgné.
- Le flux d'énergie pendant la production de lipides doit être analysé en utilisant des méthodes expérimentales.
- La production de lipides ou la productivité des lipides, qui détermine le coût de la production de biodiesel à partir de lipides, devrait être étudiée.

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

Biodiesel Production from Oleaginous Microorganisms with Organic Wastes as Raw Materials

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CHAPTER 10 OF THE BOOK: BIODIESEL PRODUCTION (ACCEPTED)

2 BIODIESEL PRODUCTION FROM OLEAGINOUS MICROORGANISMS WITH ORGANIC WASTES AS RAW MATERIALS

2.1 Résumé

Le coût du biodiesel produit à partir de micro-organismes oléagineux hétérotrophes est attribué aux substrats de haute qualité utilisés pour la culture de microorganismes oléagineux hétérotrophes. Afin de réduire le coût, les déchets organiques à faible coût ou gratuits ont été discutés pour la culture des micro-organismes hétérotrophes oléagineux. Les boues municipales et le glycérol brut se sont avérés être de bons choix pour la culture de micro-organismes hétérotrophes oléagineux. Les paramètres, qui affectent l'accumulation de lipides, ont également été examinés. Le pH, le rapport C/N et le mode de fermentation étaient les paramètres les plus importants pour une production élevée de lipides. L'utilisation de déchets organiques et la culture de micro-organismes oléagineux dans des conditions appropriées pourraient amener la production de biodiesel à partir de lipides microbiens à la pratique industrielle.

Mots clés : Biodiesel; micro-organismes oléagineux hétérotrophes; lipide; déchets organiques; paramètres.

2.2 Abstract

The cost of biodiesel produced from heterotrophic oleaginous microorganisms is attributed to the high-grade substrates used for heterotrophic oleaginous microorganisms cultivation. To reduce the cost, low cost or free cost organic wastes from agricultural wastes, industrial wastes and residual wastes were reviewed for the heterotrophic oleaginous microorganisms' cultivation. Municipal sludge and crude glycerol were found to be the good choices for heterotrophic oleaginous microorganisms' cultivation. Parameters, which affect the lipid accumulation, were also reviewed. pH, C/N ratio and fermentation mode were the most important parameters for high lipid production. Using organic wastes and cultivating oleaginous microorganisms under suitable conditions might bring the biodiesel production from microbial lipids to the industrial-scale practice.

Keywords: Biodiesel; heterotrophic oleaginous microorganisms; lipid; organic wastes; parameters.

2.3 Introduction

The current problem of biodiesel production is the high cost of feedstock, vegetable oil and animal fat. In 2014, around 35% crops (weight/weight) (almost the maximum amount can be used to produce biodiesel) of Canada had been used to produce biodiesel. Compared to 2004 (1 million tonnes of biodiesel), the production of biodiesel in 2014 increased 500 times within 10 years, and it was predicted that the production will continuously increase for a long time (around 30 years). There will be a bigger and bigger gap between the biodiesel production (which is limited by the availability of raw material) and the demand for biodiesel. Till 2024, around 900 million tonnes biodiesel will be required in Canada for satisfying the entire consumption.

In order to mitigate the large rely on the raw materials (vegetable oil or animal fat) availability, it is necessary to find some alternative feedstock to produce biodiesel. It has been widely reported that oleaginous microorganisms could be used for producing biodiesel (Sitepu et al. 2014; Munch et al. 2015; Patel et al. 2015). Oleaginous microorganisms including yeast, fungi, bacteria or microalgae, are the ones containing more than 20% lipid of the total dry biomass weight (Liang and Jiang 2013). Some of these oleaginous microorganisms could accumulate high lipid content in the cell under their optimized conditions as summarized in Table 2.1. Compared to plant seeds such as soybean, sunflower, canola, etc. which have oil content around 25 to 35% w/w, oleaginous microorganism (lipid extract up to 70% w/w) is a promising replacement to the traditional raw materials. In addition, the fatty acid compositions of the oleaginous microorganism oil are similar to that of vegetable oil or animal fat (Table 2.2). Once the lipids were extracted from the cells, they could be directly used to produce the biodiesel by the similar process of the vegetable oils and animal fat for biodiesel production. The high lipid content of oleaginous microorganisms and the convenience of direct utilization of their lipid to produce biodiesel have made the biodiesel production from oleaginous microorganisms very attractive.

The biggest obstacle to biodiesel production from an oleaginous microorganism is the high cost (Benemann et al. 2011; Amanor-Boadu et al. 2014; Santander et al. 2014). It

requires around minimum US$10 to produce each gallon of biodiesel from oleaginous microorganisms, but it is only US$3–4/gallon diesel or biodiesel produced from vegetable oil and animal fat (Davis et al. 2011; Delrue et al. 2012; Ramos Tercero et al. 2014). Autotrophic microorganisms (microalgae) use sunlight as driven power to convert carbon dioxide to lipid which requires zero cost in carbon utilization. However, the cultivation requires large land occupation and the lipid accumulation is slow (Meng et al. 2009b; Bellou et al. 2014). Heterotrophic microorganisms are promising to produce lipid due to their ability to accumulate high lipid content (Table 2.1) and rapid growth rate. The process requires carbon source (sugars) and other nutrients as raw materials. High grade/purity carbon source and nutrients are costly, which is the main cause of high biodiesel production cost (Koutinas et al. 2014). In order to lower the cost, alternative carbon and nutrient sources are desired.

Organic wastes from agriculture/other industries and residential areas are rich in carbon and/or nutrients, often available for free or with a low cost, and thus, have been used as raw materials for culturing oleaginous microorganisms. This chapter overviews the sources and properties of these organic wastes, how they have been used for growing oleaginous microorganisms, their bioconversion to biodiesel with the related technologies and processes as well as the parameters that affect lipid accumulation.

2.4 Organic wastes as raw material for oleaginous microorganism cultivation

Organic wastes can be divided into agriculture, industrial, and residential wastes. They generally contain abundant of carbon and/or nitrogen and phosphorous which are essential in microorganism growth.

2.4.1 Bioconversion of Agricultural Wastes into Biodiesel

Agricultural wastes are mainly wood and crop residues. The major compositions of these wastes are fibers (60 to 90%) which include cellulose, hemicellulose, and lignin. There are also crude proteins (3 to 12%) and a little amount of metals (Ca, K, and Na) and phosphate. Agricultural wastes have been used as bioenergy since ancient time and are

still widely used in rural areas today. The major utilization is for heating and generating power by burning (Welling and Shaw 2013). The problems in these applications are the low efficiency and particulate matter air pollution.

Pyrolysis is a popular way to use the lignocellulosic materials for bioenergy and bio-oil. Traditional pyrolysis is a well-established process mainly for producing charcoal. However, fast pyrolysis technology has been given significant attention due to its high bio-oil yield (up to 80% w/w dry matters) with by-products of char and fuel gas, and no waste stream produced (Guda et al. 2015; Yildiz et al. 2015). The bio-oil can be used in boilers, furnaces, engines, and turbines instead of diesel, as well as raw materials to obtain food flavorings, specialties, resins, agro-chemicals, fertilizers, and emissions control agents through extraction (Bridgwater 2012; Lu et al. 2013). In the process, strict control in operation is highly demanded to achieve desired products. In addition, the drying (moisture will go to products) and grinding (required a particle size of less than 6 mm) are expensive. There are also liquefaction and gasification to convert lignocelluloses to lipid or gas fuels and chemicals (Zhang et al. 2011; Kruusement et al. 2014; Elliott et al. 2015). These technologies have intensive energy demand.

The bioprocessing of agricultural wastes into bioenergy such as bioethanol and biogas are mild ways of their utilization. Consolidated bioprocessing (CBP) is the hottest topic about using lignocellulose for producing biofuel. CBP achieves the four steps including saccharolytic enzymes production, polysaccharides hydrolysis, hexose sugars fermentation, and pentose sugars fermentation, in a single reactor (Hasunuma and Kondo 2012). The engineered *Saccharomyces cerevisiae* and *Paecilomyces variotii* are the most studied ones for bioethanol production by CBP (Van Zyl et al. 2007; Khuong et al. 2014; Zerva et al. 2014). In biogas production, pre-treatment to hydrolyze the complex cellulose and hemicellulose is normally performed prior to anaerobic digestion in order to achieve biogas production (Salehian et al. 2013; Zheng et al. 2014). The optimal C/N ratio of biogas production is between 20 and 30 (Mao et al. 2015; Wang et al. 2015a). Agricultural wastes generally have a very high C/N ratio of around 500 to 600; thus, the co-digestion of the lignocelluloses with dairy manure (C/N ratio of 8:1) can be a great choice (Charles and Charles 2006).

Researchers have been inspired by the studies on bioethanol and biogas production with agricultural wastes which indicates that microorganisms could utilize agricultural wastes for cell growth and could produce lipid. The main components of agricultural biomass (cellulose; hemicellulose and lignin) have rather a low biodegradability due to their long carbon chain which is not easily be used by the microorganisms. Hydrolysis is often performed before utilization to degrade long carbon chains to comparable short carbon chains such as sugar, amino acid and fatty acids, which are an ideal carbon source for the microorganisms. There are three main hydrolysis methods: thermal hydrolysis; chemical thermal hydrolysis; and enzyme hydrolysis (Liu 2010). The simplest hydrolysis is thermal hydrolysis.

There are two thermal hydrolysis methods: mild temperature thermal hydrolysis (70 °C for 2 h to 6 h) and high temperature thermal hydrolysis (110 °C or higher for 30 min to 2 h) (Abelleira-Pereira et al. 2015; Huang et al. 2015; Urrea et al. 2015; Xue et al. 2015b). The end products of these two thermal hydrolysis methods have a little bit difference. The mechanism of thermal hydrolysis includes proton adsorption onto the waste biomass, the biomass hydrolysis reactions, soluble substances dissolution, and liquid extraction. Proton adsorption onto the biomass is the essential step in the thermal hydrolysis process. To enhance the performance, addition of protons into the solution of biomass, such as acidic thermal hydrolysis was reported (Hu et al. 2015; Mokni Ghribi et al. 2015). In acidic thermal hydrolysis, pH is generally adjusted to 2 and then subjected to high temperature (> 110°C) for 0.5 to 2 h (Hu et al. 2015). Compared to solo-thermal hydrolysis, the obvious improvement is that the acidic thermal hydrolysis breaks down the lignin more efficiently and hydrolyzes the hemicellulose into perspective monosaccharides. However, by-products such as furfural and hydroxymethylfurfural which are inhibitors of microorganisms can be generated during strong acidic thermal pre-treatment. Thus, strong acidic thermal pre-treatment is rarely performed (Ariunbaatar et al. 2014). Even though acidic thermal pre-treatment is effective and reliable, the facts that the high operation cost, high amounts of acid residues, and high amounts of alkali required to neutralize the acid have prevented this process from being widely used.

In order to reduce the cost and the consumption of chemicals, biological pre-treatment (either anaerobic or aerobic method), with the addition of specific enzymes (Table 2.3)

has been widely applied. Due to higher enzyme production in aerobic treatment, the aerobic biological pre-treatment is more commonly used than anaerobic biological pre-treatment. The critical work of enzyme hydrolysis is to obtain an optimal enzymatic mixture (generated or added during pre-treatment) which is used to degrade the agricultural biomass (Bussamra et al. 2015). Compared to thermal hydrolysis and acid thermal hydrolysis, the enzyme hydrolysis needs additional specific enzymes which may cause high cost and long fermentation time (120 h to 200 h) (Ghorbanpour Khamseh and Miccio 2012).

Once the agricultural biomass is hydrolyzed, it can be separated into the solid part (residual biomass) and a liquid portion. In the residual biomass, it contains lipid which could be extracted and used as feedstock for biodiesel production. On the other hand, the liquid part of the hydrolysis contains almost all the necessary nutrients for the growth of microorganisms (Heller et al. 2015). It indicates that no additional chemical mediums would be needed for microorganism growth. After being diluted to a proper portion, the liquid portion can be directly used to feed the microorganisms. Several studies have successfully produced lipids by cultivating oleaginous microorganisms in the liquid portion obtained by hydrolysis of agriculture biomass (Table 2.4).

Oleaginous yeast and fungi are the most employed microbes for lipid production due to their high lipid accumulation (up to 70% w/w) within short cultivated time (30–288 h) and high lipid productivity (0.33 g lipid/g waste). However, utilization of agriculture wastes for lipid production is still not widely studied as the requirement of pre-treatment is very complicated.

2.4.2 Bioconversion of industrial wastes to biodiesel

Industrial practices bring significant benefits to the society; to satisfy the increasing human demands, the industrial activities will continue to go on with a trend to increase more wastes which can cause serious environmental problems in the near future (Cheirsilp and Louhasakul 2013). Industrial wastes contain industrial waste air, wastewater and waste residue. The best way of managing or reducing industrial waste air effluent is to utilize renewable energies to replace the fossil fuels, which are currently

employed in industries. Due to the heavy dependence on the industry in our generation, the management of industrial wastewater and waste residues become great challenges.

To meet the current environmental regulations, the industrial companies have to sacrifice part of their profits to pre-treat their industrial wastes. However, the management approach is generally costly as the treatment plant is in small-scale and low efficiency. In addition, most of the treatments are aimed to fastly get rid of the waste and neglect the possibility to reuse some of them to produce value-added produces (Lee et al. 2014). Many of the wastes (water or residue) have great value as raw materials for producing new materials or products. A large proportion of the industrial wastes are organic wastes. Some of them, such as effluent from food companies, effluent from commercial farms, crude glycerol and so on (Table 2.5) have high biodegradability. It suggests that they can be used by microorganisms for growth. Studies have been performed to utilize industrial organic wastes (wastewater and waste residues) to grow oleaginous microorganisms to produce lipid (Table 2.5). The process reduces the waste amount and produces feedstock of biodiesel production.

Most of the industrial organic wastes are already ground and thermal hydrolyzed during the production process, no further pre-treatment is needed to enhance their biodegradability. However, for some special wastes, pre-treatment is required to make the wastes more suitable for growing microorganisms. For instance, alkaline thermal hydrolysis is normally used to pre-treat the piggery wastewater due to its high concentration of nitrogen (Marjakangas et al. 2015). After being pre-treated, mostly nitrogen (up to 70%) can be removed by evaporation. The residual solution can be used for oleaginous microorganism growth to produce lipid.

Due to the increase in biodiesel production, crude glycerol as an unavoidable by-product of biodiesel production through trans-esterification has gained more attention. Roughly 50 million tonnes crude glycerol was generated in Canada in 2014. Crude glycerol is a promising carbon resource for the oleaginous microorganisms; it could be facilely up-taken by these microorganisms to accumulate high amounts of lipid (Table 2.5). Using crude glycerol as a substrate to feed the oleaginous microorganisms is attractive because this process not only manages the by-product of biodiesel but also efficiently produces

more lipids for the biodiesel production. The only problem is that crude glycerol does not have all the substrates that should be provided for the growth of microbes. Some chemical nutrients or other wastes should be added to supply all the necessary nutrients.

Apart from oleaginous yeast and fungi which are widely used to produce lipids, microalgae are another type of microbes employed for lipid production by consuming industrial organic wastes (mainly organic wastewater). Organic wastewater generated in the industrial process is in large quantity. It is very difficult and costly to transfer it to some other places. Hence, it is preferable to treat/convert industrial organic wastewater to other products *in situ*. Moreover, simple process and facilities would be more attractive for industries. Compared to other types of microorganisms, microalgae need less extra facilities, and the operation process is simple. After a long cultivating time (more than 200 h), the microalgae could accumulate acceptable lipid content (40–60% w/w), but the lipid productivity is sometimes low.

2.4.3 Bioconversion of residual wastes to biodiesel

Residential waste that can be used to cultivate microorganisms includes waste cooking oils; municipal solid waste; municipal activated sludge and municipal wastewater. Waste cooking oils can be directly transferred to biodiesel using chemical method (trans-esterification) after necessary refining. Using waste cooking oils to produce biodiesel is economically attractive as the production cost is lower than the market price. In fact, waste cooking oil is also a carbon source which can be used to feed oleaginous microorganisms for producing lipid which can be then converted to biodiesel. However, biodiesel production through this process would be less attractive than that directly using waste cooking oil for biodiesel production as it costs more due to the extra process for growing oleaginous microorganism (Yahyaee et al. 2013; Karmee et al. 2015).

Municipal solid waste is normally a mixture of various organics from food processing plants, domestic and commercial kitchens, cafeterias and restaurants (Uçkun Kiran et al. 2014). The municipal solid waste contains around 40% food waste or putrescible which contains up to 30% lipid. Lipid from food waste can be separated by solvent extraction. Even though this method is effective and efficient, the recovery of solvent and the lipid

separation are costly; in addition, the solvent used to extract the lipid is harmful to the environment (Karmee et al. 2015). Nowadays, anaerobic digestion is the common method used to manage the municipal solid waste. The anaerobic digestion process could produce methanol as a source of energy, and the residues could be used for fertilization (Suwannarat and Ritchie 2015). During the anaerobic process, some organic materials were biodegraded to volatile free fatty acids, which could be used by oleaginous microorganisms to accumulate lipid in the cells. Recent research showed that a lipid productivity of 0.15 g lipid/g free fatty acid could be reached by utilization of the effluent of anaerobic digestion (Vajpeyi and Chandran 2015).

Municipal activated sludge is widely generated in the municipal wastewater treatment plant. In order to reduce the COD (chemical oxygen demand), BOD (biological oxygen demand), TN (total nitrogen), TP (total phosphorous) and trace elements in the municipal wastewater, aerobic activated microorganisms are commonly used to treat the municipal wastewater. Most of the environmental toxic elements are captured and used by microorganisms. After removing these microorganisms (sedimentation), the environmental toxic elements can be reduced, and the effluent of the municipal wastewater treatment plan can be discharged into the receiving water body. However, during wastewater treatment, there is a large quantity of wastewater sludge generated. Due to its high quantity, sludge must be treated correctly and then disposed of. Municipal activated sludge contains a very high concentration of organic waste, which can be up to 90% w/w, and hazardous metals. Directly dumping sludge to the landfill would cause high greenhouse gas emissions as there is no methane capture, and the leaches lead to groundwater pollution once they reach groundwater. The main current technology used to manage municipal wastewater sludge is anaerobic digestion. After being concentrated, at temperature 30–60 °C and oxygen-limited conditions, sludge can be bio-converted to biogas in a hermetic tank. The biogas generated can be used as heating energy. This management can reduce the quantity of sludge; however it is not efficient for three reasons: 1) even though the sludge contains high organic wastes which are mainly the cell of aerobic microorganisms, the biodegradability of the sludge is low. Thus, either some pre-treatments should be introduced or longer digestion time should be performed; 2) due to the request of long digestion time, the hermetic tank used for anaerobic digestion

usually has a large dimension; and 3) because anaerobic digestion mainly reduces organic wastes, the hazardous metals are still in the residues, and are concentrated. The further management of the residues is more difficult to prevent pollution of hazardous metals.

Studies have revealed that municipal wastewater sludge can be used as a substrate to culture oleaginous microorganisms (mainly yeast and fungi). To increase the biodegradability of the sludge, pre-treatments, such as thermal hydrolysis, acid thermal hydrolysis, enzymatic hydrolysis and alkali thermal hydrolysis, are introduced (Olkiewicz et al. 2015; Tian et al. 2015). When the concentration of the sludge used to feed the microorganism is high, the possibility of inhibition in microorganism growth is high as the amounts of metals in the sludge medium are high. Pre-treatments such as wash and centrifuge are used to remove the inhibitors and to enhance the lipid accumulation (Zhang et al. 2014a).

Since both the treatment of municipal wastewater and the management of municipal wastewater sludge are complicated, some researchers proposed to use municipal wastewater directly to cultivate oleaginous microorganisms for lipid production. Previous research has tested microalgae to accumulate the lipid using wastewater as culturing water (Cabanelas et al. 2013). However, there are still many problems in the process, such as large cultivation area, long fermentation time, and the lipid content (less than 30%) due to the low concentration of carbon and nutrients. In order to solve the problems, utilization of concentrated municipal wastewater to feed oleaginous yeast or fungi should be studied.

2.5 Parameters affecting lipid accumulation

Among all the aforementioned substrates, municipal wastewater sludge, wastes from sugar production companies and crude glycerol are the raw materials which are frequently used as feedstocks of oleaginous microorganisms, due to their promising carbon resources and high daily generation amounts. Oleaginous yeast and fungi are mostly used in the current research for lipid accumulation. As shown in Tables 2.4 to 2.6, even using the same oleaginous microbe and the same substrate, different results have

been reported of lipid content and lipid productivity. Due to the current low lipid accumulation, the lipid production by oleaginous microorganisms is still in the lab-scale. There should be some essential factors which can highly affect the lipid accumulation. These factors are determined as affecting parameters. By controlling the parameters in an optional range, the lipid accumulation could be enhanced. Therefore, understanding the effects of the parameters on lipid accumulation may take the lipid production by oleaginous microorganisms into the industrial scale.

The parameters mainly affect the lipid production in two ways: one is on the cell growth and the other is on the lipid accumulation. The bright side is, as reported, lipid is mixed-growth associated product of oleaginous microbes (Amaretti et al. 2010). More cells generated in the growth phase could lead to more lipid accumulation in the lipogenic phase. The practical method is to growth the cells as much as possible in the growth phase and to let them accumulate lipid by controlling the parameters in certain conditions. To meet these aims, many parameters should be taken in to consider. The most effective ones are pH; temperature; dissolved oxygen (DO); carbon to nitrogen (C/N) ratio; carbon and nitrogen sources; trace elements; and fermentation mode.

2.5.1 pH effects

pH is one of the most important parameters which can highly affect the cell growth of the microorganisms. Firstly, at a suitable range of pH, certain enzymes in the microorganisms are activated (Figure 2.1). In fact, the microorganisms absorb substrates fast at the suitable range of pH; thus, their division is enhanced, and more cells of microorganisms could be obtained in a shorter time. Secondly, pH can affect the form of ammonia and phosphorous. The major form of ammonia is $NH_3 \cdot H_2O$ at high pH and NH_4^+ at low pH. There are always four forms of inorganic phosphorous: H_3PO_4, $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}, and the composition of them varies with pH. As microorganisms need mostly NH_4^+, $H_2PO_4^-$ and HPO_4^{2-} to grow, the pH condition is very important to make sure these three forms are in the right proportion. Thirdly, pH can affect the concentration of heavy metals. Heavy metals exit normally as irons in the water under the acidic pH condition and as precipitates under the alkaline pH condition. Low concentration of heavy metals may enhance the cell growth of the microorganisms, but high concentration of heavy metals

could cause inhibition. Alkaline pre-treatment is a common method to eliminate the overload of heavy metal. Moreover, pH can affect the proton pumping rate. Accompany with the cell growth, lot of protons are generated in the cells, and the cells need to pump the proton out of the cells; this process contributes most of the energy generated (80%) by the cell growth. When pH is low, the electrical potential difference between the two sides of the cell (inside the cell and outside the cell) is little, and the proton pumping process consumes more energy; otherwise this process consumes less energy. Overall, it showed that pH can significantly affect the cell growth of microorganisms.

2.5.2 Temperature effects

Temperature can highly affect the cell growth of the microorganisms. As shown in Figure 2.2, all microorganisms have their own optimal growth temperature in which they grow at the highest rate. The suitable temperature varies between different types of microbes. There are five classifications of microorganisms according to their optimal growth temperature (Gentina et al. 1978):

Hyperthermophile (optimal growth temperature 80°C and up to 120°C)

Thermophile (optimal growth temperature between 50°C and 85°C)

Mesophile (optimal growth temperature between 30°C and 40°C)

Psychrotroph (optimal growth temperature between 15°C and 30°C)

Psychrophile (optimal growth temperature between 10°C and 15°C)

Regardless of the enzyme activity under optimal growth temperature, the different membrane structures are another main reason why microorganisms have different optimal growth temperature. The membrane of most microorganisms is composed of either saturated or unsaturated fatty acid. Normally, saturated fatty acid is in a solid phase, while unsaturated fatty acid is in a liquid phase at room temperature (20 °C to 25 °C). Thus, the temperature can affect the fluidity of the membrane which highly affects its function. For hyperthermophile that can undertake very high temperature, the structure of its membrane is unique, mainly composed of C5 compound, phytane, and isoprenoid substance. These substances can remain stable at very high temperature.

Temperature can affect dissolved oxygen concentration. When the temperature goes up, the solubility of most airs such as oxygen goes down (Figure 2.3). Under higher temperature, the available dissolved oxygen is lower. The further discussion of this effect is posted in DO effect (see below).

Temperature can affect the solubility of heavy metallic salts. In most cases, the solubility of heavy metallic salts has a positive correlation with the temperature. Higher temperature provides higher solubility of most heavy metallic salts. Overall, the temperature is a very important parameter which should be taken into consideration in cell growth and lipid accumulation in microbes.

2.5.3 DO – agitation and aeration effects

DO is the parameter which is affected mostly by the temperature of water (temperature goes up, DO goes down), pressure of the air (pressure goes up, DO goes up also), salinity of water (more salinity in the water cause less DO in the water), and the microorganism activities (more aerobic microorganisms could cause less DO).

To determine the DO effect on the microorganisms, two more parameters are involved: OUR (oxygen uptake rate) and OTR (oxygen transfer rate). The OUR is used to describe the activities of aerobic microorganisms. Normally the more aerobic microorganism there are in the water, the faster OUR occurs. OUR and cell mass concentration of aerobic microorganisms are in linear correlation, OUR = QO_2X (QO_2 is the specific rate of oxygen consumption and X is the cell mass concentration). OTR describes the oxygen transferring from air to water, the transportation only occurs at the interface between air and water. OTR = KLa×(C* - C), where KLa is the volumetric oxygen transfer coefficient (h^{-1}) which is determined by the interfacial resistance. C* and C is the saturated and actual oxygen concentration in the water, respectively. The actual concentration of oxygen is also the current oxygen which is available for the activity of aerobic microorganisms. The high activity of aerobic microorganisms requires that the actual concentration of oxygen remains at a high level. The relationship between the actual concentration of oxygen C and other parameters (all defined before) can be described as below (Eq. 2.1) (Schuchardt et al. 2005):

$$C = C^* + OTR - OUR \quad \text{(Eq. 2.1)}$$

From Eq. 2.1, it is obvious that there are three methods to increase the actual concentration of oxygen: 1) increase the saturated concentration of oxygen in the water; 2) increase the OTR; and 3) decrease the OUR. Firstly, the saturated concentration of oxygen is determined by the surrounding environmental conditions, it can't be changed without the modification of environmental conditions. Secondly, our main aim is to let the aerobic microorganisms grow as much as possible, so the OUR increase gradually during the growth phase. It is impossible to increase the cell of aerobic microorganisms and to decrease the OUR. Therefore, the only way to increase the actual concentration of oxygen is to increase the OTR.

To increase the OTR, there are three functional parameters, KL_a, C* and C. C* and C are fixed at each instant moment. The only parameter that can be modified is KLa. Based on the two-film theory, the oxygen is transferred from air phase to interface (a supposed phase which is between air phase and water phase) in the first step and then transferred from interface to water phase. It is recognized that the concentration of oxygen in the interface is stable and invariable. The crucial step is the mass transfer from air phase to the interface. There are several approaches to enhance this step: 1) by enlarging the interface between air phase and interface so that more oxygen in air phase can contact a larger interface. Some aeration models such as flat aeration and jet aeration have been designed based on this consideration; 2) by enhancing the concentration of oxygen in the air. For example, pure oxygen or compressed air in the air phase can lead to faster mass transfer to the interface; 3) by increasing the agitation rate to reduce the viscosity coefficient of water and make the interface between air and water thinner, and thus the transferring rate is enhanced; 4) by increasing the temperature. This is a special method. A higher temperature could enhance the OTR as temperature goes up, the water ions are activated, and thus, the viscosity coefficient of water goes down, which then enhances the transferring rate. But temperature can affect not only the viscosity coefficient of water but also solubility of dissolved oxygen, which is also a determinate parameter of oxygen transferring rate.

2.5.4 C/N ratio effects

With the increase in C/N ratio, different metabolic behaviors happen in the oleaginous cells. Under carbon limitation, lipid accumulated in the cells could be digested to generate energy for supporting cell activities. Under the balanced carbon furnishing condition, oleaginous microorganisms can rapidly generate cells (cell numbers increase) rather than lipid accumulation. In the carbon excess condition, part of the carbon is directly used for lipid accumulation. This is the ideal carbon condition to be used to generate the lipid by oleaginous microorganisms. With an overdose of carbon, inhibition occurs, and additionally, the oleaginous microorganisms use the carbon to generate high amounts of organic acid (citric acid) and only part of lipids in the cells (Azim et al. 2008; Abu Bakar et al. 2015).

Heldal et al. (1996) have reported that a C/N ratio between 5 and 10 is superior for the cell growth. It was found out that the C/N ratio of the dry cell is around 5 (153:33) at the exponential growth phase (Heldal et al. 1996) which explains why a C/N ratio of 5 to 10 enhances cell division. Similar C/N ratios (5-10) are commonly used to cultivate the oleaginous microorganisms during the cell exponential phase. However, the optimal C/N ratio for lipid accumulation is very different in different studies. Normally, the carbon sources which are easier to be used by the cell have a smaller C/N ratio. For example, the widely used C/N ratio for lipid accumulation by feeding glycerol as carbon source is between 30 and 60 (Liang et al. 2010; Duarte et al. 2013b), while it is 120 or even more if lignocellulosic waste is used as a carbon source (Ruan et al. 2012; Cheirsilp and Kitcha 2015; Patel et al. 2015).

Among all kinds of carbon sources which have been used as feedstock for lipid accumulation in the oleaginous microorganisms, glucose, glycerol and molasses are the best carbon sources for cell growth and lipid accumulation (Gautam et al. 2013). It is also observed that carbon sources with short carbon chains are superior to those with long carbon chains. Pre-treatments (thermal, acid thermal, alkaline thermal, and enzyme hydrolysis) have been often performed to break the long carbon chain to short carbon chain.

Herman Campos et al. (Campos et al. 2014) utilized five nitrogen sources, ammonium chloride (NH_4Cl), ammonium hydroxide (NH_4OH), sodium nitrate ($NaNO_3$), urea (CH_4N_2O), and a mixture of all these sources, to evaluate the biomass yield and the lipid content by the oleaginous microalgae *Nannochloropsis salina*. The highest biomass yield was observed by using sodium nitrate ($NaNO_3$) or the mixture; this was also observed by Wu et al. (Wu et al. 2013). The highest lipid content was observed by using the mixture as nitrogen sources.

In addition to the optimal C/N ratio, the components of carbon sources so as the nitrogen sources are also important factors affecting biomass and the lipid productivity of oleaginous microorganisms. For certain oleaginous microorganisms, the optimal C/N ratio can vary in a large range due to different feeding substrates.

2.5.5 Trace elements effects

Trace elements are essential micronutrients that the microorganisms require in minute quantity for their normal functions, mainly including chromium, cobalt, copper, fluorine, iodine, iron, manganese, molybdenum, selenium and zinc (Strachan 2010). These trace elements are normally essential to the cell growth of microorganisms, but some of them are also toxic to the microorganisms (Table 2.7). The constitution of each of these trace elements is generally less than 0.01% of body mass.

Of these trace elements, iron and zinc are particularly important for obtaining the optimum growth of strain. The strain cultivated with the addition of trace elements grow more and faster than that without trace elements (Zhang and Jahng 2012; Nagano et al. 2013). Even though trace elements such as iron, zinc, and magnesium are needed for cell composition, an overdose of them can also cause inhibition, because the activity of enzymes is repelled or even stopped in the high concentration of trace elements (Knežević et al. 2014). It is important to control them under proper concentrations. Alkaline precipitation is a promising method to remove overdosed trace elements.

2.5.6 Fermentation mode effects

There are three fermentation modes: batch, fed-batch and continuous fermentation. In the conventional batch process, all the nutrient substrates are filled into the reactor at the beginning, and the microorganism is inoculated into the fermenter. During fermentation, nothing except oxygen, antifoam agent, and acid or base (employed to control the pH) are added to the reactors. When product (lipid) productivity reaches the highest, the fermentation will be stopped. Microorganisms will be harvested for further processing. The conventional fermentation process is easy to be prepared and performed, but the culture age during the fermentation is not able to be changed. The strain in the batch mode goes through all the four phases: lag phase, log phase, stationary phase and death phase. The strain grows fast in the log phase, but the production mainly happens in the stationary phase. The concentration of nutrient substrates has a big difference in the growth of strain and the production. Generally, the high production needs a high concentration of nutrient substrates; however, high concentration substrates can inhibit the growth of strain. In order to achieve high cell division and high lipid accumulation in the batch process, the eclectic concentration of nutrient substrates is used. However, this eclectic concentration can neither growth maximum cells number nor accumulate maximum product. Thus, the productivity of the strain is generally not effective in the batch culture.

As to prevent nutrient depletion, to achieve high cell density and to produce high metabolite of the microorganisms, fed-batch is introduced to meet these goals. Fed-batch culture is the biotechnological process where two or more nutrient substrates are fed to the reactor during the cultivation. Normally, a basic medium is added to the reactor at the right beginning to support the cell growth, followed by the addition of nutrient substrates to maintain the concentration of nutrient in the desired level. Fed-batch culture is superior to conventional batch culture when the nutrient concentration can highly affect the productivity (in this case lipid accumulation in the cells).

For both batch culture and fed-batch culture, the culture ends at the point of harvesting. The desired product cannot be generated continuously. In spite that another run follows by the harvesting, the strain in the reactor needs to go through one more round of the four

growth phases. The lag phase and log phase are time-consuming. In continuous fermentation, the strain in the stationary phase should be maintained in order to continuously produce the product. The nutrient substrates are continuously fed into the reactor, and the broth in the reactor is withdrawn at the same flow rate. The medium in the reactor maintains in a stable volume, and the product is continuously generated and harvested. This process is rarely used in the lipid production, because the process of lipid production is critical, continuously batch culture can be forced to stop due to the contamination. Compared to batch mode, fed-batch and continuous batch usually have higher productivity, but they also have longer fermentation time than batch mode.

2.6 Case study

Based on the limited literature (Ratledge, 2004; Meng, 2009), the first and only commercial single cell oil production factory was carried out in a pilot plan in Serberberg, Austria in 1985. The oleaginous fungus *Mucor circinelloides* had been inoculated in stirred-tank fermenters of 220 m^3. Harvesting and drying of the biomass had been followed by oil extraction, refinement and purification. This factory was operated for around 6 years and finally was forced to be shut down as there was no more benefit thereafter. As the price of crude oil is the essential factor of biodiesel generation and perish, by reviewing international statistical yearbook of these years, it explained why that the plant had to be stopped: in 1979, OPEC (Organization of the Petroleum Exporting Countries) cut the production of crude oil, which directly pushed the price of crude oil to go up sharply. In less than 3 years (1979 to 1981), the price of crude oil went from around 40 US dollar/barrel to around 100 US dollar/barrel. The high price of crude oil made the production of single cell oil to be cost-effective, and thus, the first factory was appeared due to the demand in 1985. However, one year after the biodiesel production plant appearance, the OPEC chattel collapsed in 1986, which made the price of crude oil go down immediately to less than 30 US dollar/barrel. The single cell oil production company had to close the company in 1991 as the biodiesel production cost was no more attractive as petrodiesel.

Since the first single cell oil production company was shut down, no more biodiesel production from the oleaginous microorganism company has been taken in practice. The production using oleaginous microorganisms has been limited in the lab. Nevertheless, the researchers have never given up the opportunity to bring them to industrial practice. They have become more cautious. Cost and technical evaluations have been employed to guide the further research and the possibility of industrial utilization.

Among the evaluation reports, the most effective report was made by Koutinas et al. (Koutinas et al. 2014). The optimal conditions for lipid production from *R. toruloides* (Table 2.8) were employed in their evaluation. It was assumed that the optimal condition could be realized in real practice. This evaluation was aimed to discover the potential of the lowest production price of lipid in the industrial practice. The research has revealed three great factors which can highly affect the microbial lipid price: 1) the price of feedstock. It showed that the microbial lipid price is 11.3 US dollar/gal with cost-free feedstock compared to 18.3 US dollar/gal with feedstock cost of 400 US dollar/tonne; 2) the lipid productivity. The microbial lipid price can be reduced from 11.3 US dollar/gal to 5.33 US dollar/gal (with feedstock cost-free) with the lipid productivity increased five times, and 3) fermenter cost. The most costly instrument of the process is the fermentation reactor, which contributes 74% of the total instruments of the lipid production process and the operation of the reactor contributes 49% of the total operating cost.

The study indicates that there are three approaches to reducing the microbial oil production price: by replacing the nutrients with cost-free substrates; by enhancing the lipid productivity; by reducing the fermentation reactor investment (Koutinas et al. 2014). However, the study didn't revise the fermentation time which affects the microbial oil price. In fact, fermentation time can affect the fermentation reactor volume (shorter fermentation time requires smaller fermentation reactor). Thus, fermentation reactor can be an important factor in the final lipid production cost.

2.7 Challenges and future perspectives

Since the price of biodiesel produced from oleaginous microorganisms is the main obstacle which prevents it from industrial practices, the current work should focus on

reducing the production price. The feasible methods would be finding cost-free substrate replacement, enhancing lipid productivity and reducing fermentation reactor volume. If lipid productivity and fermenter volume reduction could be done at the same time, it would reduce the lipid production cost. To achieve this, the operation conditions and the microorganism growth conditions should be studied and controlled in the optimal range, so that the microorganisms may accumulate high lipid in a short time. Simplifying the operation condition may also reduce the cost in some parts. Further research can be introduced on eliminating the sterilization and on performing *in situ* lipid extract.

2.8 Summary

The major obstacle of biodiesel production from microorganism lipid is the high cost. Utilization of organic wastes generated in agriculture, industries, and human activities to grow oleaginous microorganisms is a promising strategy of reducing biodiesel production cost. Compared to agricultural wastes, industrial and residential wastes are more suitable substrates and/or nutrients for oleaginous microbe production as they generally can be directly used without pre-treatment. Enhancing lipid productivity is another way of reducing biodiesel (from lipid) production cost. By controlling the cultivation condition including pH, DO, C/N ratio, temperature, trace element, and fermentation modes, one can improve lipid productivity. The combination of using organic wastes and cultivating oleaginous microorganisms under suitable conditions would bring the biodiesel production from microbial lipid to the industrial-scale practice.

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Table 2. 1 Typical oleaginous microorganism and their lipid contents

Type	Name	Lipid content (%)	Ref.
Bacteria	*Arthrobacter sp.*	40	Meng et al. (2009a)
	Acinetobacter calcoaceticus	38	Meng et al. (2009a)
	Rhodococcus opacus	25	Meng et al. (2009a)
	Bacillus alcalophilus	24	Meng et al. (2009a)
Microalgae	*Chlorococcum sp*	32.5	Tongprawhan et al. (2014)
	Isochrysis galbana	42	Converti et al. (2009)
	Monoraphidium dybowskii	43.47	He et al. (2015)
	Ankistrodesmus falcatus	59.6	Singh et al. (2015)
	Chlorella sp.	33.6	Han et al. (2015)
Yeast	*Cryptococcus sp*	61.53	Tanimura et al. (2014)
	Pichia guilliermondii Pcla22	60.6	Wang et al. (2012a)
	Kodamaea ohmeri	53.28	Kitcha and Cheirsilp (2011)
	Yarrowia lipolytica	61.7	Tai and Stephanopoulos (2013)
	Candida sp	56.58	Duarte et al. (2013a)
Fungi	*Alternaria alternata*	40.7	Bagy et al. (2014)
	Epicoccum purpurascens	70	Koutb and Morsy (2011)
	Phaeodactylum tricornutum	57.8	Xue et al. (2015a)
	Mortierella alpina	60.4	Eroshin et al. (2000)
	Rhodotorula glutinis	47.2	Liu et al. (2015)

Table 2. 2 Fatty acid compositions in oleaginous microorganisms and vegetable oils

Name of the lipid	Chemical Component	A fraction in total (% w/w)		Ref.
		Plant seeds	Microorganisms	
Palmitic acid	C16H32O2 (C16:0)	7-16	7-37	Ykema et al. (1988); Meng et al. (2009a); Kim et al. (2010a); Gao et al. (2013); Ryu et al. (2013a); Zhang et al. (2014b)
Palmitoleic acids	C16H30O2 (C16:1)	0-1	1-57	
Stearic acid	C18H36O2 (C18:0)	3-5	1-12	
Oleic acid	C18H34O2 (C18:1)	17-75	20-81	
Linoleic acids	C18H32O2 (C18:2)	7-67	3-40	
Linolenic acid	C18H30O2 (C18:3)	1-13	1-30	
Arachidic acid	C20H40O2 (C20:0)	0-1	0-1	

Table 2. 3 Parts of enzyme hydrolysis

Enzyme name	Substrate	microbe	Type of microbe (aerobic or anaerobic)	Ref.
β-galactosidase	Lactose	*Bacillus circulans*	anaerobic	Das et al. (2015)
β-1,4-xylanases	cellulose pulps	*Aspergillus terreus*	Aerobic	Moreira et al. (2015)
Amyloglucosidase	Wheat bran	*Aspergillus niger*	anaerobic	Gupta et al.
ß-xylosidases	lignocellulose	*Neurospora crassa*	Aerobic	Meleiro et al. (2015)
Cellulolytic enzymes	Sugarcane bagasse	*Trichoderma reesei*	Aerobic	Gasparotto et al.
Cellic CTec2	Corn stover	*Trichoderma reesei*	Aerobic	Lin et al. (2015)

Table 2. 4 Agriculture wastes for lipid production with oleaginous microorganisms

Agriculture Wastes	Pre-treatment	Microbes	Time (h)	Lipid content (% w/w)	Lipid productivity (g/g wastes)	Ref.
fruit pulp	Thermal	*Rhodosporidium kratochvilovae*	144	53.2	0.008	Patel et al. (2015)
Sweet potato leaves, stems and stalks	Enzymatic hydrolysis	*Trichosporon fermentans*	168	35	0.27	Zhan et al. (2013)
Sweet potato leaves, stems and stalks +wheat straw	Acidic thermal hydrolysis	*Trichosporon fermentans*	168	65	0.23	Zhan et al. (2013)
Sugarcane bagasse	Acidic thermal hydrolysis	*Yarrowia lipolytica*	120	60	0.33	Tsigie et al. (2011)
Corncobs	Enzymatic hydrolysis	*Trichosporon dermatis*	168	40	0.17	Huang et al. (2012a)
Rice straw	Acidic thermal hydrolysis	*Trichosporon fermentans*	192	40	0.12	Huang et al. (2009)
Sugarcane bagasse	Acidic thermal hydrolysis	*Trichosporon fermentans*	192	40	0.14	Huang et al. (2012b)
Wheat straw	Acidic thermal hydrolysis	*Cryptococcus curvatus*	144	33.5	0.14	Yu et al. (2011)
Sweet sorghum	-	*Mortierella isabellina*	96	11	0.09	Economou et al. (2010)
Corn fiber	Enzymatic hydrolysis	*Mortierella isabellina*	144	46	0.18	Xing et al. (2012)
Corn cobs	Acidic thermal hydrolysis	*Trichosporon coremiiforme*	192	37.8	0.17	Huang et al. (2013)
Corn stover	Acid- and alkali- thermal followed by enzymatic hydrolysis	*Mortierella isabellina*	84	30	0.14	Ruan, (2014)
Sugar beet pulp	Enzymatic hydrolysis	*Trichosporon fermentans*	96	34.4	0.11	Wang et al. (2015)
Sugar beet pulp	Enzymatic hydrolysis	*Cryptococcus curvatus*	96	43.6	0.14	Wang et al. (2015)
Sugar beet pulp	Enzymatic hydrolysis	*Trichosporon cutaneum*	96	42.1	0.14	Wang et al. (2015)
Papaya waste	Acidic thermal hydrolysis	*Chlorella protothecoides*	110	60	0.021	Heller et al. (2015)
Corncob hydrolysate	Acidic thermal hydrolysis	*Rhodotorula glutinis*	72	36.4	0.05	Liu et al. (2015)

Sweet sorghum crops	Acidic thermal hydrolysis	*C. curvatus*	96	52.5	0.11	Cui and Liang (2015)
Sweet sorghum	-	*Lipomyces starkeyi*	120	47.3	0.131	Matsakas et al. (2014)
Cassava starch	Hydrolysis by the crude amylase preparation	*Rhodosporidium toruloides*	144	63.2	-	Wang et al. (2012c)
Algal residue	Hydrodynamic cavitation under alkaline	*Cryptococcus curvatus*	30	21	0.08	Seo et al. (2014)
Sweet sorghum bagasse	enzymatic hydrolysis	*Cryptococcus curvatus*	72	63.98	0.11	Liang et al. (2012)
Hemp seed aqueous extract	-	*R. kratochvilovae HIMPA1*	216	55.56	-	Patel et al. (2014)
Waste sweet potato vines	washed, air-dried, and milled	*Trichosporon*	288	35.6	0.128	Zhan et al. (2013)
Corncob waste liquor	Acidic thermal hydrolysis	*Aspergillus sp*	96	23.33	-	Venkata Subhash and Venkata Mohan (2011)
Lignocellulosic biomass	Acidic thermal hydrolysis	*Mortierella isabellina*	144	53	0.17	Zeng et al. (2013)
Sugarcane	Ground to powdered	*Citrobacter youngae*	72	53.9	-	Maymandi and Rahimpour (2015)
Bagasse hydrolysate	Acidic thermal hydrolysis	*T. fermentans*	288	39.9	0.117	Huang et al. (2012b)
Lignocellulosic palm	-	*TSIP9*	288	34.2	0.0375	Kitcha and Cheirsilp (2014)
Sugarcane bagasse	Acidic thermal hydrolysis	*Y. lipolytica*	168	58.5	-	Tsigie et al. (2011)
Sugarcane bagasse	thermal hydrolysis	*C. protothecoides*	192	34	0.093	Mu et al. (2015)
Corncob residues	Acidic thermal hydrolysis	*T. cutaneum*	120	29.1	0.144	Gao et al. (2014)

Table 2. 5 Industrial wastes for lipid production with oleaginous microorganisms

Industrial Wastes	Pre-treatment	Microbes	Time (h)	Lipid content (% w/w)	Lipid productivity (g/g wastes or L wastewater)	Ref.
Stearin, hydrolyzed oleic rapeseed oil	-	*Yarrowia lipolytica*	64	44	0.38	Papanikolaou et al. (2001)
Starch wastewater	-	*Rhodotorula glutinis*	60	35	3.9	Xue et al. (2010)
Monosodium glutamate wastewater	-	*Rhodotorula glutinis*	72	20	13.6	Xue et al. (2008)
Butanol fermentation wastewater	-	*Trichosporon cutaneum*	192	19	0.081	Chen et al. (2012)
Bagasse hydrolyzate	-	*activated sludge microorganisms*	168	47.3	0.139	Mondala et al. (2015)
Biodiesel-derived glycerol	-	*R. diobovatum*	120	50	0.118	Munch et al. (2015)
Biodiesel-derived glycerol	-	*R. babjevae*	120	24.2	0.04	Munch et al. (2015)
Brewer fermentation waste and crude glycerol	Thermal and filtration	*Chlorella protothecoides*	168	50.6	-	Feng et al. (2014)
Microbial lipid production wastes	-	*R. toruloides Y4*	120	60	0.19	Yang et al. (2015)
Flour-rich waste streams	-	*A. awamori 2B*	200	40.4	0.06	Tsakona et al. (2014)
Piggery wastewater	Alkalic thermal hydrolysis	*C. vulgaris*	384-480	34.7	-	Marjakangas et al. (2015)
Ketchup	-	*Ochromonas danica*	60	20	-	Lin et al. (2014)
Brewery waste	-	*Yarrowia lipolytica*	24	30.1	-	Poli et al. (2014)
Dairy wastewater		*R. opacus*	100	52	-	Kumar et al. (2015)

Pectin-derived carbohydrates	enzymatic hydrolysis	*Cryptococcus curvatus*	48	47.9	0.11	Wang et al. (2015b)
Potato processing wastewater	-	*A. oryzae*	72	40	0.11-0.22	Muniraj et al. (2013)
Effluent from seafood processing plant and molasses from Sugarcane plant	-	*Mixed culture Rhodotorula glutinis and Chlorella vulgaris*	168	61.1	0.146	Cheirsilp et al. (2011)
Crude glycerol	-	*Chlorella vulgaris*	144	50	0.007	Sarma et al. (2014)
Bioethanol wastewater	-	*Rhodosporidium toruloides Y2*	120	34.9	-	Zhou et al. (2013)
crude glycerol	Methanol and soap removal	*Cryptococcus curvatus*	288	49	0.18	Cui et al. (2012)
Brewery waste	Acidic thermal hydrolysis	*Cryptococcus curvatu*	35	31.1	-	Ryu et al. (2013b)
Starch wastewater	-	*Rhodotorula glutinis*	60	30	-	Xue et al. (2010)
Piggery wastewater	-	*Chlorella pyrenoidosa*	240	50	0.21	Wang et al. (2012b)
Brewery wastewater	thermal hydrolysis	*Chlorella vulgaris*	240	42	0.04	Farooq et al. (2013)
Nisargruna biogas plant effluent	-	*Monoraphidium sp*	624	31	-	Tale et al. (2014)

Table 2. 6 Residential wastes for lipid production with oleaginous microorganisms

Residential wastes	Pre-treatment	Microbes	Time (h)	Lipid content (% w/w)	Lipid productivity (g/g wastes or L wastewater)	Ref.
Municipal solid waste	*Acid hydrolysis followed by enzymatic hydrolysis*	*Cryptococcus aerius*	*144*	*30.6*	*0.17*	*Ghanavati et al. (2015)*
Wastewater	*-*	*Rhodosporidium toruloides*	*72*	*8*	*-*	*Xue et al. (2008)*
Primary and secondary municipal wastewater	*-*	*Chlorella vulgaris*	*168*	*33*	*-*	*Ebrahimian et al. (2014)*
Wastewater	*-*	*Chlorella*	*-*	*11*	*-*	*Chiu et al. (2015)*
Sewage sludge		*Chlorella vulgaris*	*264*	*20.1*	*0.05*	*Cho et al. (2015)*
Domestic effluent	*physical removal of large particles and fat materials*	*Chlorella vulgaris*	*432*	*27*	*-*	*Cabanelas et al. (2013)*
Food waste	*enzyme hydrolysis*	*Chlorella vulgaris*	*192*	*33.3*	*-*	*Lau et al. (2014)*
Waste Cooking oil	*Filtration and washing*	*Starmerella bombicola*	*240*	*-*	*-*	*Maddikeri et al. (2015)*

Table 2. 7 Trace elements effect on microorganisms

Name	Essential to microorganism	Toxicity	Ref.
Chromium	Not sure	yes	Knežević et al. (2014)
Cobalt	Not sure	yes	Knežević et al. (2014)
Copper	Yes	yes	Knežević et al. (2014)
Fluorine	Not sure	yes	Knežević et al. (2014)
Iodine	Not sure	Not sure	Knežević et al. (2014)
Iron	Yes	Not sure	Knežević et al. (2014)
Manganese	Yes	yes	Knežević et al. (2014)
Molybdenum	Not sure	yes	Knežević et al. (2014)
Selenium	Not sure	yes	Knežević et al. (2014)
Zinc	Yes	Not sure	Knežević et al. (2014)
Magnesium	Yes	Not sure	Knežević et al. (2014)

Table 2. 8 Fermentation conditions used in the cost evaluation (Koutinas et al. 2014)

Conditions	Specific condition	Condition effect on the production price
Microbe	*Rhodosporidium toruloides*	Not evaluated
Feedstocks	Glucose + peptone + yeast extract	Microbial oil cost 11.3 US dollar/gal (feedstocks cost-free); microbial oil cost 18.3 US dollar/gal (feedstocks cost 400 US dollar/t)
Productivity	Lipid content: 67.5 (W/W) Biomass yield: 0.35 g/g glucose Lipid yield: 0.23 g/g glucose Productivity: 0.54 g/L-h	With 5 times productivity and no feedstocks cost, the microbial oil cost can be achieved at 5.33 US dollar/gal
Fermentation time	134 h	Not evaluated
Fermentation mode	Fed-batch	Not evaluated
Operation conditions	Operation hours: 8300 h/y Production capacity:10000 t/y	Not evaluated

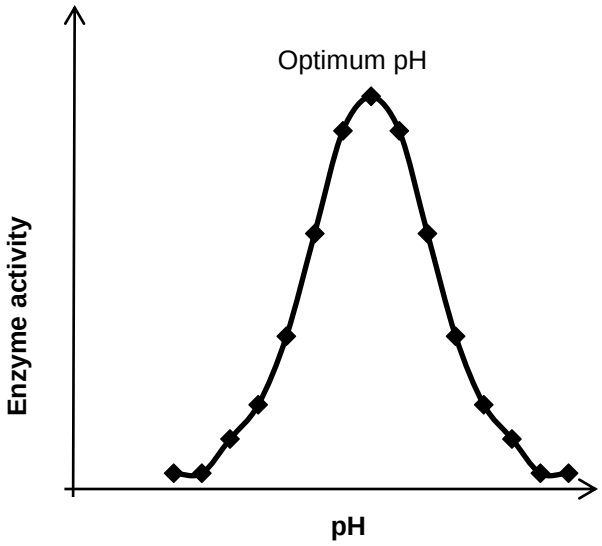

Figure 2. 1 Enzyme activity vs. pH (Grasso et al. 2015)

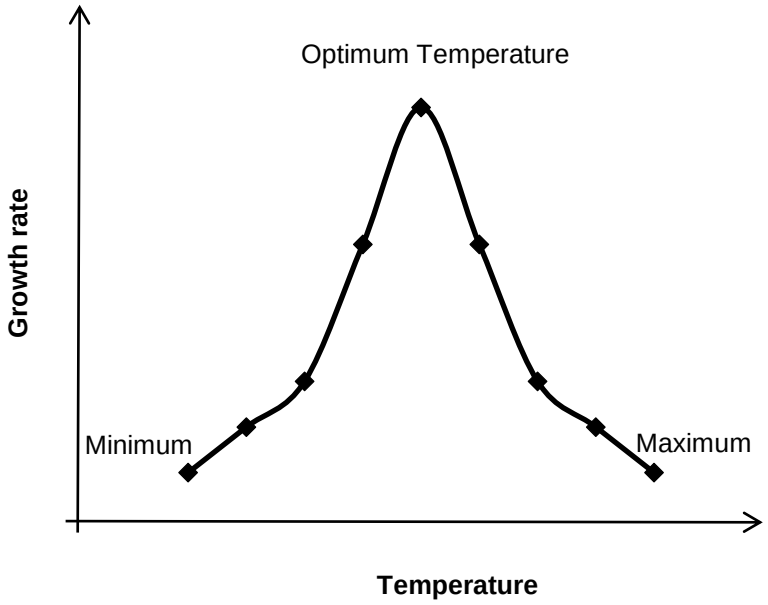

Figure 2. 2 Growth rate at different temperature (Hatfield and Prueger)

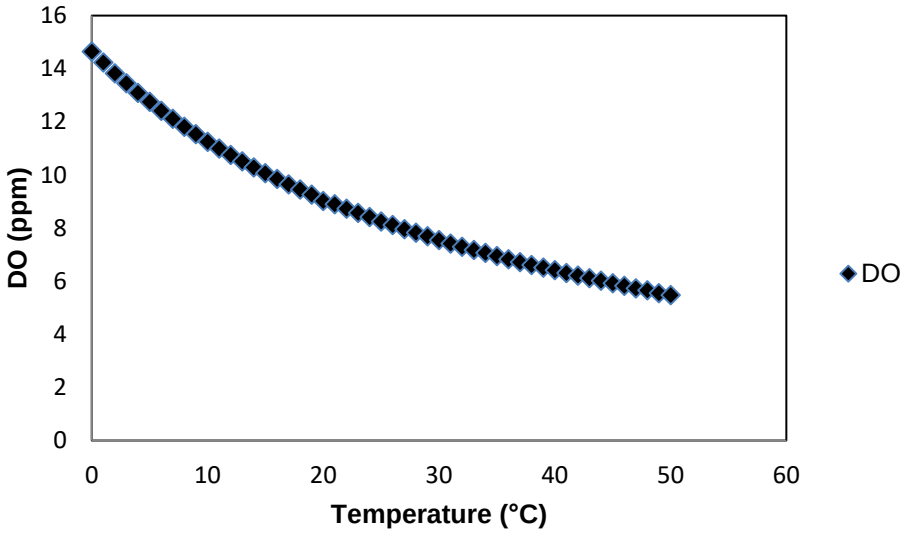

Figure 2. 3 DO under different temperature in standard atmospheric pressure

Ultra-sonication application in biodiesel production from heterotrophic oleaginous microorganisms

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3 ULTRA-SONICATION APPLICATION IN BIODIESEL PRODUCTION FROM HETEROTROPHIC OLEAGINOUS MICROORGANISMS

3.1 Résumé

La production de biodiesel à partir d'huiles de graines végétales et de graisses animales est confrontée à de grands défis en raison de l'augmentation des prix et de la pénurie d'huiles et de graisses. L'utilisation d'huiles microbiennes, normalement appelée lipides, pour la production de biodiesel a suscité un intérêt croissant. Cependant, il est encore en phase de la laboratoire en raison du coût élevé de la production. Les étapes majeures de la production de biodiesel à partir de microorganismes sont la préparation du milieu de culture, la culture de microorganismes, la récolte de biomasse, l'extraction de lipides et la trans-estérification ou trans-estérification in situ. L'utilisation de déchets organiques comme matières premières pour la croissance de microorganismes oléagineux hétérotrophes pour l'accumulation de lipides, qui est ensuite utilisée pour produire du biodiesel, a été prédite comme une méthode prometteuse pour réduire le coût. Cependant, le procédé comporte de nombreux obstacles, notamment une faible biodégradabilité des déchets organiques, une faible capacité d'accumulation de microorganismes oléagineux hétérotrophes, l'utilisation de solvants organiques toxiques pour l'extraction des lipides, des besoins en méthanol d'accès en trans-estérification et en trans-estérification in-situ. L'ultra-sonication en tant que technologie verte a été largement utilisée pour améliorer la production de bioproduits à partir de déchets organiques. Dans cet article, les applications de l'ultra-sonication dans les étapes de la production de biodiesel à partir de microorganismes oléagineux hétérotrophes ont été passées en revue. Son impact, potentiel et limitation sur les processus ont été discutés.

Mots clés: Production de biodiesel, ultra-sonication, micro-organisme oléagineux, lipide

3.2 Abstract

Biodiesel production from traditional feedstock, plant seed oils and animal fats, is facing a great challenge due to the increase in the prices and the shortage of the oils and fats. Utilization of microbial oil, normally called as lipid, for biodiesel production has gained growing interest. However, it is still in lab scale study stage due to the high cost of the production. The major steps of biodiesel production from microorganisms are cultivation medium preparation, microorganism cultivation, biomass harvesting, lipid extraction, and trans-esterification or in-situ trans-esterification which achieves the two purposes: lipid extraction and trans-esterification in one step. Employing organic wastes as raw materials to grow heterotrophic oleaginous microorganism for lipid accumulation which is then used to produce biodiesel has been predicted to be a promising method to reduce the cost. However, the process has many obstacles including low biodegradability of organic wastes, low lipid accumulation capacity of heterotrophic oleaginous microorganism while using organic wastes, great dependence on high energy consumption approach for biomass harvesting, utilization of toxic organic solvent for lipid extraction, and a large amount of access methanol requirement in trans-esterification and in-situ trans-esterification. Ultra-sonication as a green technology has been extensively utilized in enhancing bio-product production from organic wastes. In this article, ultra-sonication applications in the steps of biodiesel production from heterotrophic oleaginous microorganisms have been reviewed, and its impact, potential, and limitation on the processes have been discussed.

Keywords: Biodiesel production, ultra-sonication, oleaginous microorganism, lipid

3.3 Introduction

Currently, biodiesel production has gained significant attention due to the prediction of fossil fuel depletion. The commercial biodiesel is generally produced from plant seed oils, animal fats, and waste cooking oils. Currently, the price of biodiesel derived from plant seed oils and animal fats is around 3.03 US $/gal which is higher than that (2.46 US $/gal) of the petrodiesel. However, the prices of these oils and fats are still gradually increasing due to the great competition on the raw materials between biodiesel production and food processing. It reveals that the price of biodiesel would continue to elevate and becomes incomparable with that of diesel. Eventually, the biodiesel production will be hindered. The price of waste cooking oil is low and currently, it has been employed as feedstock for biodiesel production. However, there is no mature system in waste cooking oil collection all over the world, which leads to its low available quantity for biodiesel production.

As biodiesel demand increases, research and development on alternative feedstock oil for biodiesel production have attracted great interests. It has been widely reported that oleaginous microorganisms are the promising replacement of plant seed oils, animal fats, and waste cooking oils in biodiesel production (Sharma and Singh 2017, Soccol et al. 2017, Uprety et al. 2017, Zhu L. D. et al. 2017b). The major difficulty of microbial oil for biodiesel production is the high cost which is from the utilization of expensive substrates and nutrients in microorganism cultivation, complex lipid extraction, and large amount methanol requirement in trans-esterification (Amer et al. 2011, Wang Songmei et al. 2016a, Xavier et al. 2017). To overcome the problems and promote the biodiesel production from microorganisms, efforts have been devoted in the following aspects: searching for cheap substrates and nutrients such as organic wastes; enhancing microorganism harvesting efficiency and low the energy consumption; simplifying and increasing lipid extraction, and reducing methanol addition but not impacting on trans-esterification efficiency (Lorenz et al. 2017, Skorupskaite et al. 2016, Taskin et al. 2016, Wang Songmei et al. 2016a, Wang Yuancong et al. 2016b, Yun et al. 2017, Zheng et al. 2016).

Organic wastes including livestock waste, sugar cane, sugar beet, corn stover, rice straw, crude glycerol from biodiesel production, cheese whey, wastewater sludge have been

employed as raw material for oleaginous microorganism cultivation and lipid accumulation (Gong et al. 2016, Kumar et al. 2016, Liu et al. 2016, Park Gwon Woo et al. 2017a, Riaño et al. 2016, Soccol et al. 2017, Taskin et al. 2016, Uprety et al. 2017, Zhang Xiaolei et al. 2014c, Zhang Xiaolei et al. 2014e, Zhu L. D. et al. 2017b). However, most of the results showed that replacing expensive substrates (glucose) and nutrients (chemicals) with organic wastes in oleaginous microorganism cultivation led to low biomass and lipid production, which suggested that organic wastes were not as efficient as glucose and chemicals to grow microorganism. It was mainly due to the complex structure of the wastes (difficult to degrade) and inhibitors presenting in the wastes (Feng et al. 2014, Ma et al. 2016, Park Gwon Woo et al. 2017a, Zhu L. D. et al. 2017a). Studies on applying pre-treatment on organic wastes to increase the concentrations of simple carbon and nutrients have been extensively reported (Selvakumar and Sivashanmugam 2017, Xavier et al. 2017, Zhang Xiaolei et al. 2014c). The major effective pre-treatment technology includes thermal treatment, hydrolysis, and ultra-sonication. Apart from pre-treatment on raw materials (organic wastes), strategies through creating environmental stress during fermentation, have been applied to improve lipid production from a microorganism, and hence compensate the low efficiency of waste in lipid accumulation by the microorganism. Nitrogen limitation, phosphorus limitation, trace element addition, iron addition, temperature swing, and ultra-sonication stimulation are efficient ways of enhancing lipid production from oleaginous microorganism with organic wastes, and positive effect has been observed (Dahmen-Ben Moussa et al. 2017, Han et al. 2016b, Kim et al. 2016, Sachdeva et al. 2016, Wang Yuancong et al. 2016b).

Lipid extraction is to separate lipid from microorganism cell body. The common method is solvent extraction which employs organic solvents such as chloroform, methanol, or hexane to disrupt cell wall and membrane and dissolve lipid (Skorupskaite et al. 2016, Wang Songmei et al. 2016a). The process normally requires a large amount of solvent in order to break the cell wall, and hence causes high energy input in the solvent recovery process. To reduce the solvent addition amount, technologies which are capable of disrupting cells have been applied in lipid extraction. It was found that microwave radiation, bead milling, homogenization, and ultra-sonication were a promising method in cell disruption and had greatly reduced solvent addition amount in the process of lipid

recovery from microorganism cells (Garoma and Janda 2016, Meullemiestre Alice et al. 2016, Pan et al. 2016).

Trans-esterification is the step to convert lipid to biodiesel, which is a chemical reaction occurring between lipid and methanol in the presence of a catalyst (acid or base). In fact, the trans-esterification is mature technology in biodiesel production. However, it has an obvious problem which is the large amount excess methanol demand in order to achieve high biodiesel yield (Mathimani et al. 2015). As methanol is a cheap chemical, generally, the recovery of methanol after trans-esterification was not conducted. However, it becomes an environmental concern as the global biodiesel production amount increases. The technology which can lower the methanol addition amount and remain the high trans-esterification efficiency has been developed, which is the employment of supercritical methanol, nanomaterials, microwave, and ultra-sonication in trans-esterification (Jawaharraj et al. 2017, Mathimani et al. 2015, Mohamadzadeh Shirazi et al. 2017, Zhang Xiaolei et al. 2016).

In-situ trans-esterification combining the lipid extraction and trans-esterification has been developed in biodiesel production from oleaginous microorganisms (Choi et al. 2014, Wang Songmei et al. 2016a, Zhang Xiaolei et al. 2014b). *In-situ* trans-esterification is the process to transfer the lipid in the cell to biodiesel in local (without lipid extraction), which simplifies the production. It provides a shortcut of biodiesel production from a microorganism and has been given significant attention. However, the technology is still in the lab stage and requires intensive study before going to practice. Low reaction rate and large demand in methanol (acting as solvent as well as a reactant) amount are the main obstacles in the application of in-situ trans-esterification. To increase the reaction rate and reduce methanol addition amount, researchers have introduced microwave, ultra-sonication, the combination of microwave and ultra-sonication, co-solvent addition in in-situ trans-esterification process (Ehimen et al. 2012, Ho et al. 2016, Sivaramakrishnan and Incharoensakdi 2017, Yu et al. 2017, Zhang Xiaolei et al. 2016).

From above statement, it can be seen that ultra-sonication plays a significant role in overcoming the difficulties in biodiesel production from oleaginous microorganisms, and showed promising performance in accelerating the process to reality. It would be

attributed to the capacity of ultra-sonication to create cavitation which is to produce microbubbles and induces high pressure and temperature in local once the bubbles collapse. In addition, ultra-sonication can cause the generation of free radicals as well as high shearing forces, which can enhance decomposition of complex materials and chemical reaction rate.

Ultra-sonication as a green technology has attracted great interest in biofuel production. Its enhancement on conversion of biomass to biofuels (biogas, bioethanol, and biodiesel) has been extensively reviewed by Luo et al. (Luo et al. 2014). It covered the ultra-sonication application in biodiesel but focused on its involvement in esterification and trans-esterification of plant seed oils/animal fats and autotrophic microorganism (mainly microalgae) for biodiesel production. In fact, heterotrophic oleaginous microorganisms (bacteria, yeast, and fungus; there is also heterotrophic microalgae but not involved in this article as it rarely studied for lipid production) have shown promising potential in biodiesel production compared to autotrophic microorganism as they grow faster, could reach higher biomass concentration, don't require light and arable land, and has lower contamination risk. It is rather important to understand the impact and contribution of ultra-sonication in biodiesel production from a heterotrophic oleaginous microorganism.

This work reviewed the ultra-sonication application in biodiesel production from heterotrophic oleaginous microorganisms. It mainly covered the ultra-sonication impact on a different step of biodiesel production from oleaginous microorganism including microorganism cultivation, lipid extraction, and trans-esterification, and its impact on *in-situ* trans-esterification was discussed as well. The article was aimed to evaluate the feasibility of the technology in biodiesel production from microbes and to provide an insight of ultra-sonication potential to bring the process of biodiesel production from microbes to practice.

3.4 Ultra-sonication application in heterotrophic microorganism cultivation

Heterotrophic microorganism cultivation includes medium preparation and the fermentation process. Hence, ultra-sonication application in heterotrophic oleaginous

microorganism cultivation is mainly in raw material (substrate and nutrients) preparation and in stimulation of cells during cultivation. The details were presented below.

3.4.1 Ultra-sonication application in organic waste pre-treatment

As stated, utilization of organic wastes (food wastes, crop wastes, wastewater sludge) as raw materials is the major strategy to reduce the cost of biodiesel production from microbes and makes the production comparable with that from plant seed oils/animal fats (Gong et al. 2016, Park Gwon Woo et al. 2017a, Taskin et al. 2016, Xavier et al. 2017, Zhu L. D. et al. 2017a). However, organic waste is normally difficult to be degraded, and pre-treatment has to be demonstrated prior to being used as a medium. Ultra-sonication could efficiently break the complex and big molecules to simple and small ones, and hence increase the bioavailability of the organic waste (Bhutto et al. 2017, Iskalieva et al. 2012, Karimi et al. 2014, Martín et al. 2015). It was found that ultra-sonication could highly enhance sugar release from rice straws (Borah et al. 2016). The amounts of the release of total sugar and reducing sugar were around 2.5-fold of that without ultra-sonication (35 kHz, 30 W, 6 h) treatment. It would be due to that ultra-sonication provided cavitation, which induces hydroxyl radical (derived from water) formation, local turbulence, micro-circulation, and micro-convection in the system. Hence, ultra-sonication treatment led to the physical and chemical transformation in the rice straw to release sugar. It was observed that ultra-sonication (20 kHz, 150 W, 45 min) has improved the biodegradability of wastewater sludge to be around 80%, and increased the biogas production to 172 mL/g VS from 88 mL/g in the anaerobic digestion (Martín et al. 2015). It was reported that ultra-sonication significantly enhanced above 65% yield of hydrogen from food wastes (Elbeshbishy et al. 2011, Gadhe et al. 2014). In fact, many reports have revealed that the bioproduct production from complex organic wastes had been improved with the ultra-sonication pretreatment on the wastes (Table 3.1). It was due to that ultra-sonication was capable of decomposing complex organic wastes and increasing their bioavailability, and thus enhancing the desired product production through biotechnology.

The combination of ultra-sonication with other technology such as alkaline condition, enzyme hydrolysis, chemical addition, and heat has also been reported in organic waste treatment (Ramadoss and Muthukumar 2016, Wu et al. 2017). The release of reducing

sugar after treated with ultra-sonication under alkaline condition was significantly higher than that with solo ultra-sonication treatment (Borah et al. 2016, Wu et al. 2017). Ultra-sonication pretreatment on sugarcane bagasse in the presence of titanium dioxide achieved 94.98 % holocellulose recovery which was only 55% in the pre-treatment without titanium dioxide (Ramadoss and Muthukumar 2016). It revealed that a proper combination of ultra-sonication and other treatments would be better than any single technology application due to the synergistic effect.

Recently, a study on utilization of thermo-chemo-sonic to pre-treat wastewater sludge which was then used as a medium in oleaginous microbe cultivation showed positive influence on lipid accumulation (Selvakumar and Sivashanmugam 2017). The thermo-chemo-sonic process led to a 30% increment in the solubilisation of carbon-oxygen demand (COD) which was just 14% with the solo thermo-chemical treatment (Selvakumar and Sivashanmugam 2017). COD solubilisation is an important indicator of bioavailability of the materials (wastes). The increase of COD solubilisation after applying ultra-sonication to the thermo-chemical process indicates that ultra-sonication positively impact on the bioavailability, and hence enhanced the microorganism growth and lipid production. Combining ultra-sonication with other treatments would be an efficient and effective technology of enhancing lipid production from heterotrophic microorganism fermentation with organic wastes.

Generally, the ultra-sonication performance is determined by power, frequency, mode (with or without pulse), and energy input of ultra-sonication. It could be seen that most of the studies had employed 20 kHz frequency in raw material pre-treatment (Table 3.1). It would be attributed to that 20 kHz frequency has high penetrability, can make the energy concentrated and transport long distance. The ultra-sonication power refers to the energy added to the system, but that how much the energy has added to the system can be described with the energy density (Table 3.1). Compared to power, the energy density is far more efficient to demonstrate the energy intensity of the ultra-sonication to the acceptor (organic wastes). Higher energy intensity tends to provide better results on the pre-treatment (Table 3.1). However, it is not always following to the relation as the structure of organic wastes and system efficiency have a significant impact on the performance as well (Hay et al. 2015, Wu et al. 2017).

Overall, ultra-sonication displayed great potential in assisting to elevate the bioavailability of organic wastes, which could improve the growth of heterotrophic oleaginous microorganism and lipid production from the wastes. In order to further enhance the performance, combination of ultra-sonication with thermal, chemical, and biological treatment can be considered to treat the organic wastes. As low frequency (20 kHz) provides concentrated energy in local and high energy density promotes the disruption of the complex materials (organic wastes), selection on frequency, power, and ultra-sonication time should be well investigated according to the type and texture of wastes.

3.4.2 Ultra-sonication application in microorganism lipid accumulation

It has been well reported that environmental stress such as nutrient depletion could induce lipid accumulation in cells of oleaginous microorganisms (Ahmad et al. 2015, Fortela et al. 2016, Ren et al. 2014, Sachdeva et al. 2016, Venkata Subhash and Venkata Mohan 2014, Xin et al. 2010, Zhang Huaiyuan et al. 2014a). Oleaginous microorganism assimilates substrates to support life activities as well as to accumulate lipid which is stored in cell body as an energy source. Oleaginous microorganisms are sensitive to the environmental condition. Any change/disturbance could cause the insecure sense of the microorganism and hence stimulates it to store energy in the cell body.

Ultra-sonication generates turbulence, micro-convection, high temperature and pressure in local as well as free radicals. Ultra-sonication has been used to stimulate various bioprocesses and microbial productivity enhancement has been observed (Avhad and Rathod 2014, Budiman et al. 2017, Sun Chun Xiao et al. 2017, Wang Feng et al. 2013). It was reported that ultra-sonication (20 kHz, 0.085-0.5 kWh/L, 20 min, 3 s on 5 s off) stimulation on *Rhodobacter sphaeroides* increased hydrogen production by 52.2% to 200% (Budiman et al. 2017, Zhu Xun et al. 2011). Ultra-sonication improvement in microbial productivity could be summarized into two major reasons: increasing the permeability of cell membranes and enhancing/inhibiting the critical enzyme formations. The permeability of cell membrane impacts on the transportation of substrates and nutrients from medium to the inside of cells. Due to the increase of the permeability of the membrane, the substances that enter cells requiring the assistance of carrier proteins (active transport) could accomplish the transportation without the dependence on the carriers. It would

accelerate the intake of substrate or nutrient by cells and progress cell metabolism, and hence improve the microbial product production. The pathway of any microbial product formation is complicated and involves the participation of enzymes. The addition of ultra-sonication during fermentation could alter the production of certain enzymes which enhances/inhibits some intermediate products and thus effects on the final product production. Applying ultra-sonication (25 kHz, 160W, 5 min, 1 s on 4 s off) on *Bacillus sphaericus* had increased the productivity of fibrinolytic enzyme to 201 from 110 U/mL (control) (Avhad and Rathod 2014). In laccase production, a similar trend has been observed that the laccase production reached 588.9 U/L with the stimulation of ultra-sonication (40 kHz, 120W, 10 min: two times 5 min ultra-sonication within 12 h), which was 327.1 U/L in control (Wang Feng et al. 2013).

So far, the effect of ultra-sonication on lipid accumulation of heterotrophic oleaginous microorganism has not been studied. However, recently, utilization of ultra-sonication to stimulate autotrophic oleaginous microbes for lipid production has been reported (Han et al. 2016a, Han et al. 2016b). With the ultra-sonication (40 kHz, 200W, 20 min) treatment on the culture (*Scenedesmus quadricauda*) at the end of the logarithmic phase, lipid accumulation was highly increased from 24.0% w/w (control) to 37.8% w/w. When the treatment was applied at the beginning of fermentation of *Anabaena variabilis*, the lipid content also improved which were 32.12% and 46.90% w/w in without and with ultra-sonication treatment, respectively (Han et al. 2016a). Apart from lipid, protein production was also enhanced. In the formation of lipid, several enzymes including CoA, acetyl-CoA, and NADPH play significant roles. Enzymes are mostly proteins. The increase of protein could be due to the increase of CoA, acetyl-CoA, and NADPH or the enzymes related to the formation of them, and thus the high lipid accumulation has been led. Though the substrate required by autotrophic (CO2) and heterotrophic (sugars) microbes are different, the basic mechanism of lipid synthesis is similar, which is highly affected by critical enzyme formation during fermentation (Ratledge 2004, Rossi et al. 2011). It indicates that ultra-sonication could be an efficient method to enhance lipid production from heterotrophic oleaginous microorganisms.

Unlike, utilization of ultra-sonication on pre-treatment of complex organic wastes, which generally requires low frequency and high power, to enhance the microbial product

production by ultra-sonication stimulation, the utilized frequency (normally 40 kHz) is high and the power (100 - 200 W) is low (Avhad and Rathod 2014, Han et al. 2016a, Wang Feng et al. 2013). High frequency and low power provide gentle rub on cells and could preserve cell integrity and viability, and hence no significant damage to cells would occur (Lecina Martí et al. 2017). It hence prevents the death of cells but causes the increase of permeability of cells. Thus, substrate and nutrient transportation would be improved, and further enhanced metabolism of microorganisms for microbial productivity.

Ultra-sonication applying stage to the culture (lag, logarithmic, and stationary phase) could be also important in microbial product formation. The microbial product can be divided into growth associated, non-growth associated, and mixed mode one according to their relation to cell growth. Growth associated product is formed simultaneously with the growth of cells. For non-growth associated production, product formation is unrelated to cell growth but is determined by cell concentration. Mix mode product formation is a result of the combination of growth rate and cell concentration. It suggests that logarithmic phase is essential for growth associated product formation, the stationary phase has more soundly effect on the product formation compared to other phases, and both logarithmic and stationary phases are important in mixed mode product formation. For the growth associated product formation, applying ultra-sonication on cells at logarithmic phase would accelerate cell growth and indirectly enhance the product formation. For the non-growth associated product, if ultra-sonication induces the progress of cell division which leads to a high final cell concentration, ultra-sonication should be applied to the cells at logarithmic phase. For the mixed mode product, the ultra-sonication application on cells at logarithmic phase could highly impact on product formation. So far, there is no clear statement on whether lipid is growth associated, non-growth associated, and mixed mode microbial product. However, from above discussion, it can be seen that logarithmic phase is an essential period for the production of microbial products, thus applying the ultra-sonication treatment on cells at logarithmic phase could improve lipid production from heterotrophic microorganisms.

3.5 Ultra-sonication application in microorganism harvesting

Normally, the fermentation will be forced to stop when the lipid production reaches the maximum. Thereafter, harvesting will be performed. Harvesting is aimed to recover the biomass from the broth with the minimum loss of the product (lipid). As mentioned, the lipid is an energy source for cells and will be decomposed by the cells to support cell activities when the external substrate is deficient. In industrial scale production, biomass separation from broth may not be performed immediately after the fermentation due to the availability of the separation equipment (mainly referring to centrifugation). During the period waiting to be separated from the broth, the cells remain alive but settled on the bottom of the container and are not able to access the substrates; however, cells require energy to support their basic activities such as respiration. It suggests that there is the possibility of lipid decomposition during the period which should be completely prevented in order to avoid the reduction of lipid. In fact, our previous study has observed that the lipid consumption occurred after the fermentation stopped for 3 h (Zhang Xiaolei et al. 2015a).

To prevent the lipid consumption by cells themselves, the best choice is to inactivate or kill the cells right after the fermentation was ceased. Many strategies could achieve the purpose, for instance, exposing the cells to extremely low or high pH and temperature, high salinity, high shearing force, and radiation of UV, microwave, and ultra-sonication (Jones et al. 2012, Zhang Xiaolei et al. 2015a). Among all, ultra-sonication is considered as potential one due to its high efficiency on cell inactivation and accurate energy targeting (Buldakov et al. 2014, Feril Jr and Kondo 2005). Our previous studies have found that ultra-sonication (20 kHz, 750 W, amplitude 30%, 8 min) was an efficient and effective way to inactivate/kill oleaginous microorganism *Trichosporon oleaginosus* and didn’t impact on downstream processing and the yield and properties of final product (biodiesel) (Zhang Xiaolei et al. 2015a). After cells were inactivated/killed, the risk of the internal consumption of lipid by cells would be prevented. Cell separation from broth can be sequentially performed without the concern of lipid loss during the waiting period.

The current technologies of cell separation from broth, also called dewatering, are filtration, sedimentation and centrifugation. Among all, centrifugation is the most efficient

one for cell dewatering but also requires intensive energy deposition compared to others (Danquah et al. 2009, Lecina Martí et al. 2017). Compared to centrifugation, filtration is more favorable as it could achieve high efficiency of cell dewatering but less demand for energy input. However, centrifugation and filtration are only cost acceptable for the broth with high biomass concentration (greater than 20 g/L) (Nurra et al. 2014, Sahoo et al. 2017, Semiyaga et al. 2017). Sedimentation is the most energy and cost-efficient method of cell dewatering as it naturally occurs as long as the density of cells is higher than water. Microorganism cells normally have gas vesicles and oleaginous microorganism has a large content of lipid. It leads to that the density of cells was normally low (close to water). Hence it is difficult to obtain cell dewatering by sedimentation.

The addition of flocculants have been introduced to improve the settling of cells which had a similar density as water but the performance highly depended on the number of extracellular polymer substances (EPS) secreted by the cells (Lecina M. et al. 2016). Some researchers reported that applying high energy ultra-sonication (frequency in the range of MHz) to the broth could induce cells to agglomerate into large aggregates and thereafter led to high-efficiency sedimentation; however, the energy input required in the ultra-sonication was around 16-24 kWh/m^3 (Bosma et al. 2003). Recently, studies by Lecina et al. have employed low energy ultra-sonication to improve the sedimentation efficiency of cells in broth and positive results have been observed (Lecina Martí et al. 2015, Lecina Martí et al. 2017). In their studies, ultra-sonication frequency, power, and energy density used were 20 kHz, 80 W, and 1.1-2.2 kWh/m^3, respectively. After the treatment, in the following sedimentation, a 90% biomass recovery was achieved, which was similar as the performance with the high energy ultra-sonication (Bosma et al. 2003, Lecina Martí et al. 2017). Low energy ultra-sonication caused the structure change of the cells and ruptured the gas vesicles, which reduced the buoyancy of the cells and enhanced the sedimentation.

Due to the low efficiency of sedimentation, it hasn’t been considered for cell recovery from broth in practice. However, the addition of ultra-sonication prior to sedimentation has shown promising performance. It indicates that low energy ultra-sonication treatment followed by sedimentation provided an alternative method of cell recovery from the

fermentation broth. It may replace centrifugation and filtration which are currently applying in biomass dewatering in industrial scale microbial product productions.

As stated above, in order to prevent lipid consumption by cell self during the waiting period to be dewatered, ultra-sonication can be utilized to inactivate/kill cells. However, the power of ultra-sonication used in the cell inactivating/killing was normally 500-750 W which was much higher than that employed in the dewatering process (ultra-sonication followed by sedimentation) (Feril Jr and Kondo 2005, Zhang Xiaolei et al. 2015a). In addition, the low energy ultra-sonication seemed not capable of killing the cells, but its inactivation effect on the cells was not indicated and reported. Hence, studies are required to investigate if the low energy (around 80 W) ultra-sonication could inactive the cells and avoid the lipid self-consumption. If the low energy ultra-sonication can accomplish the inactivation of cells, thus, sedimentation could be a promising technology of oleaginous cell recovery from fermentation broth as it could highly enhance biomass sedimentation. However, if the low energy ultra-sonication could not achieve the inactivation of oleaginous cells, investigation of a suitable ultra-sonication which can inactivate cells as well as reduce the buoyancy of cells but not lead to lipid release to broth from cells should be conducted.

3.6 Ultra-sonication application in lipid extraction

Lipid extraction is the process to separate the lipid droplets from the cells of oleaginous microorganisms. Solvent extraction with methanol and chloroform is the most applied approach for lipid extraction (Mathimani et al. 2017, Xie et al. 2017). As chloroform is toxic as well as inflammable, there is great concern on its application, which also hinders biodiesel production from oleaginous microorganisms.

Lipid droplets are the intercellular production of oleaginous microorganisms. The extraction of lipid includes the disruption of cell wall and membrane and escapes of the lipid drops from cells. Solvent extraction is to depend on methanol and chloroform to disrupt cell wall and membrane and then chloroform (non-polar) to dissolve the lipid drops, and hence accomplish the lipid separation from cell debris. It suggests that great portion of the solvents used in the extraction plays a role in cell disruption and probably just small

parts of the solvents are to dissolve the lipid droplets. If the cell disruption can be replaced by another method, the solvent amount required in the extraction could be largely reduced. Moreover, hexane (non-toxic and commercially being used in oil extraction from plant seeds) is similar to chloroform and can be employed to dissolve lipid droplets. It implies that if the proper method can be found to disrupt the cells, lipid extraction can be achieved without the utilization of chloroform.

As mentioned, ultra-sonication provides cavitation phenomena. Microscopic bubbles at various nucleation sites in the fluid would be formed during ultra-sonication. The ultra-sonication effect occurs in two phases: rarefaction and compression phase. The bubbles grow during the rarefaction and are compressed during the compression phase. The microscopic bubbles collapse in the compression phase and thus a violent shock wave was formed which leads to the generation of tremendous heat, pressure, and shear in local.

Ultra-sonication as a green technology has been applied for cell disruption for decades (Harrison 1991, Palma and Bucalon 1987, Save et al. 1994). 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). Ultra-sonication utilization in lipid extraction from microalgae has also been extensively reported (Garoma and Janda 2016, Lee et al. 2017, Mubarak et al. 2015, 2016, Natarajan et al. 2014). Studies have evidenced that ultra-sonication could attack the integrity of cell walls, rupture the linkages between the phospholipid molecules, and break the cells (Abdullah et al. 2016, Sun Jing-Xia et al. 2004). Normally, to obtain a similar extent of lipid recovery, ultra-sonication assisted lipid extraction required less than 1 h but traditional chloroform and methanol extraction needs several hours (Metherel et al. 2009). It can be seen that the lipid extraction was accelerated, moreover, the solvent amount required was reduced as well, and the lipid compositions (fatty acid profiles) remain intact with the assistant of ultra-sonication (Mubarak et al. 2016, Natarajan et al. 2014, Zhang Xiaolei et al. 2014d). In traditional solvent extraction system, the water content of biomass has a greatly negative impact on lipid recovery as water presence leads to the dilution of the solvent concentration. Hence, drying is normally conducted prior to the extraction to eliminate the water in order to achieve high lipid recovery. Ultra-sonication assisted lipid

extraction has no strict requirement on water content. It was reported that lipid recovery from wet biomass reached to 98% w/w total biomass with the assistance of ultra-sonication (Lee et al. 2017, Mubarak et al. 2016).

Ultra-sonication assisted lipid extraction can be divided into two types: one is the ultra-sonication as pre-treatment, which means ultra-sonication only used to disrupt cells (in water); the other is ultra-sonication participating in extraction, which means ultra-sonication used to disrupt cells (in solvent) as well as assist/enhance solvent to transfer to lipid droplets in the cells. Compared to the utilization of ultra-sonication as pre-treatment, directly applying ultra-sonication in the extraction is normally much more efficient (Table 3.2). There are also exceptions. For instance, it was observed that most of the lipid recovery efficiency could reach above 98% except the cases which applied low energy (power) ultra-sonication (80 W and 300 W) (Table 3.2). It was found that high power led to high lipid extraction efficiency (Halim et al. 2013). Apart from power, ultra-sonication time, temperature, and used solvent also play a significant role in the lipid recovery efficiency (Araujo et al. 2013, Metherel et al. 2009). High temperature also could improve lipid recovery (Table 3.2). The mixture of chloroform and methanol seemed better than hexane (Table 3.2). In most of the cases, ultra-sonication frequency was set at between 20 to 40 kHz and high lipid recovery efficiency was obtained, which suggests that it would be a suitable value for lipid extraction (Luo et al. 2014).

So far, ultra-sonication application in lipid extraction from a heterotrophic oleaginous microorganism (mainly yeast and fungus) is still limited compared to that from autotrophic one (microalgae) (Meullemiestre A. et al. 2017). It would be due to that more researchers and engineers consider microalgae have more potential as feedstock for biodiesel production compared to heterotrophic one. Thus, more efforts and research interest have been devoted to microalgae for biodiesel production. However, as stated above, heterotrophic oleaginous microorganism has shown advantages compared to autotrophic one. Hence, the work to investigate ultra-sonication application in lipid extraction from a heterotrophic oleaginous microorganism is necessary and highly required for the development of biodiesel production from heterotrophic oleaginous microorganisms. However, for the lipid extraction from heterotrophic microorganisms (here mainly referring yeast and fungus) with the ultra-sonication, the conditions including ultra-sonication

frequency, power, energy density, temperature, and operation time, cannot completely adopt from that employed in autotrophic microorganisms (here mainly referring microalgae). The major reason is that their cell walls are different. Microalgae cell walls are made of made of glycoproteins and polysaccharides, but those of fungus and yeast are made of glucosamine polymer chitin. Compared to microalgae, the cell wall of fungus and yeast are more rigid and tough to disrupt. Then to achieve similar cell disruption with ultra-sonication, higher energy (power) may have to be provided in the case of yeast and fungus compared to that in microalgae case. Energy consumption is an important factor in the feasibility of technology application. High energy requirement on lipid extraction with ultra-sonication might become an obstacle of its application in practice. The combination of ultra-sonication with low energy input technology such as mild heating could be a solution to reduce the energy consumption.

3.7 Ultra-sonication application in trans-esterification and in-situ trans-esterification

3.7.1 Ultra-sonication application in trans-esterification

Trans-esterification is the process where oil/fat/lipid reacts with short chain alcohol, normal methanol, in the presence of a catalyst to form biodiesel. In fact, the process is well established and commercially utilized in biodiesel production. However, there are still some problems needed to be solved. In theory, one molar of oil/fat/lipid requires 3 molars of methanol; however, in order to increase the conversion efficiency, normally, the excess methanol by 2 times (6 molar methanol for per molar oil/fat/lipid) is added, and temperature of 50-60 ºC is applied. The excess methanol can be recovered after the reaction but intensive energy requirement makes the recovery unfavorable. In addition, methanol is cheap material, and hence, it leaves in the by-product stream. Generally, trans-esterification efficiency is around 95 to 98% w/w total oil/fat/lipid, which implies that there is some feedstock is not converted to biodiesel. The unconverted oil/fat/lipid tends to remain in the phase of synthesized fatty acid methyl esters (FAMEs, biodiesel), and hard to separated if expensive techniques are not applied. On the other hand, the conversion efficiency of trans-esterification has a great impact on the profit of biodiesel

industries, thus, an increase of the conversion efficiency is an important task. Thus, many researchers have been trying to enhance the conversion efficiency of oil/fat/lipid to biodiesel (Yu et al. 2017). In fact, the conversion efficiency is mainly determined by the mass transfer between oil/fat/lipid and methanol. Oil/fat/lipid and methanol are immiscible which affects on the mass transfer. Poor mass transfer normally results in low conversion rate and efficiency. It suggests that the key is to improve mass transfer in order to increase trans-esterification conversion rate and efficiency.

Ultra-sonication generates sinusoidal mechanistic waves which induce the cavitation phenomena in a liquid system. Cavitation bubbles could play a role of microreactor in trans-esterification of oil/fat/lipid and methanol. With the collapsing of the bubbles which induces high pressure (10-500 MPa) and temperature (1000-10000 K) in the microreactors, reactant (methanol and lipid) turns to vapor and then involved into the coming formed bubbles (microreactor), and effective mass transfer occurred. Thus, trans-esterification reaction rate and efficiency would be highly enhanced.

Because of the advantage of ultra-sonication to improve mass transfer, it has been extensively studied in the trans-esterification step (Hingu et al. 2010, Santos et al. 2009, Vyas et al. 2011). With the application of ultra-sonication in trans-esterification, to achieve similar efficiency, the molar ratio of methanol to oil/fat/lipid can be reduced from 12:1 to around 6:1, the reaction time could be high decreased from around hours to less than 30 min, and the reaction temperature could also reduce to 35 ºC from around 50-60 ºC (Hingu et al. 2010, Teixeira et al. 2009, Vyas et al. 2011).

In fact, except the common ones: molar ratio of methanol to lipid and catalyst type and amount, reaction time, as well as temperature, ultra-sonication energy intensity (power input in per volume liquid) and ultra-sonication mode (continuous or pulse) also have a great impact on the performance of ultra-sonication in trans-esterification. High energy intensity could provide aggressive mixing to improve mass transfer. It was reported that the conversion efficiency of trans-esterification turned to 99% at a power density of 5.1 W/mL but was 90% at a power density of 3.4 W/mL (Martinez-Guerra and Gude 2015). But the study also showed that the trend was only suitable to a certain value of energy intensity, and a decrease of conversion efficiency was observed once it was beyond the

value. It was predicted that too high energy intensity produced a large amount of microbubbles, and these bubbles could merge to form bigger and more stable bubbles, which could present as barriers of acoustic energy transfer. Ultimately, the conversion efficiency of trans-esterification would be affected.

Ultra-sonication mode includes with a pulse and without a pulse. It was revealed that the conversion efficiency of trans-esterification was high in pulse system than that in the continuous system when the reaction time was long (more than 20 min), otherwise continuous ultra-sonication was more efficient in trans-esterification (Ji et al. 2006, Martinez-Guerra and Gude 2015, Stavarache et al. 2007). Short pulse allows the immiscible reactants (methanol and lipids) to partially separate and then better mixed when ultra-sonication was provided again, and hence improve the trans-esterification reaction.

To apply ultra-sonication in trans-esterification, ultra-sonication energy intensity and mode should be well selected in order to let ultra-sonication positively lead the reaction. However, so far, no systematic study has been found to elevate these parameters effect on converting microbial lipid to biodiesel, and moreover, the energy and cost consumption of the ultra-sonication application in trans-esterification for biodiesel production from oleaginous microorganisms have not been reported. The relative work is demanded.

3.7.2 Ultra-sonication application in in-situ trans-esterification

As stated, biodiesel production from heterotrophic oleaginous microorganism includes microorganism fermentation, biomass harvesting, lipid extraction, and trans-esterification. Among all, lipid extraction is the step that introduces toxic organic substance (chloroform) to the environment. Moreover, it is a high energy and cost requiring process (Amer et al. 2011, Davis et al. 2011). Recent years, *in-situ* trans-esterification has been developed. The *in-situ* trans-esterification directly converts oil located in oil-bearing substances (seeds and microbes) to biodiesel without affecting on biodiesel profile (FAMEs composition) (Samuel and Dairo 2012). *In-situ* trans-esterification grabs growing interest due to the prevention of toxic substance utilization and simplification the process of the biodiesel production from a heterotrophic oleaginous microorganism.

However, the technology is still in lab scale which is mainly due to the long reaction time (over 10 h) and high alcohol lipid ratio (300:1 to 900:1) demand (Haas and Scott 2007, Ho et al. 2016, Samuel and Dairo 2012). Either long reaction time or large amount methanol addition is mainly due to the presence of cell wall and membrane which act as a barrier of reactant (methanol) transferring to the inside of cells to contact with lipid. In order to promote the application of in-situ trans-esterification, the technology providing assistance on cell disruption such as co-solvent, homogenization, ultra-sonication, and microwave irradiation has been employed in the in-situ trans-esterification (Ehimen et al. 2012, Koutsouki et al. 2015, Sivaramakrishnan and Incharoensakdi 2017, Zhang Xiaolei et al. 2014b, Zhang Xiaolei et al. 2016).

Ultra-sonication assisting *in-situ* trans-esterification is considered as one of the most potent technology as it is easy to be realized in industrial scale (ultra-sonication has been widely utilized in industrial practice in many fields). Ultra-sonication addition in the *in-situ* trans-esterification is to create vigorous mixing as well as disrupt cell membrane and cell wall and thus enhances mass transfer. The microbubbles 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 ultra-sonication aided in-situ trans-esterification of microalgae biomass to biodiesel (24 kHz, 200 W) and only 32% conversion was obtained for normal in-situ trans-esterification (Ehimen et al. 2012). It was mainly due to that the ultra-sonication led to the high permeability of cells and promoted the methanol transportation to the inside of cells, and hence enhance biodiesel yield of *in-situ* trans-esterification.

In fact, many parameters are impacting on the performance of ultra-sonication in *in-situ* trans-esterification, which includes ultra-sonication energy intensity (power input in per volume liquid), ultra-sonication mode (continuous or pulse), reaction time, co-solvent, temperature, pressure, and mixing. High energy intensity would provide violent shearing force, high pressure, and high temperature in local, and hence effectively disrupt the cells and facilitate the methanol to transfer inside of cells to react with the lipid droplets. However, as discussed in the section of the ultra-sonication application in trans-esterification, the conversion efficiency could be negatively impacted when the energy

intensity reached certain value attributed to the formation of the big and stable bubbles. However, no related work has been reported on this point.

In trans-esterification, ultra-sonication mode (with or without pulse) can be selected according to the difference in ultra-sonication time. Normally, pulse one was better if the reaction has to last a long time (over 20 min), in contrary, the one without a pulse is better. However, it is known that the ultra-sonication function in trans-esterification and *in-situ* trans-esterification is different as ultra-sonication is expected to provide a high mass transfer between lipid and methanol in trans-esterification but to disrupt cells to increase the permeability of cell wall and membrane to allow the methanol to enter and excess the lipid for reacting in *in-situ* trans-esterification. Thus, ultra-sonication without pulse could continuously supply a shearing force on cells and produce the intensive and large amount of collapsing on microbubbles and enhance the process of *in-situ* trans-esterification. Most of the reported studies on the ultra-sonication application in *in-situ* trans-esterification were employed continuous ultra-sonication (without pulse) regardless of the operation time (Table 3.3). The application of ultra-sonication with a pulse in in-situ trans-esterification should be studied to evaluate its performance and compare with the continuous one in order to optimize ultra-sonication application in the process.

Methanol is polar but lipid is non-polar. It indicates that methanol is very difficult to approach lipid and react with it. It becomes much more difficult in the *in-situ* trans-esterification compared to the normal trans-esterification as barriers (cell wall, cell membrane, as well as water in the cells) exist in the system. In order to break the barriers, ultra-sonication can be provided. However, the polarity nature of methanol and lipid remains the same; hence, a non-polar solvent such as hexane and chloroform is normally added as co-solvent in *in-situ* trans-esterification in order to increase the contacting chance between lipid and methanol. Study showed that the combination of ultra-sonication and co-solvent addition (n-pentane) could increase conversion efficiency to 99% with much less methanol addition (methanol oil molar ratio of 79:1) compared to the one without co-solvent (93% biodiesel yield with methanol oil molar ratio of 315:1) (Ehimen et al. 2012). In most of the *in-situ* trans-esterification, co-solvent is utilized (Table 3.2).

Providing high temperature, pressure, and mixing is the most common way to enhance mass transfer in chemical reactions, thus, they are employed in *in-situ* trans-esterification as well. However, high temperature could cause the oxidation of unsaturated fatty acids and thus increase the saturation degree (increases the viscosity) of the biodiesel. In addition, high temperature can cause the loss of methanol if the reaction takes place in an open system. Therefore, the temperature of in-situ trans-esterification is generally controlled under around 60 ºC. Ultra-sonication can elevate the system temperature up to 100 ºC if it is not well manipulated, thus special attention should be given in the temperature adjustment in ultra-sonication assisted in-situ trans-esterification. High pressure can cause great concern in safety of the operation, thus very few studies have investigated the pressure impact on ultra-sonication assisting in-situ trans-esterification (Table 3.3). It was observed that with the high pressure (300 kpa) application, the reaction time could be significantly reduced to achieve a high conversion efficiency of biodiesel in-situ trans-esterification in which 99% conversion efficiency was obtained within 2 min reaction time (Ehimen et al. 2012). Mixing has also shown a great impact on the conversion efficiency of in-situ trans-esterification. Ultra-sonication produces vigorous mixing in local, but it cannot promote the mass homogenization in the reactor. Thus, macromixing is important in the system. Studies have revealed that mixing enhanced the efficiency of in-situ trans-esterification (Ehimen et al. 2012, Suganya et al. 2014); however, applying mixing in the ultra-sonication assisted in-situ trans-esterification is still limited. More efforts should be made in this aspect as it would be a way to promote in-situ trans-esterification into reality.

It can be seen that almost all the biomass used in the in-situ trans-esterification has been dried (Table 3.3). It is because of that water presence can inhibit the contact between methanol and lipid, and thus interfere the conversion of lipid to biodiesel (Park Jeongseok et al. 2017b, Zhang Y. et al. 2015b). However, drying is a high energy demand process, utilization of wet biomass for biodiesel production by in-situ trans-esterification should be performed in order to lower the energy input which influents on the cost of the process.

Ultra-sonication has shown great potential to enhance in-situ trans-esterification, however, it was also observed that it could impact on the quality of the final product (biodiesel) (Koutsouki et al. 2015, Zhang Xiaolei et al. 2016). When the biomass obtained from the

fermentation contains no impurities, ultra-sonication can be applied to assist the in-situ trans-esterification in order to achieve high conversion of biodiesel. However, if impurities are present in the biomass, the ultra-sonication application has to be well determined as ultra-sonication may induce certain reactions between lipid, methanol, or biodiesel with impurities and hence cause the formation of other products which might mix in the biodiesel. Moreover, ultra-sonication may also extract the impurities into the system and finally presenting in the biodiesel, which is normally wouldn't occur without ultra-sonication as they are hard to be separated from the solids.

3.8 Summary

Biodiesel production from heterotrophic oleaginous microorganisms includes the cultivation of the microorganisms, biomass harvesting from the fermentation broth, lipid extraction, and trans-esterification or in-situ trans-esterification which is the combination of lipid extraction and trans-esterification. Ultra-sonication has been employed throughout the process of biodiesel production from heterotrophic oleaginous microorganisms to enhancing the biodiesel yield. Ultra-sonication has been found capable of stimulating lipid accumulation in microorganisms during cultivation. However, the mechanism has not been revealed. Work is demanded to investigate how the lipid accumulation is enhanced and what are the ways to further optimize the accumulation in order to maximize lipid production. Study on ultra-sonication employment in biomass harvesting is very few which would be due to unattractive benefit on the harvesting. However, in fact, ultra-sonication can improve sedimentation which is the most cost-effective harvesting method. It is still worthy to give attention to the application of ultra-sonication in biomass harvesting. Among all, its application on lipid extraction, trans-esterification, and in-situ trans-esterification has been widely studied compared to in other steps. Overall, improvements in lipid recovery efficiency of extraction and biodiesel yield of trans-esterification and in-situ trans-esterification with the assistance of ultra-sonication have been observed compared to the controls. In-situ trans-esterification is considered as the promising replacement of lipid extraction and trans-esterification. Ultra-sonication would advance the application of in-situ trans-esterification in practice, but the optimal conditions (ultra-

sonication intensity, frequency, operation time, temperature etc.) seem case depending, and the trend is not obvious. There is no reliable reference to direct the application in large scale. Efforts should be made in the aspect.

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Table 3. 1 Ultra-sonication enhancement on biodegradability of organic waste

Organic wastes	Target product	Ultra-sonication conditions						Performance	Ref.
		Equipment	Frequency (kHz)	Power (W)	On-off cycle	Time (min)	Energy density (kWh/L)		
Food waste (rice, vegetables, fruit peels and other residues)	H2	Ultrasonic probe	20	1200	-	12	1.5	H2 yield: 149 ml/g VS (in control: 85 ml/g VS)	(Gadhe et al. 2014)
Food waste (from food production industry)	H2	Ultrasonic probe	20	500	-	-	1.10	H2 yield: 97 ml/g VS (in control: 42 ml/g VS)	(Elbeshbishy et al. 2011)
Palm oil and pulp and paper mills	H2	Ultrasonic probe	20	-	-	-	3.77	H2 yield: 8.72 mL/mL medium (in control: 4.67 ml/ mL medium)	(Budiman and Wu 2016)
Pulp and paper mills	H2	Ultrasonic probe	20	700	-	45	7.77	H2 yield: 5.77 mL/mL medium (in control: 1.10 ml/ mL medium)	(Hay et al. 2015)
Rice straw	-	Ultrasonic probe	22	300	2 s on, 4 s off	60	1.24	Reducing sugar concentration: 2.91 g/L (in control: 0.85 g/L)	(Wu et al. 2017)
Olive pomace	Xylanase and cellulase	Ultrasonic probe	20	750	-	10	0.61	Xylanase and cellulase production increased 3.6-fold and 1.2-	(Leite et al. 2016)

								fold, respectively, compared to control.	
Weeds	Ethanol	Ultrasonic bath	35	35	-	600	17.5	To achieve similar reducing sugar concentration (40 g/L), the treatment time was reduced from 120 h to 10 h.	(Borah et al. 2016)
Wastewater sludge	Methane	Ultrasonic bath	20	100	60 s on, 30 s off	144	7.2	CH4 production: 0.54 L/L·day (0.39)	(Cho et al. 2013)

Table 3. 2 Ultra-sonication assisted lipid extraction

Oleaginous microbes	Biomass concentration during ultra-sonication (g/L)	Ultra-sonication for cell disruption						Solvent	Biomass: solvent (g/mL)	Lipid recovery (% w/w total lipid	Ref.
		Type	Frequency (kHz)	Power (W)	Time (min)	Energy density (kWh/L)	Tem. (°C)				
Chlorella vulgaris (microalgae)	20	Pre-treatmenta	20	750	20	-	30	2:1 v/v chloroform: methanol	1:20	66	(Park Ji-Yeon et al. 2015)
Chlorella vulgaris (microalgae)	Dry cells	Extractionb	24	-	5	-	50	1:1 v/v chloroform: methanol	-	-	(Derakhshan et al. 2014)
Chlorella sp. (microalgae)	2	Pre-treatment	20	1000	-	0.8	20	-	-	75	(Nataraj an et al. 2014)
Mixed culture of autotrophic microorganisms	Dry cells	Extraction	40	80	40	11.22	-	1.5:1.25:1 v/v/v chloroform: methanol/water	1:9.5	39	(Florentino de Souza Silva et al. 2014)
Thalassiosira Fluviatilis (microalgae)	Dry cells	Extraction	40	400	20	6.67	0	Hexane	1:5	95	(Neto et al. 2013)
Tetraselmis suecica (microalgae)	8.4	Pre-treatment	40	130	25	0.27	20	0.7:3.3 v/v hexane and isopropanol	1:24	98	(Halim et al. 2013)
Rhodosporidium toruloides (yeast)	50	Pre-treatment	20	1000	30	25 W/cm2	55	16 v/v hexane: ethanol	1:100	27	(Meullemiestre A. et al. 2017)
Trichosporon oleaginosus (yeast)	Dry cells	Extraction	0.05	2800	15	16.8	25	1:1 v/v chloroform: methanol	1:20	100	(Zhang Xiaolei et al. 2014d)

Yarrowia lipolytica (yeast)	Dry cells	Extraction	20	300	30	3	20	1:2 v/v chloroform: methanol	1:5	60	(Halim et al. 2013)
Mixed culture of heterotrophic microorganism	32	Pre-treatment	-	600	20	1	20	Hexane	-	100	(Olkiewicz et al. 2015)

[a] Pre-treatment refers to the ultra-sonication is used to disrupt cells, after the treatment, the cells are recovered and exposed to solvent for lipid extraction;

[b] Extraction refers to the ultra-sonication is used to disrupt cells in the solvent.

Table 3. 3 Ultra-sonication application in *in-situ* trans-esterification

Microbe	Raw material	Pre-treatment	Ultra-sonication				Other combination	Methanol /lipid ratio	Catalyst (w/w oil)	Mixing (rpm)	Temperature (ºC)	Yield (%)	Ref.
			Frequency (kHz)	Power (W)	Mode (pulse/ continuous)	Time (min)							
Botryococcus sp.	-	Drying	30	200	continuous	360	-	-	Lipase	-	40	78	(Sivaramakrishnan and Incharoensakdi 2017)
Chlorella sp.	-	Drying	20	150	continuous	100	Co-solvent: chloroform	83:1	10 % H_2SO_4	-	60	81.20	(Karimi 2017)
Chlorella sp.	-	Drying	25	490	continuous	6	-	250:1	2% NaOH	-	60	96.20	(Martinez-Guerra et al. 2014)
Chlorella sp.	-	Drying	24	-	continuous	2	Co-solvent: n-pentane and diethyl ether; Pressure 300 kPa	315:1	3.78% H_2SO_4	500	60	99.00	(Ehimen et al. 2012)
Enteromorpha compressa	-	Drying	40	1200	continuous	90	Co-solvent: Tetrahydrofuran	5.5:1	10 % H_2SO_4	600	65	98.89	(Suganya et al. 2014)
Yarrowia lipolytica	Crude glycerol	N-Lauroyl sarcosine	20	750	continuous	35	Co-solvent: Hexane	360:1	3.5 % H_2SO_4	-	25	94.30	(Yellapu et al. 2017)

Scenedesmus sp.	-	Drying	22.5	100	continuous	20	-	60:1	4%	-	50	71.37	(Guldhe et al. 2014)
Trichosporon oleaginosus	Crude glycerol	Drying	20	750	continuous	20	Co-solvent: Hexane	60:1	1% NaOH	-	25	92.10	(Zhang Xiaolei et al. 2014b)
Trichosporon oleaginosus	Sludge	Drying	20	750	continuous	60	Co-solvent: Hexane	60:1	3% H2SO4	-	55	95.24	(Zhang Xiaolei et al. 2016)

Lipid production from fed-batch fermentation of crude glycerol directed by the kinetic study of batch fermentations

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4 LIPID PRODUCTION FROM FED-BATCH FERMENTATION OF CRUDE GLYCEROL DIRECTED BY THE KINETIC STUDY OF BATCH FERMENTATIONS

4.1 Résumé

La levure oléagineuse *Trichosporon oleaginosus* a été cultivée avec le glycérol brut comme substrat pour la production de lipides dans un fermenteur de 15 L. L'impact du rapport carbone sur azote (C/N), y compris 20, 30, 45 et 60, sur la production de lipides a été étudié dans des fermentations discontinues. La plus forte concentration de biomasse et de lipides de 24,80 g/L et de 12,14 g/L a été observée durant la fermentation au rapport C/N de 45. Le rapport C/N de 30 montre une concentration comparable de biomasse (23,70 g/L) et lipide (11,26 g/L). Basé sur l'étude cinétique des fermentations discontinues, la fermentation fed-batch a été réalisée. Dans la fermentation fed-batch, la concentration maximale de biomasse et de lipides était de 43,82 g/L et de 21,87 g/L, respectivement, au rapport C/N de 45.

Mots clés: levure oléagineuse; le glycérol brut; rapport C/N; étude cinétique; fermentation fed-batch.

4.2 Abstract

Oleaginous yeast *Trichosporon oleaginosus* was cultivated in crude glycerol as a substrate for lipid production in a 15 L fermenter. The impact of weight carbon to nitrogen ratio (C/N) including 20, 30, 45 and 60 on lipid production from the strain was investigated in batch fermentations. The highest biomass and lipid concentration of 24.80 g/L and 12.14 g/L, respectively, was observed during the fermentation at C/N ratio of 45. C/N ratio of 30 showed a comparable concentration of biomass (23.70 g/L) and lipid (11.26 g/L). Based on the kinetic study of the batch fermentations, fed-batch fermentation was designed and conducted. In the fed-batch fermentation, the maximum concentration of biomass and lipid was 43.82 g/L and 21.87 g/L, respectively at C/N ratio of 45.

Keywords: oleaginous yeast; crude glycerol; C/N ratio; kinetic study; fed-batch.

4.3 Introduction

Net energy production and carbon dioxide emission are the two most important factors for evaluating the sustainability of new energy sources [1]. Biodiesel production from plant seed oils provides positive net energy and replacement of diesel by biodiesel reduces carbon dioxide emission [2]. It indicates that biodiesel is a promising new energy source. Nowadays, the biodiesel is mainly produced from plant seed oils by a chemical reaction called trans-esterification [3]. However, biodiesel production is restrained by the availability of plant seed oils because they are also important food items. Researchers discovered that some oleaginous microorganisms could accumulate high lipid (microbial oil) content in their cells and the composition of lipids was similar to that of plant seed oils [4-8]. Therefore, microbial oils might replace plant seed oils for biodiesel production [9]. However, the cost of biodiesel produced from microbial oil is still very high, which is mainly attributed to the high substrate cost required for oleaginous microorganism cultivation [10].

In order to reduce the cost, studies have focused on using organic wastes instead of high-grade substrates for oleaginous microorganism cultivation [11]. Among the organic wastes, crude glycerol has attracted special attention as it is an unavoidable by-product of biodiesel production [12]. The utilization of crude glycerol for lipid production may reduce biodiesel production cost and could lead to biodiesel production from microbes, a zero waste discharge process [13]. However, cultivation of oleaginous microorganism with crude glycerol normally leads to low lipid production, especially when crude glycerol has high soap content [14]. Due to the shortage of plant seed oils and animal fats, waste cooking oil has become the main feedstock of biodiesel production, which generates high soap content crude glycerol.

With the aim of increasing lipid production, molar (or weight) C/N ratios have been widely investigated [15, 16]. Researchers found that most of the oleaginous microorganism started to accumulate lipid at molar C/N ratio of 20 and the lipid production increased with increasing molar C/N ratio. The lipid production did not vary much within the range of molar C/N ratio of 50 to 100. Inhibition of lipid synthesis was observed when molar C/N ratio was higher than 100 [17, 18]. In addition, the kinetic studies were also

performed to understand the product production pattern of microorganism and to find out the route of enhancing the product production [19-21].

In this study, the crude glycerol solution from biodiesel production industry containing low glycerol but high impurities (methanol, soap and catalyst) was used for lipid production using *T. oleaginosus*. As reported, high methanol concentration could inhibit the growth of the strain [22], thus methanol was evaporated from crude glycerol before being used. Soap and glycerol served as carbon sources from crude glycerol solution. The C/N ratios (w/w) of 20, 30, 45 and 60 were investigated to evaluate its impact on lipid production of *T. oleaginosus*. A kinetic study was then performed to explore the relation between the biomass and lipid production and the substrate utilization by *T. oleaginosus*. Based on the kinetic study, a three-step fed-batch fermentation was designed and employed to enhance the biomass and lipid production.

4.4 Materials and methods

4.4.1 Materials

4.4.1.1 Crude glycerol characterization

Crude glycerol was collected from a local biodiesel production industry which employed waste cooking oil as feedstock and base as catalyst. It was stored at room temperature (20 °C). Each time before utilization, the crude glycerol solution was well mixed. A 5 mL of crude glycerol was diluted 10 times to obtain a 50 mL of solution, and 0.5 mL of sample was withdrawn and plated in yeast peptone dextrose agar plates. The plates were incubated at 28 °C and observation was performed every 24 h. No microorganisms were detected in the plates up to 120 h. It suggested that no biodegradation occurred in the crude glycerol while storing at room temperature. It would be due to the high methanol concentration in the crude glycerol.

The crude glycerol density was measured by weighing 10 mL crude glycerol solution. The pH of the crude glycerol solution was measured with a pH meter and glycerol content was measured according to Bondioli and Bella [23]. Methanol content was obtained by evaporating 100 mL of crude glycerol solution at 60 °C for 15 minutes. The weight loss of

the solution was considered due to the methanol evaporation. To determine soap content, the pH of 50 mL crude glycerol solution was lowered to pH 1 by adding 85% H_3PO_4 to convert the soap to free fatty acids (FFA). The solution obtained was centrifuged at 5000 rpm for 20 minutes. The top layer (FFA) was collected and weighted. The amount of soap was then calculated according to Eq. 1:

Soap amount (g) =304×FFA amount/282 (1a)

Soap content (% w/v) = g of soap/50 mL of crude glycerol solution×100% (1b)

Where 304 represents average soap molar mass and 282 is average FFA molar mass.

The crude glycerol used in the study was generated in the trans-esterification with NaOH as a catalyst. To determine the catalyst (NaOH, 40 g/mol) content, 10 mL crude glycerol solution was adjusted to pH 7 with 1M HCl solution and the volume (V) used was recorded. The catalyst content (% w/v) was equal to 1×V×40/10×100%. Ash content was obtained by heating 10 mL crude glycerol solution sample at 750 °C for 3 h and then the residue (ash, Wash) was weighted. Ash content (% w/v) was equal to Wash/10 ×100%.

The component (glycerol, methanol, soap, catalyst, ash, water) concentration in crude glycerol solution is listed in Table 1.

4.4.1.2 Strain

Oleaginous yeast *Trichosporon oleaginosus* (ATCC 20905) was employed in the study for lipid production from crude glycerol.

4.4.2 Fermentation

4.4.2.1 Pré-culture

A loop full of *T. oleaginosus* colony preserved on malt extract agar plate was inoculated in 1L sterilized YPD medium (20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract). The inoculated medium was incubated at 30 °C, 180 rpm for 24 h in an incubating shaker. After 24 h of incubation, the pre-culture was then used to inoculate fermenters.

4.4.2.2 Batch fermentation

Crude glycerol solution was sterilized at 121 °C for 15 min before being used as carbon source. It is assumed that methanol evaporated during the sterilization process. The fermentation medium (per L) contained 2.7 g KH_2PO_4, 0.95 g Na_2HPO_4, 1.616 g NH_4Cl, 0.1g EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g peptone, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.0055 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4 \cdot 7H_2O$, 0.00076 g $MnSO_4 \cdot H_2O$ [24], and sterilized crude glycerol solution. The amount of crude glycerol added to the fermenter content was determined according to the C/N (w/w) ratio used.

The nitrogen in the medium was added in the form of peptone and NH_4Cl. The nitrogen concentration in the medium was 0.4 (g/L peptone)×0.15 (percentage of nitrogen in peptone)+1.616 (g/L NH_4Cl) ×0.28 (percentage of nitrogen in NH_4Cl) = 0.51 g/L. According to the crude glycerol composition (Table 4.1), 1 L crude glycerol solution contained 235.8 g soap and 132.4 g glycerol, which contributed 235.8 (g soap)×0.75 (g carbon/g soap) +132.4 (g glycerol)×0.39 (g carbon/g glycerol) =228.49 g carbon per L of crude glycerol solution. Since methanol was evaporated, carbon source due to methanol was not taken into consideration. The investigated C/N (w/w) ratios were 20, 30, 45, and 60. Therefore, to obtain the C/N (w/w) ratio of 20, 30, 45 and 60, 45 mL, 67 mL, 101 mL and 133 mL crude glycerol solution (non-sterilized), which provided 10.28 g, 15.30 g, 22.84 g and 29.69 g carbon from glycerol and FFA, were required, respectively. Hence, 45 mL (30 mL after sterilization), 67 mL (45 mL after sterilization), 101 mL (67.5 mL after sterilization) and 133 mL (90 mL after sterilization) crude glycerol solution were sterilized and then added to per liter medium to achieve the C/N (w/w) ratio of 20, 30, 45 and 60, respectively. The organic carbon concentration of the glycerol media was determined with TOC analyzer to check the calculation. The analyzed results were similar as calculated, which was 10.53 g , 15.62 g, 23.07 g and 29.91 g with analysis and 10.28 g, 15.30 g, 22.84 g and 29.69 g with calculation for the media prepared from 45 mL, 67 mL, 101 mL and 133 mL crude glycerol solution (non-sterilized), respectively.

Fermentation was carried out in a stirred tank 15 L fermenter (working volume: 10 L, Biogenie, Que., Canada) equipped with accessories and programmable logic control (PLC) system for dissolved oxygen (DO), pH, anti-foam, impeller speed, aeration rate and

temperature. The software (iFix 3.5, Intellution, USA) allowed automatic set-point control and integration of all parameters via PLC.

Before each sterilization cycle, the polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4 and 7 (VWR, Canada). The oxygen probe was calibrated to zero with 5% Na_2SO_3 solution and 100% with air-saturated water. Subsequently, fermenters were charged with fermentation nutrient medium (C/N (w/w) ratio of 20, 30, 45 and 60) and sterilized. Then the DO probe was recalibrated to zero by sparging N2 gas and 100% saturation by sparging air at an agitation rate of 500 rpm. Thereafter, 1 L pre-culture was transferred to the medium and fermentation started.

During fermentation, DO was maintained above 35% saturation by adjusting agitation rate (300-500 rpm) and air flow rate (0.2-2 L/min). The temperature was maintained at 30 °C by circulating water through the fermenter jacket. Fermentation pH was controlled automatically at 5±0.1 through computer-controlled peristaltic pumps by the addition of pH control agents: 4M NaOH /4M H_2SO_4. Dissolved oxygen and pH were continuously monitored by means of a polarographic dissolved oxygen probe and of a pH sensor (Mettler-Toledo, USA), respectively.

Samples (150 mL) were withdrawn at an interval of about 6 h to 8 h during the fermentation under the sterilized condition for sample analysis.

4.4.3 Kinetic study

The specific cell growth rate (μ) was calculated with Eq. 2:

$$\mu = \frac{\ln \frac{CFU_2}{CFU_1}}{t2 - t1} \quad (2)$$

where CFU_1 and CFU_2 were the CFU concentration of the sample at time t_1 and t_2, respectively.

Biomass productivity and lipid productivity were calculated with the following equations (Eq. 3and Eq. 4):

Biomass productivity (g/L/h) = $dX/dt = (X_2-X_1)/(t_2-t_1)$ (3)

Lipid productivity (g/L/h) = $dp/dt= (P_2-P_1)/(t_2-t_1)$ (4)

Where X_1, X_2 are the total biomass (lipid free biomass + lipid) concentration at time t_1 and t_2; P_1, P_2 are the lipid concentration at time t_1 and t_2.

The fermentations were performed at pH 5. The soap in crude glycerol was converted to FFA at this pH. Hence, the substrates in the system were glycerol and FFA. Glycerol and FFA consumption rates were calculated based on Eq. 5 and Eq. 6, respectively:

$$\text{Glycerol consumption rate (g/L/h)} = d(\text{glycerol})/dt = (S_{glycerol1}-S_{glycerol2})/(t_2-t_1) \quad (5)$$

$$\text{FFA consumption rate (g/L/h)} = d(\text{FFA})/dt = (S_{FFA1}-S_{FFA2})/(t_2-t_1) \quad (6)$$

Where $S_{glycerol1}$, $S_{glycerol2}$ are the glycerol concentration at time t_1 and t_2; S_{FFA1}, S_{FFA2} are the soap concentration at time t_1 and t_2.

4.4.4 Fed-batch fermentation

In the fed-batch fermentation, per liter medium nutrient contained 5.4 g KH_2PO_4, 1.9g Na_2HPO_4, 3.232 g NH_4Cl, 0.1g EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.8 g peptone, 0.04g $CaCl_2 \cdot 2H_2O$, 0.0055g $FeSO_4 \cdot 7H_2O$, 0.001g $ZnSO_4 \cdot 7H_2O$, 0.00076g $MnSO_4 \cdot H_2O$ and 30 mL of sterilized crude glycerol solution, which is equivalent to 10.28 g/L carbon. The weight C/N ratio of the basic medium was 10.

Similar to batch fermentations, after sterilization and inoculation, the fed-batch fermentation started. Extra sterilized crude glycerol solution was fed three times at 16 h, 24 h and 32 h fermentation time to increase the weight C/N ratio to 20, 30 and 45, respectively. During the fermentation, DO was kept above 35%, the temperature was maintained at 30 °C, pH was controlled at 5. Samples (150 mL each sample) were withdrawn at an interval of about 8 h during the fermentation under sterilized condition.

4.4.5 Analytical technologies

4.4.5.1 Biomass, glycerol and soap concentration

Unless stated, the item “biomass” refers to the sum of the lipid-free biomass (LFB) and lipid throughout the text. To determine biomass concentration, 10 mL sample was centrifuged at 8000 rpm for 15 min. The supernatant was collected and stored at 4 °C to determine glycerol and soap concentration. The resulting solids (pellet) were washed with

distilled water until a clear supernatant was obtained. Thereafter, the solid residue (after being washed) was transferred to a pre-weighed aluminum cup and dried at 105 °C until the weight was constant. The weight difference of the aluminum cup with and without solid residue was the weight of the solid residue. The biomass concentration was calculated by using the weight of solid residue (g) divided by 0.01 L.

Glycerol and soap concentrations were estimated with the similar method mentioned above in crude glycerol characterization.

4.4.5.2 Lipid content determination

The 30 mL of fermentation broth was centrifuged at 8000 rpm for 15 min. The biomass pellet obtained was washed twice with distilled water. The resulting solids were mixed with 30 mL of the mixture of chloroform and methanol (2:1 v/v) and 6 mL Zirconia beads (1 mm diameter) in a 50 mL solvent proof tube. The solution was subjected to a continuous shaking for 12 h in a wrist action shaker (Burrell Model 75). After being centrifuged at 6000 rpm for 15 min, the bottom layer (lipid dissolved in chloroform) was collected and filtered. The filtrate was collected. 20 mL mixture of chloroform and methanol (1:1 v/v) was then added to the residual biomass and beads for the second time extraction. After shaking for 2 h, the solution was treated in a similar way as the first extraction. After uniting the filtrates of the first and the second extraction, the mixture was centrifuged at 6000 rpm for 15 min. The bottom layer was collected and transferred to a pre-weighed glass tube and subjected to nitrogen gas evaporation. After the glass tube weight was constant, the lipid content was calculated according to Eq. 7:

$$\text{Lipid content (\% w/w)} = (W_L - W_e)/(SS \times V) \times 100\% \quad (7)$$

Where W_L was the weight of the glass tube with sample after evaporation (g); W_e was the weight of the empty glass tube (g); SS was the biomass concentration (g/L); V was the volume of the sample (30 mL or 0.03 L).

All analysis was conducted in duplicate samples.

4.5 Results and discussion

The crude glycerol used in this study contained high methanol (31.14±2.02% w/v) and soap (23.58±1.53% w/v) content but low glycerol (13.24±0.63% w/v) content (Table 4.1). This crude glycerol solution is of very low glycerol concentration, which has not been reported for lipid production with oleaginous microorganisms. Crude glycerol reported for oleaginous microorganism cultivation generally had glycerol content between 30 to 80% w/v [25, 26].

4.5.1 Batch mode experiments at different C/N ratio

A lag phase of 0 h to13 h or 16 h was observed after inoculation (Fig. 4.1a). The lag phase might be caused by the change of growth environment from pre-culture to the fermenter. The lag phase is generally induced by a sudden variation of the growth medium and/or growth conditions [27]. In this study, it would be due to the medium change. The maximum cell concentration of 6.0×10^{9} CFU /mL (C/N ratio of 20), 5.75×10^{10} CFU /mL (C/N ratio of 30), 7.8×10^{10} CFU /mL (C/N ratio of 45), 1.21×10^{10} CFU /mL (C/N ratio of 60) were observed at around 60 h.

The maximum biomass and lipid concentration were 13.40 g/L (at 63 h) and 3.19 g/L (at 42 h), 23.70 g/L and 11.26 g/L (at 54 h), 24.80 g/L and 12.14 g/L (at 56 h), and 19.30 g/L and 10.04 g/L (at 56 h) in the fermentation with C/N ratio of 20, 30, 45, and 60, respectively (Fig. 4.1b and c). The fermentation with C/N ratio of 20 was the only case where the maximum biomass and lipid concentration occurred at the different time. The biomass concentration increased with C/N ratio from 20 to 45 and decreased with further increase of C/N ratio to 60. The maximum biomass concentration at C/N ratio of 45 (24.80 g/L) was slightly higher than C/N ratio of 30 (23.70 g/L). This increase was mainly due to the increase of lipid concentration from 11.26 g/L (C/N ratio of 30) to 12.14 g/L (C/N ratio of 45), because the maximum concentration of lipid-free biomass (LFB = biomass minus lipid) corresponding to maximum lipid concentration at around 56h of fermentation was approximately same (Fig. 4.1d). Thus, C/N ratio of 30 was sufficient for biomass production and C/N ratio of 45 was somewhat favorable for lipid production. A similar

observation was also reported by other researchers that the optimal C/N ratio for *T.oleaginosus* was around 30 or 40 [24, 28, 29].

The highest lipid content (around 70% w/w) occurred at about 13 h or 16 h irrespective of C/N ratio (Fig. 4.1e). As discussed above, during the period of 0 h to 13 h or 16 h, the culture was in a lag phase in which the cell numbers were almost constant (Fig. 4.1a). It indicates that during this period the cell division might not take place. As mentioned above, the cultivation medium composition had a great difference when the culture was transferred from pre-culture medium to fermentation medium, which could have caused the lag phase. The shock is, in fact, one type of environmental stress for microorganisms. It was reported that oleaginous microorganisms generally intend to accumulate lipid content when stress is encountered [30, 31]. That is why the high lipid content had been observed during 0 to 16 h (Fig. 4.1e). From 13 h or 16 h to 24 h, the lipid content rapidly decreased and reached the minimum (Fig. 4.1e). During the time period of 13 h or 16 h to 24 h, the lipid-free biomass rapidly increased (Fig. 4.1d). However, lipid concentration was almost constant from 16 h to 24 h (Fig. 4.1c). Thus, the lipid content decreased to a minimum at 24 h due to increase in lipid-free biomass (Fig. 4.1d). After 24 h, the lipid concentration started to increase until 54 h or 56 h (except C/N ratio of 20). The lipid content at 54 h or 56 h fermentation was 22.98%, 47.50%, 48.95% and 52.02% (w/w) at C/N ratio of 20, 30, 45 and 60, respectively. This indicated that higher C/N ratio was favorable for *T. oleaginosus* to accumulate higher lipid content. A similar trend was observed when oleaginous yeast *Yarrowia lipolytica* was cultivated in fructose medium, lipid content constantly increased from 12.8% (w/w) to 55.7% (w/w) with increasing C/N ratio from 15 to 90 [32].

In this study, glycerol and soap were the carbon sources in the medium. In fact, the fermentation was conducted at pH 5 and soap at this pH was converted to free fatty acid (FFA). From 0 h to 8 h, the concentration of glycerol and FFA was slightly decreased (Fig. 4.1f and g) followed by a rapid decrease from 8 h to 40 h (Fig. 4.1f and g). From 40 h to the end of fermentation, the glycerol and FFA concentration decreased slowly (Fig. 4.1f and g). From 0 h to 8 h, biomass increase was slow and correspondingly the glycerol and FFA consumption was slow as well. Additionally, the increase of biomass and reduction of glycerol and FFA became concomitantly rapid from 8 h to 36 h (Fig. 4.1b, f, g). At C/N

ratio of 20, both glycerol and FFA were almost depleted at 42 h of fermentation (Fig. 4.1f and g). It indicated that after 42 h *T.oleaginosus* grew in a carbon limiting condition. As a result, the lipid concentration slightly decreased after 42 h (Fig. 4.1c). The lipid accumulation is accomplished in carbon-rich condition and the accumulated lipid in the cell could be decomposed and used to support the growth in carbon limiting condition [33]. At C/N ratio of 30, the depletion of glycerol and FFA occurred at 54 h (Fig. 4.1f and g). Thus, the lipid concentration after 54 h rapidly decreased (Fig. 4.1c). It was reported that a reduction of lipid concentration and lipid content was due to the utilization of lipids as an energy source after the depletion of carbon source in the medium [33]. At C/N ratio of 45, 2.35 g/L of glycerol and 2.98 g/L of FFA were left in the medium at the end of fermentation (Fig. 4.1f and g). However, in spite of the presence of carbon source, the lipid concentration still slightly decreased after 54 h (Fig.4.1c). This could be due to nitrogen limitation after 54 h, which is critical in cell metabolism [34]. At C/N ratio of 60, 5.46 g/L of glycerol and 5.32 g/L of FFA were left in the medium at the end of fermentation but still the lipid concentration slightly decreased after 60 h. It could be attributed also to nitrogen deficiency in the medium [34].

4.5.2 Kinetic study based on the batch mode experiments

4.5.2.1 Specific growth rate at different C/N ratio

CFU (Fig. 4.1a) was used to calculate the specific growth rate (μ) according to the Eq. 2. It was observed that approximately similar value of maximum specific growth rate (0.6 h^{-1}) was observed at C/N ratio of 20, 30 and 45 (Fig. 4.2). All fermentations were conducted under the similar conditions of pH, DO and temperature and the same concentrations of nitrogen, phosphorous and trace elements. The only difference was the carbon source concentration (glycerol and FFA concentration). Therefore, a similar value of the maximum specific growth rate observed at C/N ratio of 20, 30 and 45 indicated that the carbon source was sufficient to support the high specific growth rate even in the case of low C/N ratio of 20. It has been reported that *Cryptococcus curvatus* had high specific growth rate (maximum specific growth rate 0.43 h^{-1}) and was only slightly impacted by the variation of glycerol concentration when the carbon source was sufficient in the medium [35]. Apart from the carbon source, nutrients including nitrogen and phosphorus

were important for the growth of microorganisms, and the cell growth would be limited when these nutrients are deficient. A lower value of maximum specific growth rate (around 0.4 h^{-1}) at C/N ratio of 60 (Fig. 4.2) may be due to growth inhibition by excessive carbon source concentration.

4.5.2.2 Biomass productivity, lipid productivity and substrate consumption rate at different C/N ratio

The biomass productivity (dx/dt), lipid productivity (dp/dt), glycerol consumption rate (d(glycerol)/dt), and FFA consumption rate (d(FFA)/dt) were calculated according to the Eq. 3, Eq. 4, Eq. 5, and Eq. 6, respectively. The biomass, lipid, glycerol, and FFA data used in equations (Eq. 3, 4, 5, and 6) were used and presented in Fig. 4.1b, c, f, and g.

In all cases (C/N ratio of 20, 30, 45, and 60), the maximum biomass productivity occurred between 16 h and 32 h (Fig. 4.3a,b,c,d). The maximum biomass productivity of 1.08 g/L/h and 0.98 g/L/h at C/N ratio of 30 and 45, respectively, was not much different (Fig. 4.3b and c). The maximum biomass productivity of 0.39 g/L/h was lower in the case of C/N ratio of 20 due to carbon limitation (Fig. 4.3a) and 0.85 g/L/h in the case of C/N ratio of 60 due to growth inhibition by high substrate (carbon source) concentration (Fig. 4.3d).

The maximum lipid productivity reached around 24 h and stayed at the highest level until 48 h of fermentation at C/N ratio of 30, 45 and 60 (Fig. 4.3b,c and d). The lipid productivity reached a maximum after maximum biomass productivity occurred between 16 h and 24 h (Fig. 4.3b,c and d). At C/N ratio of 30 (0.28 g/L/h) and 45 (0.29 g/L/h) a similar lipid productivity was observed (Fig. 4.3b and c). At C/N ratio of 60, the lipid productivity (0.2 g/L/h) was higher than that at C/N ratio of 20 (0.14g/L/h) but lower than that at C/N ratio of 30 and 45 (Fig. 4.3b and c). Thus, it suggested that C/N ratio of 30 and 45 were suitable for lipid production, but the carbon concentration present in the medium was not sufficient to induce lipid production at C/N ratio of 20 and at C/N ratio of 60 carbon concentration was too much and inhibited cell growth as well as lipid production rate.

In this study, two carbon sources (glycerol and FFA) in the medium played important role in biomass and lipid production. Glycerol could be transported across the cell membrane directly by microorganisms through passive diffusion[36]. Glycerol consumption rate was associated with the biomass productivity [37, 38]. In other words, high biomass

productivity led to a high glycerol consumption rate. FFA was consumed through the reaction pathways of ω- and β-oxidation [39]. FFA consumption rate was found to depend mainly on the metabolic activity of microorganisms, pH and temperature [40].

At C/N of 20, 30 and 45, both glycerol and FFA were rapidly consumed during 16 h to 32 h imparting a high biomass productivity (Fig. 4.3 a,b,c and d). However, the consumption rate of FFA and glycerol kept increasing at C/N ratio of 60 even after the growth rate (dx/dt) started decreasing from 16 h (Fig. 4.3d). It could be due to the diversion of the substrate for the production of other products such as citric acid, which would be generated in the presence of excess substrate (carbon source) condition [34]. At C/N ratio of 20 and 30, maximum FFA consumption rate (13 h or 16 h) occurred before the maximum biomass productivity (24 h) (Fig. 3a b). At C/N ratio of 45, maximum FFA consumption rate (24 h) occurred almost at the same time with the maximum biomass productivity (24 h) (Fig. 4.3c). At C/N ratio of 60, maximum FFA consumption (32 h) rate took place after the highest biomass productivity (24 h) (Fig. 4.3d). Thus, at low C/N ratio, FFA consumption rate reached maximum before the maximum biomass productivity and at high C/N ratio, maximum FFA consumption attained after the maximum biomass productivity.

4.5.3 Fed-batch fermentation

4.5.3.1 Fed-batch fermentation design

As discussed above, an increase of nitrogen and phosphorus concentration of the fermentation medium might increase the cell growth. Thus, a fermentation with double nitrogen (0.51 g/L in batch fermentation and 1.02 g/L in fed-batch fermentation) and phosphorous concentration (0.81 g/L in batch fermentation and 1.62 g/L in fed-batch fermentation) was conducted in order to enhance biomass and lipid production.

From batch fermentations, it was found that C/N ratio of 45 was favorable for biomass and lipid production. The carbon concentration needed would be 45.9 g/L in order to achieve the C/N ratio of 45 with the nitrogen concentration of 1.02 g/L. However, the carbon concentration of 29.69 g/L was proved to be excessive and caused inhibition (Fig. 4.1 a,b,c,d and Fig. 4.2). Hence, it would not be proper to perform fermentation at C/N

ratio of 45 when the nitrogen and phosphorous concentration were doubled in the medium in batch mode. However, unlike batch fermentation that substrates and nutrients were added once for all before fermentation, fed-batch fermentation is a solution to avoid the inhibition of excessive carbon concentration as the carbon source could be added separately during fermentation [41].

It was observed that high biomass productivity led to a high carbon (glycerol and FFA) consumption rate. Thus, carbon source should be sufficient when the high biomass productivity occurred, which was during 16 h to 32 h (Fig. 4.3 a,b,c and d). It indicated that the carbon source should be fed during 16 h to 32h. To minimize the excess carbon source inhibition, the carbon source feeding was divided into three steps, but the C/N ratio was always calculated based on the initial nitrogen concentration. The fed-batch fermentation started with carbon concentration (contributed by glycerol and FFA) of 10.28 g/L, which was proved to be sufficient for the growth (C/N ratio of 10 in batch fermentation, Fig. 4.2). At 16 h, 10.28 g/L of carbon source (from glycerol and FFA) was fed to the medium to increase the C/N ratio to 20. The C/N ratio was calculated based on initial nitrogen concentration of 1.02 g/L at 0 h and the total carbon source of 20.56 g/L (10.28 g/L at 0 h and 10.28 g/L at 16 h) after a feed. At 24 h, 10.28 g/L of carbon (glycerol and FFA) was fed to the fermenter for the second time and the C/N ratio was increased to 30 (calculated same way as mentioned above). At 32 h, 15.42 g/L of carbon (from glycerol and FFA) was fed to the medium for the third time and the C/N ratio became 45 (calculated same way as mentioned above). The feed dilution of the fermenter medium was not taken into consideration because the sterilized crude glycerol solution used as feed was very less compared to the working volume of the fermentor.

4.5.3.2 Fed-batch fermentation performance

From 0 h to 8 h, biomass and lipid concentration gradually increased as glycerol and FFA concentration slowly decreased (Fig. 4.4a). A similar observation was found in batch fermentations with all C/N ratios (20, 30, 45, and 60). After 8 h, the biomass (with lipid) concentration started to rapidly increase. At 16 h, the concentration of biomass and lipid were 15.84 g/L and 1.54 g/L, respectively. It indicated that the carbon sources were mainly used for lipid-free biomass production and a small amount of lipid was produced

during 0 h to 16 h. Sterilized crude glycerol solution was added at 16 h, 24 h and 32 h to provide glycerol and FFA to feed the growing microorganisms, which substantially increased the biomass concentration (Fig. 4.4a) compared to the batch process at same C/N ratio (45) (Fig. 4.1b). The increase of biomass and lipid concentration was rapid from 16 h to 32 h, and correspondingly the consumption of glycerol and FFA was also rapid (Fig. 4.4a). At 32 h, the total biomass (with lipid) concentration increased to 34.76 g/L and lipid concentration increased to11.16 g/L. It suggested that the strain was fast producing biomass as well as lipid during 16 h to 32 h (Fig.4.4b). From 32 h to 52 h, the biomass concentration increased by 9.06 g/L (from 34.76 g/L to 43.82 g/L) and the lipid concentration increased by 10.71 g/L (from 11.16 g/L to 21.87 g/L). It indicated that the biomass increase from 32 h to 52 h was due to the lipid accumulation. It was observed that the LFB concentration reached a maximum at 24 h and from 36 h to 52 h the LFB concentration was slightly decreased (Fig. 4.4a). At 52 h, the maximum biomass concentration of 43.82 g/L, the maximum lipid content of 49.89% (w/w), and the maximum lipid concentration of 21.87 g/L were simultaneously achieved (Fig. 4.4a). Thereafter, biomass concentration and lipid concentration slightly decreased. It would be due to the deficiency of carbon source because glycerol and FFA were almost exhausted (Fig. 4.4a). In fact, lipid production might continue if the sufficient carbon source was present.

According to the kinetic parameters estimation, the maximum biomass productivity and lipid productivity in the fed-batch fermentation was 1.55 g/L/h and 0.97 g/L/h (Fig. 4.4b), which was much higher than 0.98 g/L/h and 0.29 g/L/h in batch fermentation (Fig. 4.3c), respectively at the same C/N ratio of 45. The high productivity of biomass and high consumption rate of FFA and glycerol simultaneously occurred during 10 h to 32 h in the fed-batch fermentation (Fig. 4.4b), which were similar to those observed in the bath process at C/N ratio of 45 (Fig. 4.3c). Compared with batch fermentations (Table 4.2), the fed-batch fermentation designed based on the kinetic parameters of batch fermentations had significantly improved the biomass and lipid production as well as the biomass and lipid productivity. It has been widely reported that fed-batch fermentation normally needed a longer time to reach higher and the maximum biomass and lipid production compared with batch fermentation [24, 29, 42-44]. In this study, the maximum biomass and lipid production during fed-batch fermentation were almost twice as much as those obtained

in batch fermentation at same C/N ratio of 45 (Table 4.2). However, the fermentation time was similar, which was 52 h for fed-batch fermentation and 54 h for the batch fermentation at C/N ratio of 45. The enhancement of biomass and lipid production was observed in fed-batch compared to the batch one (Fig. 4.3c and Fig. 4.4b), which could be attributed to the increase of the initial concentration of nitrogen (final C/N ratio was same as in batch culture C/N ratio of 45) and phosphorus in the fed-batch process. In addition, substrate inhibition was minimized by dividing the feed in three shots in the fed-batch fermentation, which was only one feed in batch fermentation. Based on this study, 1 L crude glycerol solution could produce 109.35 g lipids which could synthesize 103.88 g biodiesel by assuming the conversion rate of lipid to biodiesel was 95%.

It was reported that crude glycerol with a high impurity (FFA, methanol and catalyst) could impact the biomass and lipid production [24] (Table 4.3). However, this study showed that the high biomass and lipid production could be achieved without the impact of high impurities content of crude glycerol. In addition, the lipid productivity obtained in this study was similar to that obtained with pure glycerol [45] and even comparable with those obtained with glucose (Table 4.3) [42-44]. It indicated that crude glycerol solution even with high impurity could be employed for lipid production as long as the fermentation was well designed and operated. As mentioned, the current problem of biodiesel production from microbial oils (lipids) was the high cost of the raw material (carbon source) for oleaginous microorganism cultivation. However, this study provided a way of lipid production with crude glycerol to obtain similar lipid productivity as that employing glucose. It would be a method to promote the biodiesel production from microbial oils.

4.6 Conclusions

This work studied the lipid production from oleaginous yeast *T. oleaginosus* with crude glycerol solution having low glycerol content but high soap and methanol content. The optimal C/N ratio was found to be around 45. Based on the kinetic study, a fed-batch fermentation was designed to enhance the biomass production and lipid production. The average lipid productivity in the designed fed-batch fermentation was up to 0.42 g/L/h, which was comparable with those using glucose as substrate. It was confirmed that crude

glycerol even with high impurities (soap, free fatty acids) could replace glucose for lipid production when proper fermentation was employed. This study indirectly suggests that a suitable fermentation can enhance the lipid production of oleaginous microorganisms.

4.7 Acknowledgment

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4.8 References

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Table 4. 1 Crude glycerol composition

Component name	Concentration of the component (% w/v)
Density	0.877±0.05 (g/mL)
pH	8.32 (Temperature 20.0 °C)
Methanol	31.14±2.02
Soap	23.58±1.53
Glycerol	13.24±0.63
Water	10.37±0.81
Ash	3.00±0.10
Catalyst (NaOH)	3.10±0.14

Table 4. 2 Comparison of bath fermentations with fed-batch fermentation

Fermentation mode	C/N ratio (w/w)	Carbon concentration (g/L)	Nitrogen concentration (g/L)	Maximum biomass concentration (g/L)	Lipid concentration at maximum biomass (g/L)	Lipid content % (w/w)	Average biomass productivity (g/L/h)	Average lipid productivity (g/L/h)	Fermentation time (h)
Batch	20	10.28	0.51	13.40	3.08	22.98	0.21	0.05	63
Batch	30	15.30	0.51	23.70	11.26	47.50	0.44	0.21	54
Batch	45	22.84	0.51	24.80	12.14	48.95	0.44	0.22	56
Batch	60	29.69	0.51	19.30	10.04	52.02	0.34	0.18	56
Fed-batch	45	46.26	1.02	43.82	21.87	49.89	0.84	0.42	52

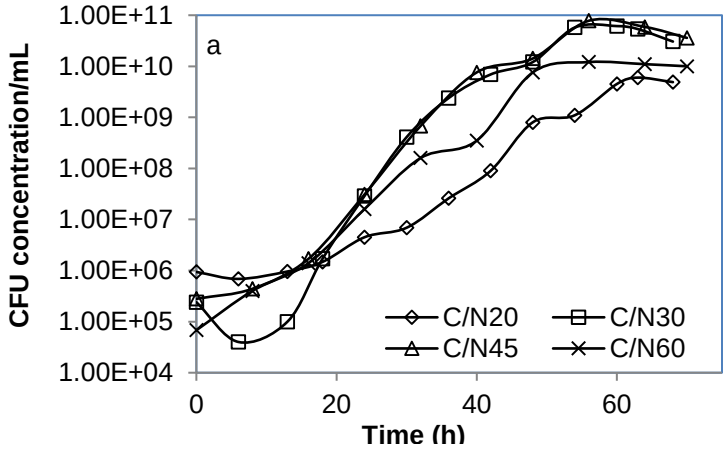
Average biomass productivity =maximum biomass concentration (g/L)/fermentation time (h)

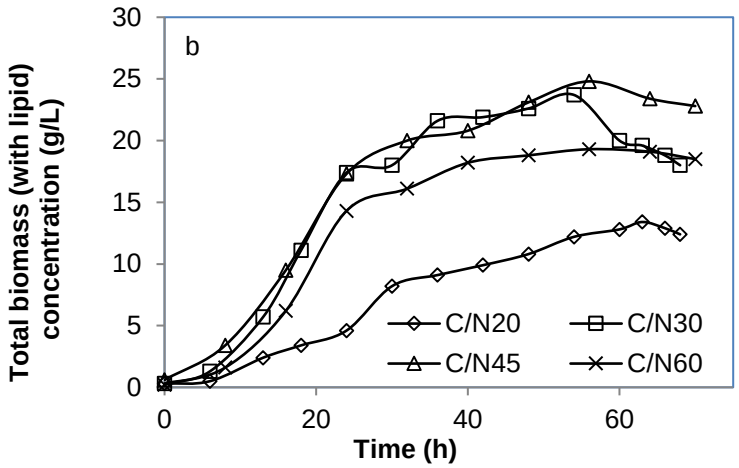
Average lipid productivity=maximum lipid concentration (g/L)/fermentation time (h)

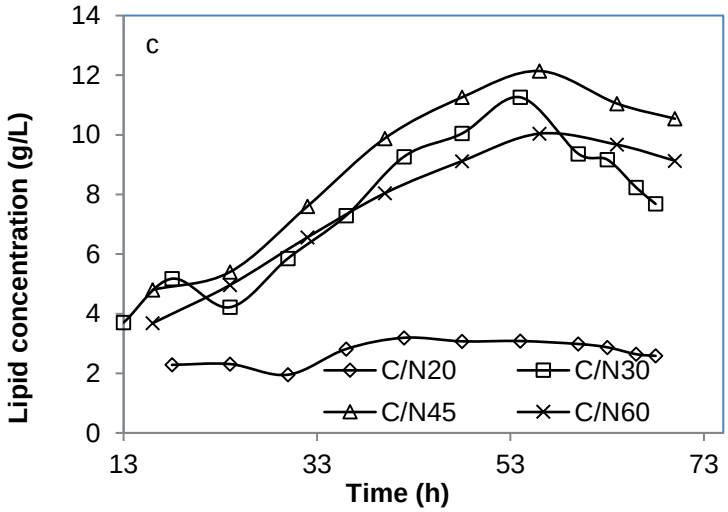
Table 4. 3 Biomass and lipid production of oleaginous yeast using different substrates

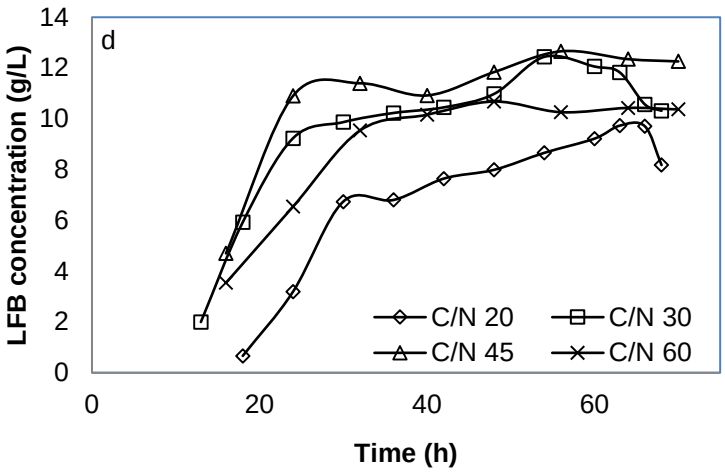
Microbe	Cultivation mode	Substrate	Biomass (g/L)	Lipid (g/L)	Lipid content % (w/w)	Average lipid productivity (g/L/h)	Ref.
Trichosporon oleaginosus	Fed-batch	Crude glycerol	43.82	21.87	49.89	0.42	This study
Cryptococcus curvatus	Fed-batch	Crude glycerol	32.9	17.40	52.9	0.06	[24]
Yarrowia lipolytica	Continous	Glycerol	59.8	24.2	40.0	0.43	[45]
Lipomyces starkeyi	Fed-batch	Glucose and xylose	77.4	36.7	49.9	0.32	[42]
Rhodosporidium toruloides Y4	Fed-batch	Glucose	106.5	71.88	67.5	0.54	[43]
Cryptococcus curvatus O3	Fed-batch	Glucose	104.1	86.09	82.7	0.47	[44]

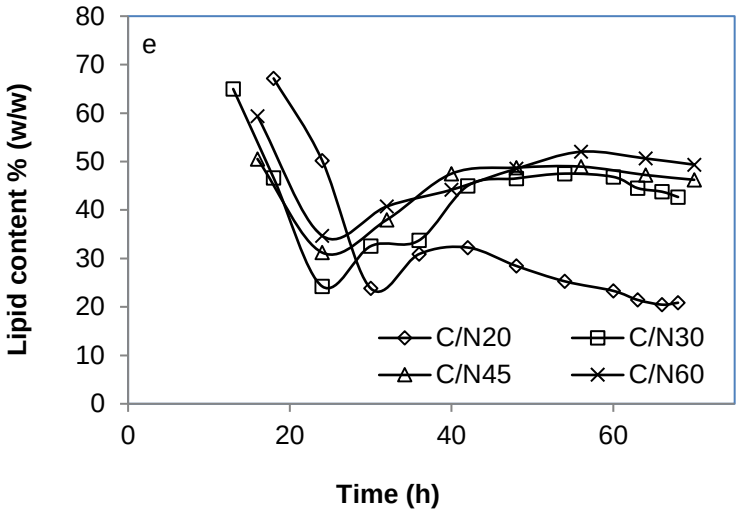
Average lipid productivity= Maximum lipid production (g/L)/fermentation time

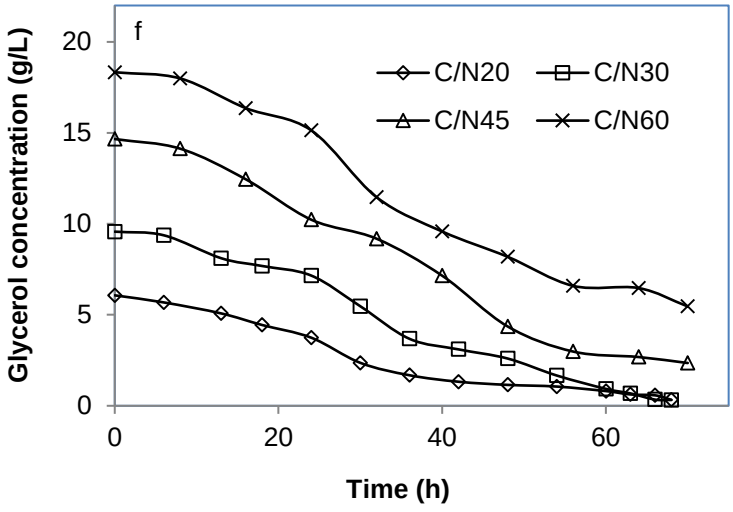

a
CFU concentration/mL
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
0
20
40
60
Time (h)
C/N20
C/N30
C/N45
C/N60


b
Total biomass (with lipid) concentration (g/L)
30
25
20
15
10
5
0
0
20
40
60
Time (h)
C/N20
C/N30
C/N45
C/N60


c
Lipid concentration (g/L)
14
12
10
8
6
4
2
0
13
33
53
73
Time (h)
C/N20
C/N30
C/N45
C/N60


d
LFB concentration (g/L)
Time (h)
C/N 20
C/N 30
C/N 45
C/N 60


e
Lipid content % (w/w)
Time (h)
C/N20
C/N30
C/N45
C/N60


f
Glycerol concentration (g/L)
Time (h)
C/N20
C/N30
C/N45
C/N60

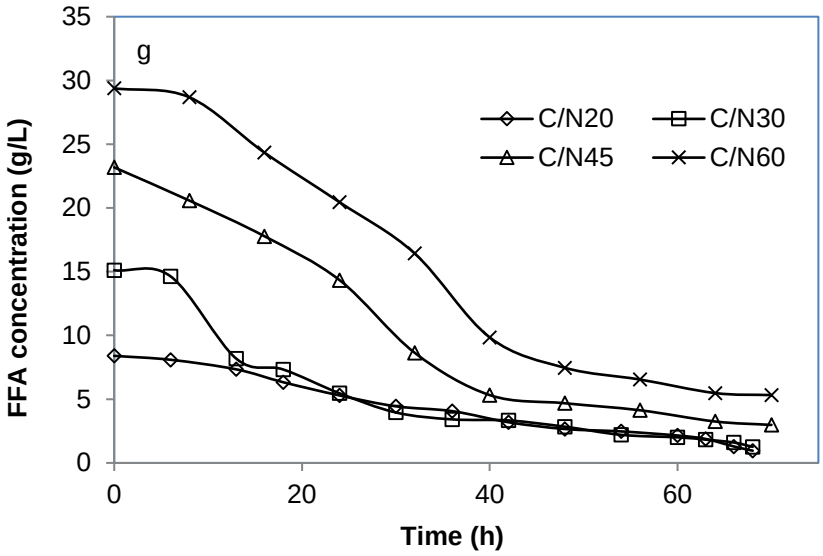


Figure 4. 1 Batch fermentations at different C/N ratio

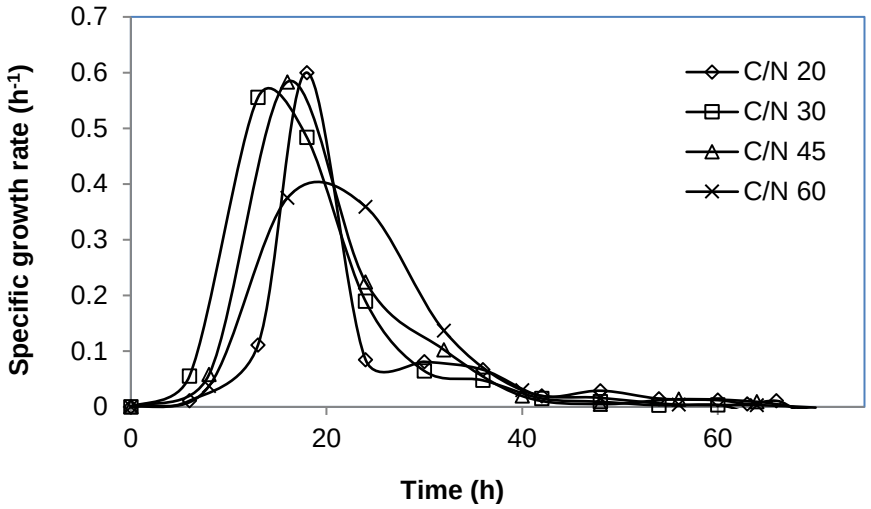
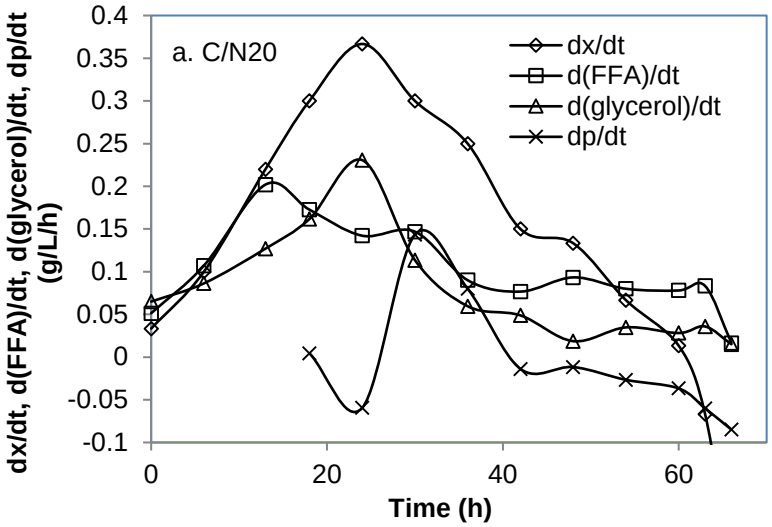
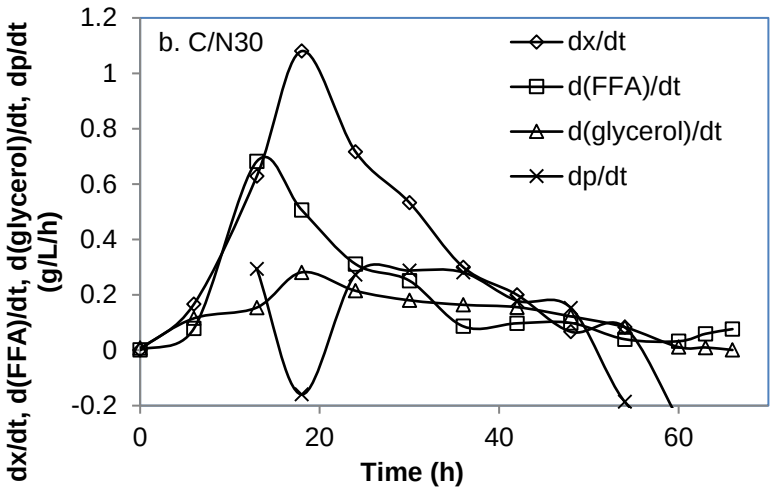
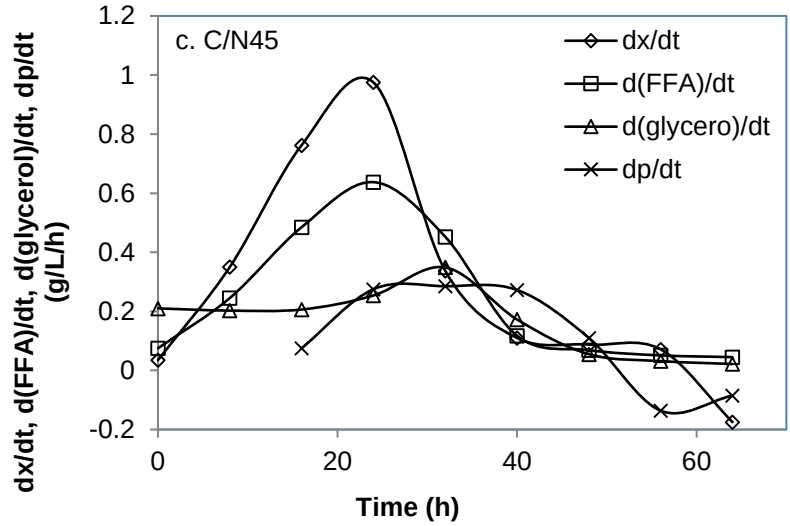


Figure 4. 2 Specific growth rates at different C/N ratios


a. C/N20
dx/dt
d(FFA)/dt
d(glycerol)/dt
dp/dt
dx/dt, d(FFA)/dt, d(glycerol)/dt, dp/dt (g/L/h)
Time (h)


b. C/N30
dx/dt
d(FFA)/dt
d(glycerol)/dt
dp/dt
dx/dt, d(FFA)/dt, d(glycerol)/dt, dp/dt (g/L/h)
Time (h)


c. C/N45
dx/dt
d(FFA)/dt
d(glycero)/dt
dp/dt
dx/dt, d(FFA)/dt, d(glycerol)/dt, dp/dt (g/L/h)
Time (h)

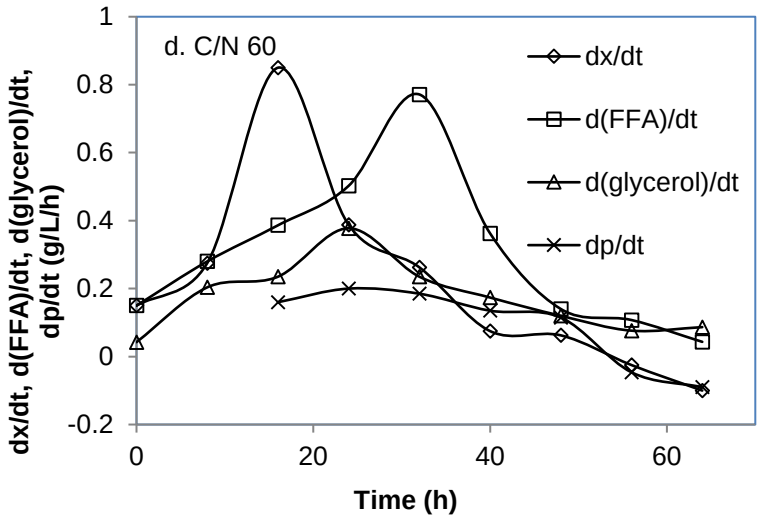


Figure 4. 3 Kinetic study on biomass and lipid production, nutrients consumption

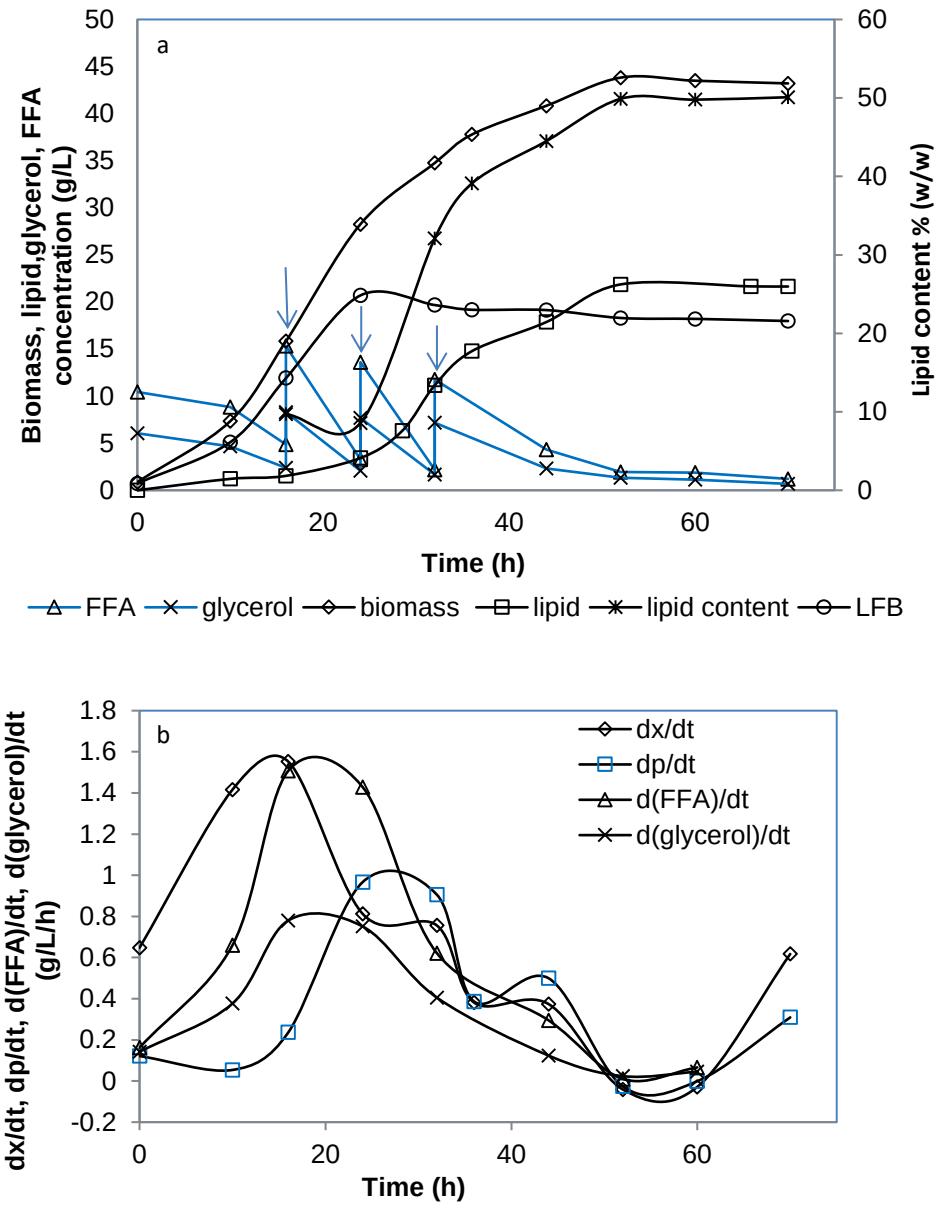


Figure 4. 4 **The biomass and lipid productivities in fed-batch fermentation (FFA is free fatty acids; LFB is lipid free biomass)**

Utilization of methanol in crude glycerol to assist lipid production in non-sterilized fermentation from *Trichosporon oleaginosus*

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5 UTILIZATION OF METHANOL IN CRUDE GLYCEROL TO ASSIST LIPID PRODUCTION IN NON-STERILIZED FERMENTATION FROM *TRICHOSPORON OLEAGINOSUS*

5.1 Résumé

Dans ce travail, le méthanol dans une solution de glycérol brut a été utilisé pour aider la levure oléagineuse *Trichosporon oleaginosus* à produire des lipides dans des conditions non stérilisées. La concentration de methanol étudiée était de 0%, 1,4%, 2,2%, 3,3% et 4,4% (poids/volume). Les résultats ont montré que le méthanol jouait un rôle important dans la fermentation non stérilisée pour la production de lipides. La concentration optimale de méthanol était d'environ 1,4% (p/v) dans laquelle la croissance de *T. oleaginosus* était favorisée et surmontait celle des contaminants. La fermentation fed-batch non stérilisée avec une concentration en méthanol de 1,4% (p/v) a ensuite été réalisée et une production élevée de biomasse (43,39 g/L) et une production élevée de lipides (20,42 g/L) ont été atteintes.

Mots clés : la production de lipides; levure oléagineuse; fermentation non stérilisée; le méthanol; solution de glycérol brut.

5.2 Abstract

In this work, methanol in crude glycerol solution was used to assist the lipid production with oleaginous yeast *Trichosporon oleaginosus* cultivated under non-sterilized conditions. The investigated methanol concentration was 0%, 1.4%, 2.2%, 3.3% and 4.4% (w/v). The results showed that methanol played a significant role in the non-sterilized fermentation for lipid production. The optimal methanol concentration was around 1.4% (w/v) in which the growth of *T. oleaginosus* was promoted and overcame that of the contaminants. The non-sterilized fed-batch fermentation with initial methanol concentration of 1.4% (w/v) was then performed and high biomass production (43.39 g/L) and lipid production (20.42 g/L) were achieved.

Keywords: lipid production; oleaginous yeast; non-sterilized fermentation; methanol; crude glycerol solution.

5.3 Introduction

As an alternative to petrodiesel, biodiesel has been given increasing interest. It is mainly due to that biodiesel can be directly utilized in diesel engines without any modification with less emission of hydrocarbon (5.76–6.25%) and carbon dioxide (0.47–0.58%) (Mohsin et al., 2014). A rapid development in biodiesel production technology is due to the advantages associated with the safe environment. Presently, vegetable oils and animal fats are the common feedstocks of biodiesel production(Gerpen, 2005). However, their prices keep increasing due to their high demand for food production. Consequently, biodiesel production from these traditional feedstocks gradually becomes unattractive (Ma & Hanna, 1999). It is necessary to find an alternative replacement, which should be abundant and cost compatible.

Previous researchers found that some microorganisms could accumulate high lipid amount in their cells (Eroshin et al., 2000; Koutb & Morsy, 2011; Tai & Stephanopoulos, 2013; Tanimura et al., 2014). In addition, the compositions of the lipids are similar to the plant seed oils. Thus, lipids are considered as good candidates of traditional feedstocks for biodiesel production (Sitepu et al., 2014; Zhang et al., 2014b).

Currently, the heterotrophic oleaginous microorganism is gaining great attention for lipid production because of its fast growth and high lipid productivity (Koutb & Morsy, 2011; Tai & Stephanopoulos, 2013). However, the lipid production is still limited to the lab scale owing to the high production cost and complicated operation processes (Koutinas et al., 2014).

To ensure the high lipid content of microbial cells, sterilization of the medium and fermenter before fermentation is necessary and maintenance of the sterilized condition during the fermentation is important (Zhao et al., 2008). However, the sterilization requires immense energy input and high facilities investment, which make the lipid production from heterotrophic oleaginous microorganism not feasible for industrial practice (Koutinas et al., 2014). To avoid the sterilization during lipid production, strategies including high substrates concentration, low fermentation temperature and low fermentation pH were individually or conjunctively applied (Santamauro et al., 2014; Taskin et al., 2016; Taskin et al., 2015). However, studies revealed that these strategies somehow negatively

affected the lipid production. Work is required to develop a new method to achieve similar lipid production from heterotrophic oleaginous microorganism under non-sterilized conditions as under sterilized condition.

Previous researchers revealed that the maximum methanol concentration that microorganism can tolerate is strain depending (Dewez et al., 2003; Salakkam & Webb, 2015). Normally, bacteria have less tolerance toward methanol concentration (which is 0.1-1% w/v) than yeasts (1-4% w/v) (Chen et al., 1976). Yeast like *Saccharomyces Cerevisiae* can even tolerate the methanol concentration up to 12% (w/v) (Ramos et al., 2013). In addition, methanol could promote or inhibit the growth of the microorganisms by varying its concentration (Chen et al., 1976). When the methanol concentration is in the range of promoting oleaginous yeast growth but inhibiting that of the contaminants (mainly bacteria), lipid production from oleaginous yeast could be achieved in non-sterilized conditions by manipulating methanol concentration in this range.

Commercially, biodiesel is synthesized by trans-esterification of vegetable oils. In the reaction, alcohol is the other reactant. Methanol is currently used due to the cost consideration. To convert every 1 mole of vegetable oils requires three moles of methanol; however, in the industrial practice, methanol addition is normally overdosed to maximize the conversion efficiency of the vegetable oils to biodiesel (Musa, 2016). Generally, biodiesel production industries are reluctant to recover the excess methanol, due to the low price of methanol and high energy demand for the recovery (Kiss & Ignat, 2012). As a result, the overdosed methanol would be left in the by-product of biodiesel production, which is crude glycerol. Thus, crude glycerol generally has high methanol content. Apart from glycerol and methanol, some other materials including soap, water, catalyst (acid or base), etc. were also present in the crude glycerol (Kitcha & Cheirsilp, 2011; Saenge et al., 2011; Signori et al., 2016). It was reported that twenty-three out of 113 yeast strains showed comparable or even higher specific growth rate, biomass production and biomass yield on crude glycerol compared to those on glucose regardless of the impurities of the crude glycerol (Taccari et al., 2012). The effect of four impurities in crude glycerol (methyl oleate, sodium oleate, NaCl and methanol) on lipid production by oleaginous yeast *Rhodosporidium toruloides* was investigated (Gao et al., 2016). Except for methanol, the other three impurities were found able to enhance lipid production. Methanol inhibition

occurred at a concentration higher to 14 g/L and lipid production was decreased. However, methanol inhibition was neglected when methanol was mixed with other impurities (Gao et al., 2016). Similarly, methanol in crude glycerol showed minor inhibition on lipid production by oleaginous yeast *Lipomyces starkeyi* at the concentration of 14 g/L (Liu et al., 2017). Oleaginous yeast *Rhodosporidium toruloides* grew well and showed good lipid production at high methanol concentration of 1.5% (w/v) in the presence of various impurities from crude glycerol (Uprety et al., 2017). Hence, if the methanol concentration is well manipulated, methanol in the crude glycerol may be used to control the contamination in order to produce lipid in non-sterilized fermentation, hence reduce the lipid production cost which in turn decrease the cost of biodiesel produced from the lipid.

In this study, a crude glycerol with high methanol content from a local biodiesel production company was employed for lipid production from oleaginous yeast *Trichosporon oleaginosus*. To evaluate the impact, the fermentation of *T. oleaginosus* with methanol-free crude glycerol was conducted in sterilized and non-sterilized fermentation. The necessity of methanol and the optimal methanol concentration presented in the fermentation medium were studied for lipid production in non-sterilized conditions. Studies have revealed that methanol concentration of 1.1% - 1.3% (w/v) could promote the growth of some microorganisms and no growth was observed for the bacteria after the methanol concentration beyond 4.6% (w/v) (Chen et al., 1976; Wayman & Tseng, 1976). Thus, in this study, methanol concentration of 1.4% (w/v), 2.2% (w/v), 3.3% (w/v) and 4.4% (w/v) were utilized to investigate the optimal methanol concentration on the lipid production from *T. oleaginosus* in the non-sterilized fermentation. A fed-batch fermentation starting with the optimal methanol concentration in the medium was conducted under non-sterilized conditions to enhance biomass and lipid production.

5.4 Materials and methods

5.4.1 Materials

5.4.1.1 The strain

In the study, oleaginous yeast *Trichosporon oleaginosus* (ATCC 20905) was employed as lipid producer.

5.4.1.2 Crude glycerol solution collection and composition

Crude glycerol solution from a local biodiesel production company was collected and stored at room temperature (20 °C) for further utilization. The crude glycerol was characterized by the similar methods exhibited in our previous research (Chen et al., 2017).

5.4.2 Contamination impact on lipid production from *T. oleaginosus* under non-sterilized conditions

5.4.2.1 The pre-culture medium

The pre-culture medium was prepared by dissolving 50 g YPD (20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract) in 1 L tap water in a 6 L flask. The medium was sterilized at 121 °C for 15 min. After cooling down to about 25 °C, a loop full of *T. oleaginosus* colony preserved in malt extract agar plate at 4 °C was inoculated to the medium and incubated at 30 °C and180 rpm in a shaker. After 24 h, the pre-culture was used as a seed for fermentation.

5.4.2.2 Lipid production from methanol-free crude glycerol solution under sterilized and non-sterilized conditions

Around 2 L of crude glycerol was sterilized at 121 °C for 15 min to remove methanol, and then the 0.45 L of the methanol free crude glycerol was taken to prepare 9 L of the fermentation medium. Other chemicals including 27g KH_2PO_4, 9.5 g Na_2HPO_4, 16.16 g NH_4Cl, 1g EDTA, 2 g $MgSO_4 \cdot 7H_2O$, 4 g peptone, 0.4 g $CaCl_2 \cdot 2H_2O$, 0.055 g $FeSO_4 \cdot 7H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$, and 0.0076 g $MnSO_4 \cdot H_2O$ were dissolved in 8.55 L tap water, and then directly mixed with the 0.45 L crude glycerol (methanol free) to cultivate *T.*

oleaginosus for lipid production in the non-sterilized fermentation. In sterilized fermentation, the 8.55 L nutrient solution was transferred to the fermenter and sterilized at 121 °C for 15 min, thereafter, the 0.45 L crude glycerol (methanol free) was added to the fermenter prior to the fermentation.

All the fermentation experiments were conducted in a 15 L fermenter with a working volume of 10 L. For the sterilized fermentation, after the nutrient solution (8.55 L) was sterilized, cooled down to 28 °C, and well mixed with 0.45 L sterilized crude glycerol (methanol free), the 1 L pre-culture was inoculated. The fermentation was operated at pH 5, temperature 30°C, agitation 300 to 500 rpm, and dissolved oxygen (DO) concentration above35% (v/v). At an interval of 6h or 8h, Samples (110 mL/sample) were taken during the fermentation for the analysis.

In the non-sterilized fermentation, the 1 L pre-culture was inoculated to the 9 L medium containing 8.55 L of the non-sterilized nutrient solution and 0.45 L methanol free crude glycerol. Fermentation conditions were similar to the sterilized one (pH 5, temperature 30°C, agitation 300 to 500 rpm, and dissolved oxygen (DO) concentration＞ 35% (v/v)). Samples were taken in a similar way as described in the sterilized fermentation.

5.4.3 Optimization of methanol concentration for lipid production in non-sterilized fermentation

5.4.3.1 The pre-culture medium

The pre-culture medium was prepared similar to that been presented above.

5.4.3.2 Fermentation

Crude glycerol containing methanol was directly used to prepare fermentation medium. The 9 L fermentation media contained similar chemical composition as that been described above and 450 mL, 700 mL, 1050 mL or 1400 mL crude glycerol to obtain the final (10 L) methanol concentration of 1.4% (w/v), 2.2% (w/v), 3.3% (w/v) or 4.4% (w/v), respectively. The 1 L pre-culture was added to the medium prior to the fermentation.

The fermentations were operated under similar condition as the sterilized and non-sterilized fermentation with methanol-free crude glycerol. Similarly, during the fermentation, the samples (110 mL/sample) were collected every 6 h or 8 h for analysis.

5.4.4 Non-sterilized fed-batch fermentation for the enhancement of biomass and lipid production

5.4.4.1 The fed-batch fermentation medium

The 9 L fed-batch fermentation medium was prepared with tap water dissolving 54 g KH_2PO_4, 19 g Na_2HPO_4, 32.32 g NH_4Cl, 1g EDTA, 2 g $MgSO_4 \cdot 7H_2O$, 8 g peptone, 0.4 g $CaCl_2 \cdot 2H_2O$, 0.055 g $FeSO_4 \cdot 7H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.0076 g $MnSO_4 \cdot H_2O$ and 450 mL of crude glycerol (containing methanol) (Chen et al., 2017).

5.4.4.2 The fed-batch fermentation

In the fed-batch fermentation, the fermenter and the fermentation medium were not sterilized. After the inoculation of 1 L pre-culture, the fermentation started. The 300 mL, 300 mL and 450 mL methanol free crude glycerol solution was fed at 16 h, 24 h and 32 h fermentation time, respectively. During the fermentation, DO was kept above 35% (v/v), the temperature was maintained at 30 °C, pH was controlled at 5. Samples (110 mL) were withdrawn at an interval of about 8h during the fermentation.

5.4.5 Analytical techniques

The samples collected during the fermentations were used to determine the colony forming units (CFU), biomass, lipid, soap, glycerol and methanol concentration analysis. The analytical techniques were similar as described in our previous study (Chen et al., 2017). To estimate the lipid content in the biomass, 30 mL of the fermentation broth was taken, centrifuged and washed twice with distilled water. The resulting solids after washing were mixed with 30 mL of the organic solvent mixture of chloroform and methanol (2:1 v/v) and about 6 mL Zirconia beads (1 mm diameter) in a 50 mL solvent proof tube. The solution was continuously subjected to shaking for 12 h and then centrifuged at 6000 rpm for 15 min. After collecting the bottom layer (lipid dissolved in chloroform), 20 mL of the solvent mixture of chloroform and methanol (1:1 v/v) was added to the residual

biomass and beads for the second time lipid extraction. The bottom layer of the first and the second extraction was combined and transferred to a pre-weighed glass tube. The solvent was evaporated under nitrogen gas stripping. When the weight of the glass tube was constant, the residual (the lipid) in the glass tube was weighed. The lipid extracted from biomass reacted with methanol in the presence of 1% H_2SO_4 (v/v methanol) as a catalyst at 50 °C for 12 h to form fatty acid methyl esters (FAMEs). The methanol to lipid molar ratio was adjusted to 6:1. The composition of FAMEs was then determined with Gas Chromatography linked to Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500) (Zhang et al., 2014a).

5.5 Results and discussion

The crude glycerol solution utilized in this study contained 31.14% (w/v) of methanol, 26.80% (w/w) of soap, 15.05% (w/w) of glycerol, 11.78% (w/w) of water and 3.41% (w/w) of ash.

5.5.1 Contamination impact on lipid production from *T. oleaginosus* under non-sterilized conditions

Fermentation medium is normally prepared from tap water; however, tap water contains bacteria. In addition, fermentation facilities can also bring contaminant to the fermentation if sterilization is not performed. However, the contaminants impact on the lipid production from *T. oleaginosus* in non-sterilised fermentation is still not clear.

The results of the non-sterilized fermentation were discussed and compared with the sterilized one. Details were presented below.

5.5.1.1 The cell growth in sterilized and non-sterilized fermentation

In both the sterilized and non-sterilized fermentation, a lag phase (0 to 8 h) was observed, which would be due to the sudden cultivation environment change (from pre-culture medium to the fermentation medium) for *T. oleaginosus* (Fig. 5.1a) or the pre-culture was not yet in exponential phase (Muñoz-Cuevas et al., 2010). However, no lag phase was observed for the contaminants in the non-sterilized fermentation (Fig. 5.1a). It might be attributed to that the contaminants were mostly contributed by the tap water used to

prepare the fermentation medium, thus, the contaminants already adapted the growth environment. After the lag phase, *T. oleaginosus* started to grow and the cell count of *T. oleaginosus* increased in both the sterilized and non-sterilized fermentation (Fig. 5.1a). The maximum CFU concentration of *T. oleaginosus* was 4.96×10^{10} CFU/mL obtained at 54 h in sterilized fermentation and 3.80×10^{9} CFU /mL obtained at 40 h in non-sterilized one (Fig. 5.1a). In the non-sterilized fermentation, the CFU of *T. oleaginosus* was more than that of the contaminants from 0 h to 40 h (Fig. 5.1a). However, the contaminants rapidly grew and the CFU of the contaminants took over that of *T. oleaginosus* after 40 h in non-sterilized fermentation (Fig. 5.1a). The maximum CFU concentration of the contaminants was 8.40×10^{9} CFU /mL at 56 h, which was more than two times higher than that of the *T. oleaginosus* (Fig. 5.1a). Therefore, growth termination of *T. oleaginosus* (CFU became stable) after 40 h could be attributed to the competition of high growth of the contaminants (bacteria). The co-products of the synthesis of the bacteria, such as organic acids, proteinaceous compounds, cyclic peptides and hydroxylated fatty acids could perform as toxic substances and inhibit the growth of the yeast (Beckner et al., 2011). In addition, the fast growth of bacteria competed for the space, substrates, and nutrients with the yeast during the fermentation (Dong et al., 2015). Thus, the growth of the yeast was inhibited and the lipid production was affected. Strategies, such as low temperature (Santamauro et al., 2014), high initial yeast cell density (Ling et al., 2013), or low fermentation pH (Yen et al., 2015), were used to control the undesired contaminants and assisted oleaginous yeast to overcome the inhibition of the contaminant.

CFU concentration presented in Fig. 1a was used to calculate the specific growth rate (μ) based on the Eq. 1.

$$\mu=\frac{\ln\frac{CFU_2}{CFU_1}}{t2-t1} \qquad (1)$$

where CFU_1 and CFU_2 was present the colony formation unit concentration at fermentation time t_1 and t_2, respectively.

It was found that the maximum specific growth rate of *T. oleaginosus* in non-sterilized and sterilized fermentation was 0.24 (h^{-1}) and 0.40 (h^{-1}), respectively, whereas, the maximum

specific growth rate of the contaminants in non-sterilized fermentation was 0.45 (h^{-1}). This suggested that the contaminants grew faster than *T. oleagnosus* in the non- sterilized fermentation and the growth of *T. oleaginosus* was inhibited due to the presence of contaminants.

5.5.1.2 The biomass and lipid production in sterilized and non-sterilized fermentation

The highest biomass and lipid concentrations obtained in sterilized fermentation were 23.32 g/L at 54 h (Fig. 5.1b) and 11.26 g/L at 54 h (Fig. 5.1b), respectively; while the maximum biomass and lipid concentrations were only 6.90 g/L at 56 h (Fig. 5.1b) and 3.05 g/L at 48 h (Fig. 5.1b), respectively, in the non-sterilized one. It demonstrated that sterilization was significantly important to ensure high biomass and lipid production of *T. oleaginosus* cultivation. It was observed that the highest biomass and lipid concentrations occurred simultaneously at 54 h in the sterilized fermentation (Fig. 5.1b). In the non-sterilized fermentation, the highest biomass concentration occurred at 56 h but the highest lipid concentration and content occurred at 48 h (Fig. 5.1b). The biomass increase would be mainly due to the growth of contaminants after 48 h (Fig. 5.1a and 5.1b). In addition, it was found that the lipid concentration decreased after 48 h, which indicated the lipid consumption (Fig. 5.1b). However, there is still substrate (soap and glycerol) present in the medium after 48 h (Fig. 5.1c). Thus, it would be due to the fact that a faster growth of contaminants has aggressively consumed the substrate and restricted *T. oleagnosus* to utilize substrates (soap and glycerol) (Wilson, 2014), then, left *T. oleagnosus* no choice but to consume the stored lipid as energy source to maintain the basic cell activity (Fig. 5.1b).

As has been discussed above, the environment change from pre-culture to the fermentation gave the stress to *T. oleaginosus*, which urged *T. oleaginosus* to accumulate lipids in the cells (Lee et al., 2015; Wang et al., 2016). As a result, the maximum lipid content was observed at the end of the lag phase both in sterilized and non-sterilized fermentations, which was 64.96% (w/w) at 13 h and 49.42% (w/w) at 16 h, respectively (Fig. 5.1b). Due to the existence of the contaminants, which contributed lipid-free biomass, the lipid content at the end of the lag phase was lower in the non-sterilized fermentation than in sterilized fermentation (Fig. 5.1a and 5.1b). In both sterilized and non-sterilized

fermentation, the lipid content rapidly decreased after the lag phase even though *T. oleaginosus* started to grow faster (Fig. 5.1a and 5.1b). It was suspected that *T. oleaginosus* produced lipid-free biomass faster and earlier than the lipid (Chen et al., 2017). Apart from the first peak achieved during lag phase (Fig. 1b), another peak of lipid content was observed at 54 h and 48 h in sterilized (48.28% w/w) and non- sterilized fermentation (48.62% w/w), respectively. It indicated that the contaminants didn't prevent *T. oleaginosus* from accumulating high lipid content in non-sterilized fermentation. Moreover, the contaminants did not contribute a large fraction of the total biomass regardless of its higher CFU concentration than *T. oleaginosus* because the cell mass of the contaminants (bacteria) was much less than *T. oleaginosus* (yeast) (Posten & Cooney, 2008).

5.5.1.3 The substrates utilization in sterilized and non-sterilized conditions

As has been observed and discussed in our previous study, the utilization of both soap and glycerol was highly associated with the biomass production (Chen et al., 2017). The biomass production in sterilized fermentation was much greater than that in non-sterilized fermentation (Fig. 5.1b). It would be the cause of the greater consumption of soap and glycerol in sterilized fermentation than in non-sterilized fermentation (Fig. 5.1c). It was found that the high growth of contaminants inhibited the growth of the *T. oleaginous* in non-sterilized fermentation, which caused the lower biomass and lipid production as well as substrates utilization compared to that in sterilized fermentation. However, in fact, *T. oleaginous* is capable of accumulating high lipid content in non-sterilized fermentation. The low lipid production was mainly due to the growth of contaminants, which inhibited the growth of *T. oleaginous.* It suggests that the lipid production could be enhanced in non-sterilized fermentation as long as the growth of contaminants was controlled.

5.5.2 The optimal methanol concentration for lipid production in non-sterile conditions

As discussed, lipid production from heterotrophic oleaginous microorganisms generally required sterilization in order to avoid the negative impact of contaminants on the growth; however, sterilization demands high energy and cost investment. To investigate if methanol could be used to control the growth of contaminant in lipid production from *T.*

oleaginosus under the non-sterilized condition, fermentations of *T. oleaginosus* with crude glycerol containing methanol were performed. As mentioned above, different strains have different tolerance to methanol concentration. Thus, by manipulating methanol concentration in the medium, it might be possible to control the contaminant growth in the non-sterilised fermentation of *T. oleaginosus* for lipid production.

5.5.2.1 The methanol concentration effect on the cell growth of *T. oleaginosus* and contaminants

The highest CFU concentrations of both *T. oleaginosus* and contaminants were observed at methanol concentration of 1.4% (w/v) which was 1.20×10^{10} CFU/mL and 7.00×10^{8} CFU/mL, respectively (Fig. 5.2a). In the fermentation with methanol concentration of 2.2% (w/v), the maximum CFU concentration of both *T. oleaginosus* and contaminants were lower than that in the fermentation with methanol concentration of 1.4% (w/v) (Fig. 5.2a). Once methanol concentration was increased to 3.3% (w/v) in the medium, there was no growth of *T. oleaginosus* (Fig. 5.2a). Further increase of methanol concentration to 4.4% (w/v) in the fermentation medium, it was found that the CFU of *T. oleaginosus* and contaminants gradually declined after 0 h (after inoculation) (Fig. 5.2a). It indicates that methanol concentration of 4.4% (w/v) was above the tolerance limit of *T. oleaginosus* as well as contaminants and caused their death. A similar trend has been reported by other researchers where methanol concentration of 4.6% (w/v) was fatal to most of the microorganisms (Caldwell, 1989; Chen et al., 1976).

CFU count from Fig. 5.2a was used to calculate the specific growth rate of *T. oleaginosus* and contaminants according to Eq. 1. To reveal the methanol effect on the cell growth of *T. oleaginosus* and contaminants, a comparison of the maximum specific growth rate of *T. oleaginosus* and contaminants at different methanol concentration was presented in Table 5.1. The maximum specific growth rate of *T. oleaginosus* was 0.23 and 0.36 h^{-1} with 0 and 1.4% (w/v) methanol concentration, respectively. It revealed that the growth of *T. oleaginosus* was promoted at methanol concentration of 1.4% (w/v) compared with methanol concentration of 0% (w/v). The growth of contaminants was inhibited in the fermentation with methanol concentration of 1.4% (w/v) (maximum specific growth rate 0.32 h^{-1}) compared to that of 0% (w/v) (maximum specific growth rate 0.45 h^{-1}). *T.*

oleaginous grew faster than the contaminants at methanol concentration of 1.4% (w/v) (Table 5.1). In the fermentation with methanol concentration of 1.4% w/v, the CFU count of *T. oleaginous* was much higher than that of the contaminants throughout the fermentation due to the high initial CFU count of *T. oleaginosus* (transferred from pre-culture) as well as the higher growth rate (Fig. 5.2a). At methanol concentration of 2.2% (w/v), it was observed that the inhibition of growth of *T. oleaginosus* and contaminants severely occurred (Fig. 5.2a). At methanol concentration of 3.3% (w/v), the growth of *T. oleaginosus* and contaminants almost completely stopped (Fig. 3a and 3b). At methanol concentration of 4.4%, the growth of *T. oleaginosus* and contaminants was inhibited to a larger extent (Fig. 5.2a) (Table 5.1). In addition, it was found that the contaminants were more sensitive towards methanol concentration between 1.4% (w/v) and 2.2% (w/v) compared to *T. oleaginosus* (Table 5.1). Thus, the optimal methanol concentration for *T. oleaginosus* cultivation in non-sterilized fermentation would be in the range of 1.4% (w/v) to 2.2% (w/v). Oki et al. used methanol concentration of 1.76% (w/v) and a low pH to prevent the growth of bacteria during yeast incubation (Oki et al., 1972). In this study, a methanol concentration of 1.4% (w/v) seemed to be acceptable and employed in the following experiments as the growth of *T. oleaginosus* was promoted and higher than that of the contaminants.

5.5.2.2 The methanol concentration effect on the biomass and lipid production

The highest biomass concentration of 12.46 g/L was obtained at the methanol concentration of 1.4% (w/v) which was 5.66 g/L, 1.47 g/L and 0.83 g/L at methanol concentration of 2.2% (w/v), 3.3% (w/v) and 4.4% (w/v), respectively (Fig. 5.2b). Even though a high biomass concentration was observed at methanol concentration of 1.4% (w/v), the maximum lipid concentration at methanol concentration of 1.4% (w/v) was only 3.29 g/L, which was 2.63 g/L at methanol concentration of 2.2% (w/v) (Fig. 5.2b). Since the biomass concentration was too low at methanol concentration of 3.3% (w/v) and 4.4% (w/v), the lipid concentration was low as well. The aim of the work was to produce high lipid concentration from *T. oleaginous*. Thus, the lipid, soap, glycerol and methanol concentration in the fermentation with methanol concentration of 3.3% (w/v) and 4.4% (w/v) was not performed in this study.

Even though the maximum biomass concentration (12.46 g/L) at methanol concentration of 1.4% (w/v) was high, the highest lipid concentration (3.29 g/L) was low (Fig. 5.2b). The CFU count of the contaminants was much less than *T. oleaginosus* at methanol concentration of 1.4% (w/v) (Fig. 5.2a), important to mention here that the cell mass of the contaminants is much less than the yeast. Thus, it is suggested that the presence of contaminants in the fermentation was not responsible for the low lipid production. At methanol concentration of 1.4% (w/v), the fermentation started with a substrate concentration of 8.40 g/L soap, 6.07 g/L glycerol and 12.58 g/L methanol. However, it was found that methanol was barely used during the fermentation (please see below). The total carbon source from 8.40 g soap/L and 6.07 g glycerol/L was only 8.67 g carbon/L (8.40 g soap/L×0.75 g carbon/g soap +6.07 g glycerol/L×0.39 g carbon/g glycerol =8.67 g carbon/L). However, in our previous research it was found that the total carbon source from crude glycerol (soap and glycerol) in the medium should be more than 15.62 g carbon/L but less than 29.91 g carbon/L for high lipid production from *T. oleaginosus* cultivation when no environment stress restrain its growth (Chen et al., 2017). Thus, the low lipid production would be due to the lack of carbon sources at methanol concentration of 1.4% (w/v). Generally, high lipid accumulation generally occurred in carbon excess condition (Chen & Johns). Moreover, the lipid concentration quickly dropped after 42 h fermentation in the case of methanol concentration of 1.4 % (w/v). It could be due to the reason that the lipid accumulated in the cell was reused to support the cell growth under the carbon source deficient condition (Fig. 5.2a and 5.2b).

Apart from the lag phase, another the highest lipid content during the fermentation with methanol concentration of 1.4% (w/v) was only 39.09% (w/w) which was 46.45% (w/w) with methanol concentration of 2.2% (w/v) (Fig. 2b). The higher lipid content at methanol concentration of 2.2% (w/v) could be due to higher carbon source concentration at methanol concentration of 2.2% (w/v) compared to that at methanol concentration of 1.4% (w/v).

5.5.2.3 The methanol concentration effect on substrates (soap, glycerol and methanol) utilization

In the fermentation with methanol concentration of 1.4% (w/v), almost all the soap and glycerol were used at the end of the fermentation (1.46 g/L of soap and 0.53 g/L of glycerol), which suggested that *T. oleaginosus* might have grown in carbon limiting condition. At methanol concentration of 2.2% (w/v), lots of soap and glycerol were left in the medium at the end of the fermentation (7.03 g/L of soap and 6.07 g/L of glycerol). It indicated that *T. oleaginosus* had been growing in carbon excess condition which could be the reason for high lipid content of biomass (46.45% w/w) was obtained (Fig. 5.3). Even though almost all the soap and glycerol were consumed; there was still a lot of methanol left in the medium at the end of the fermentation with methanol concentration of 1.4% (w/v) (11.78 g/L). It indicated that methanol might not be used by *T. oleaginosus* and contaminants. So far, only a few microorganisms have been reported to utilize methanol. Hazeu et al. reported that only 16 out of 422 tested species were able to utilize methanol (Hazeu et al., 1972). Oki et al. also tested 192 strains, no methanol-utilizing representative was found (Oki et al., 1972). The strains should have C1(one-carbon) metabolism enzymes to utilize methanol (Witthoff et al., 2013; Yurimoto et al., 2011). Thus, it was supposed that the methanol reduction during the fermentation was due to methanol evaporation as methanol was volatile and the fermentation was operated with very high aeration and agitation speed in this study. Thus, the outlet of air flow might go through cold water to recapture the evaporated methanol when methanol was used to control the contaminants in the large-scale vessel. In conclusion, a methanol concentration of 1.4% (w/v) was considered to be acceptable for lipid production using *T. oleaginosus* in non-sterilized fermentation. However, the lipid production was only 3.29 g/L. To enhance the lipid production, a non-sterilized fed-batch fermentation with initial methanol concentration of 1.4% (w/v) was conducted.

5.5.3 Fed-batch fermentation starting with methanol concentration of 1.4% (w/v) for high biomass and lipid production

It was found that a high biomass and lipid production of 43.22 g/L and 20.78 g/L with a lipid content of 48.09% (w/w) was achieved at 60 h in the fed-batch fermentation with

initial methanol concentration of 1.4% (w/v) in non-sterilized fermentation (Fig. 5.3). Comparing with our previous fed-batch study in sterilized fermentation, the biomass and lipid production so as the lipid content were not highly affected in non-sterilized fermentation (Table 5.2) (Chen et al., 2017). Thus, it stated that high lipid production using *T. oleaginosus* could be achieved in non-sterilized fermentation with the assistance of methanol concentration of 1.4% (w/v) but a little bit longer fermentation time was needed to reach the maximum lipid production in non-sterilized fermentation compared to sterilized fermentation (Table 5.2). Similarly, as the fermentation with methanol concentration of 1.4% (w/v) in batch fermentation, methanol concentration gradually reduced during the fermentation. A large amount of methanol was left in the medium despite that soap and glycerol were almost completely utilized at the end of the fermentation (Fig. 5.3). Similar results were also reported in the research of Liang et al, where there was still much of methanol present in the medium after the depletion of glycerol when crude glycerol was used to cultivate oleaginous yeast *Cryptococcus curvatus* for lipid production (Liang et al., 2010).

The lipid extracted from biomass produced in non-sterilized fed-batch fermentation was transferred to fatty acid methyl esters (FAMEs). The purity of biodiesel obtained after trans-esterification was 98.9% and the trans-esterification efficiency was 95%. The compositions of FAMEs were measured with Gas Chromatography linked to Mass Spectroscopy (GC-MS). It was found that the FAMEs mainly consisted of long-chain fatty acids with 16 and 18 carbon atoms (C16:0 of 29.99% w/w; C16:1 of 0.44% w/w; C18:1 of 45.99% w/w; C18:2 of 20.43% w/w), which were comparable with vegetable oils. The fatty acid compositions of FAMEs indicated that lipid produced from the non-sterilized fed-batch fermentation could be used as feedstock for biodiesel production.

5.6 Conclusions

The study showed that methanol could be used to control contaminant growth in non-sterilized fermentation instead of high energy consumption sterilization (121 °C for 15 min). The presence of methanol in the medium impacted both the growth of lipid-producing strain (*T. oleaginosus*) as well as contaminants, but it was highly related to the

concentration of methanol. Therefore, the concentration of methanol is needed to be well determined when methanol was employed to control contaminant growth in oleaginous microorganism cultivation for lipid production. In this study, it was confirmed that the methanol concentration of 1.4% (w/v) was suitable to assist *T. oleaginosus* to overcome the impact of the contaminants for high biomass and lipid production in non-sterilized fermentation.

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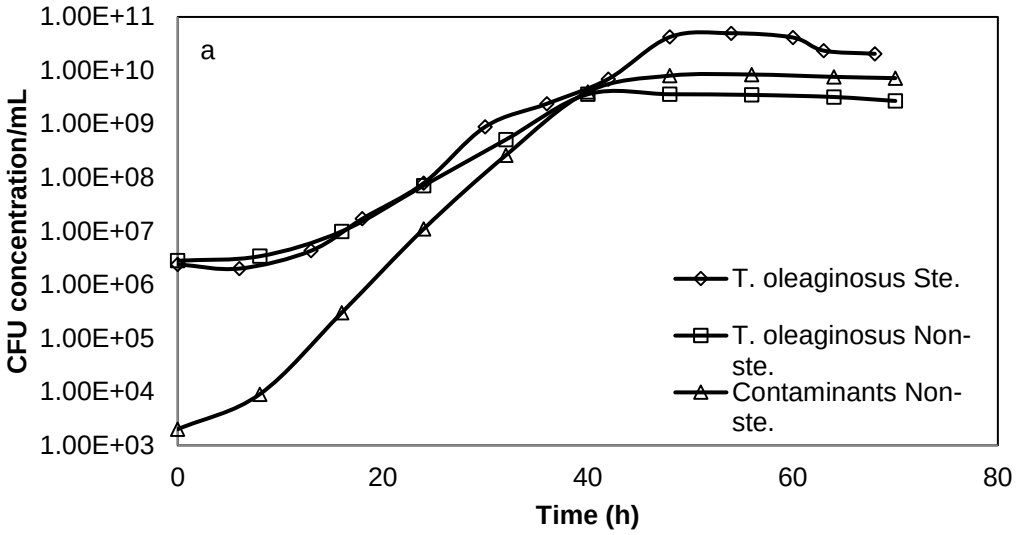
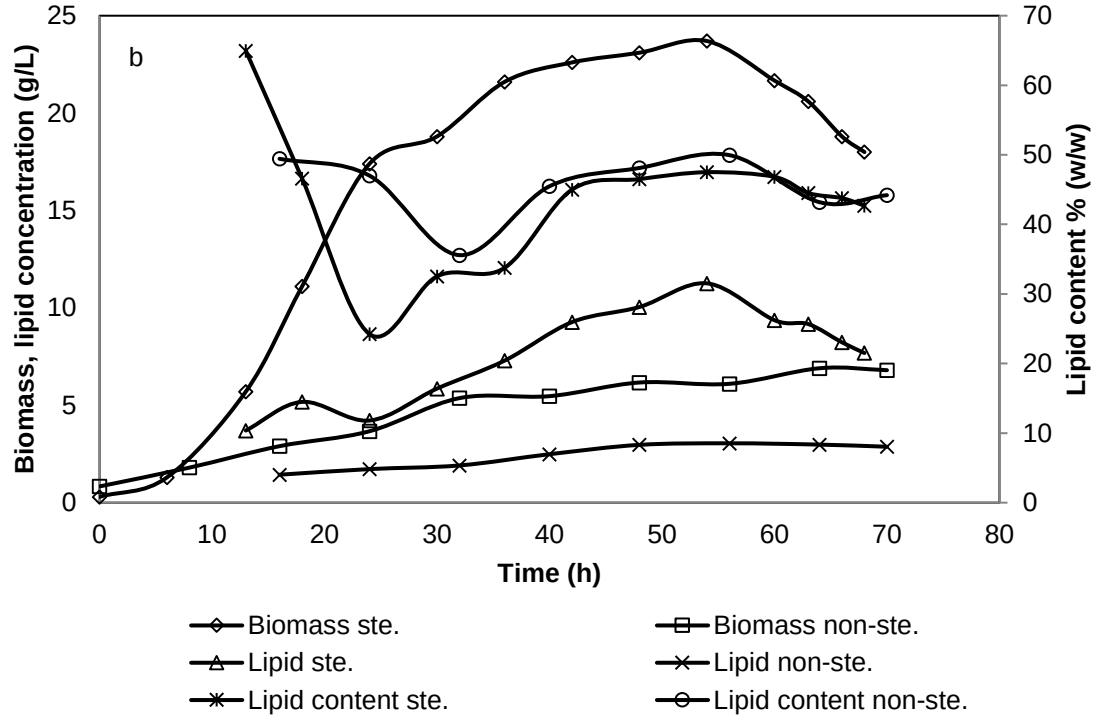
Table 5. 1 The maximum specific growth rate of *T. oleaginosus* and contaminants at different methanol concentration

Methanol (% w/v)	Maximum specific growth rate (h^{-1})	
	T.oleaginosus	**Contaminants**
0	0.24	0.45
1.4	0.36	0.32
2.2	0.22	0.19
3.3	0.012	0.09
4.4	-0.0185	-0.036

Table 5. 2 Comparison of the non-sterilized and the sterilized fed-batch fermentation

Fermentation condition	Biomass production (g/L)	Lipid production (g/L)	Lipid content (% w/w)	Fermentation time (h)	Ref.
Non-sterilized	43.22	20.78	48.09	60	This study
Sterilized	43.82	21.87	49.89	52	[30]


a
CFU concentration/mL
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
0
20
40
60
80
Time (h)
T. oleaginosus Ste.
T. oleaginosus Non-ste.
Contaminants Non-ste.


b
Biomass, lipid concentration (g/L)
Lipid content % (w/w)
25
20
15
10
5
0
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
Time (h)
Biomass ste.
Biomass non-ste.
Lipid ste.
Lipid non-ste.
Lipid content ste.
Lipid content non-ste.

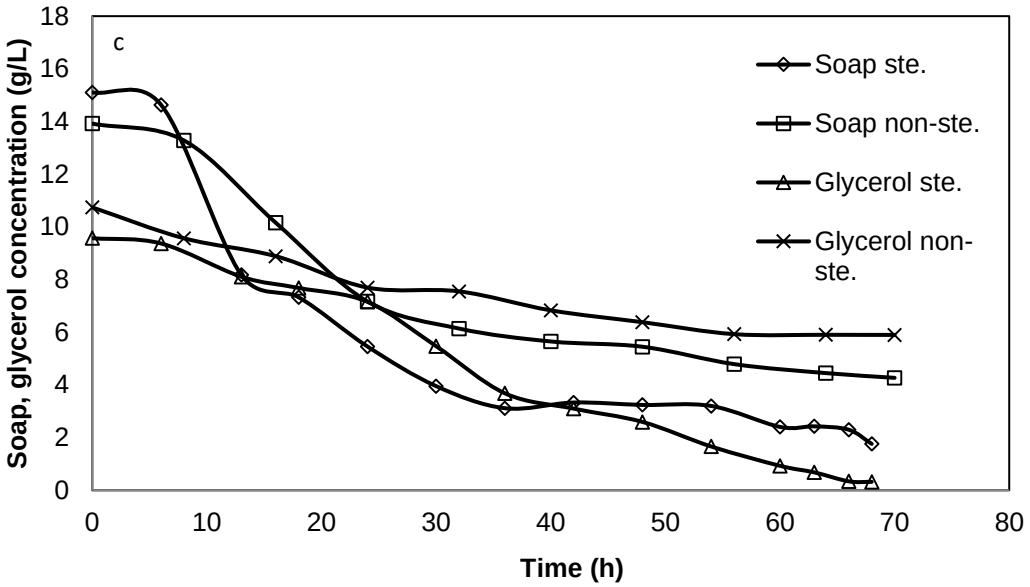


Figure 5. 1 The sterilized and non-sterilized fermentation with methanol-free crude glycerol synthetic medium (Ste.=sterilized, Non-ste.=non-sterilized)

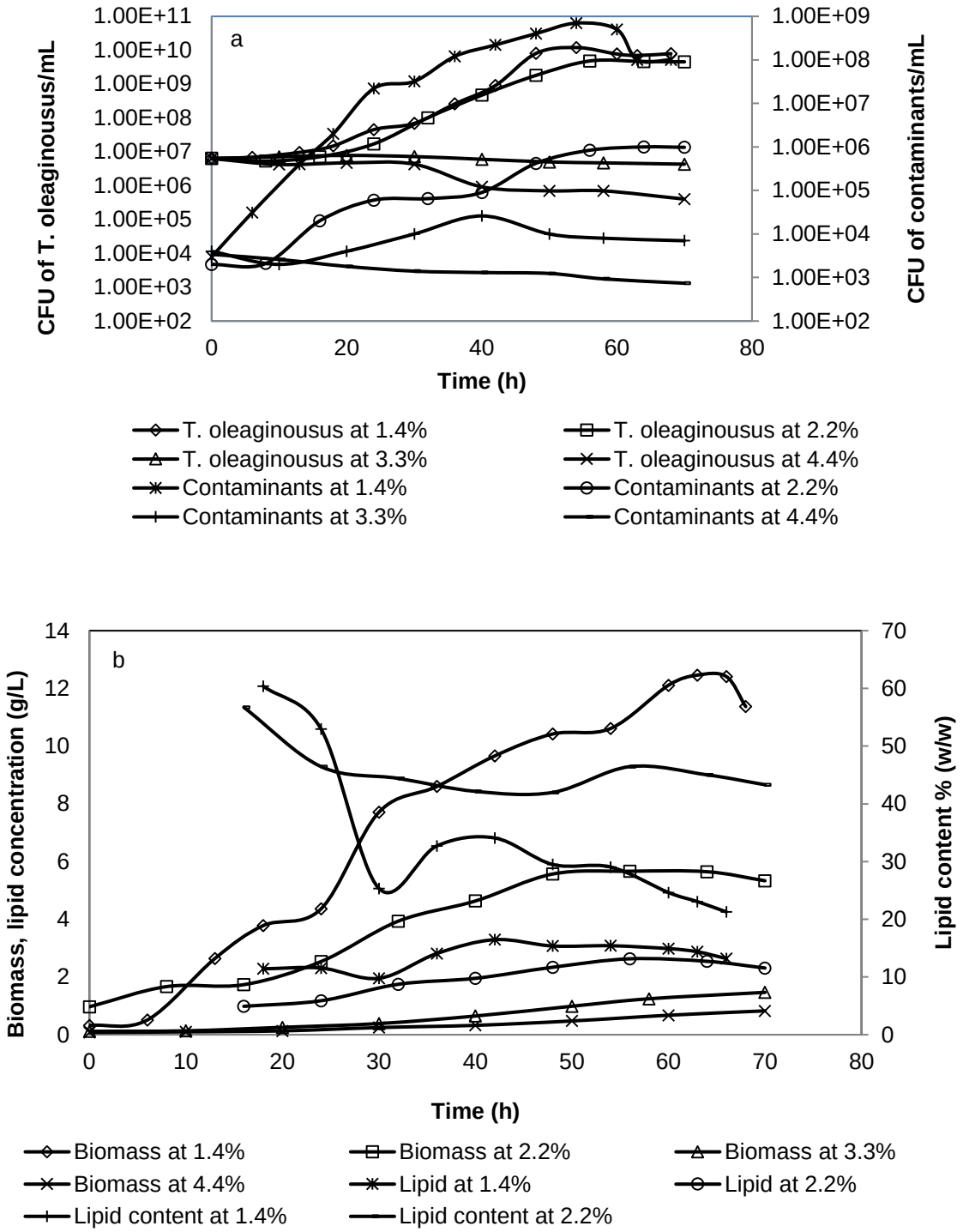


Figure 5. 2 ***T. oleaginosus*** **cultivation in non-sterilized fermentations at different methanol concentration (1.4%, 2.2%, 3.3%, 4.4% indicated at methanol concentration of 1.4% w/v, 2.2% w/v, 3.3% w/v, 4.4% w/v, respectively)**

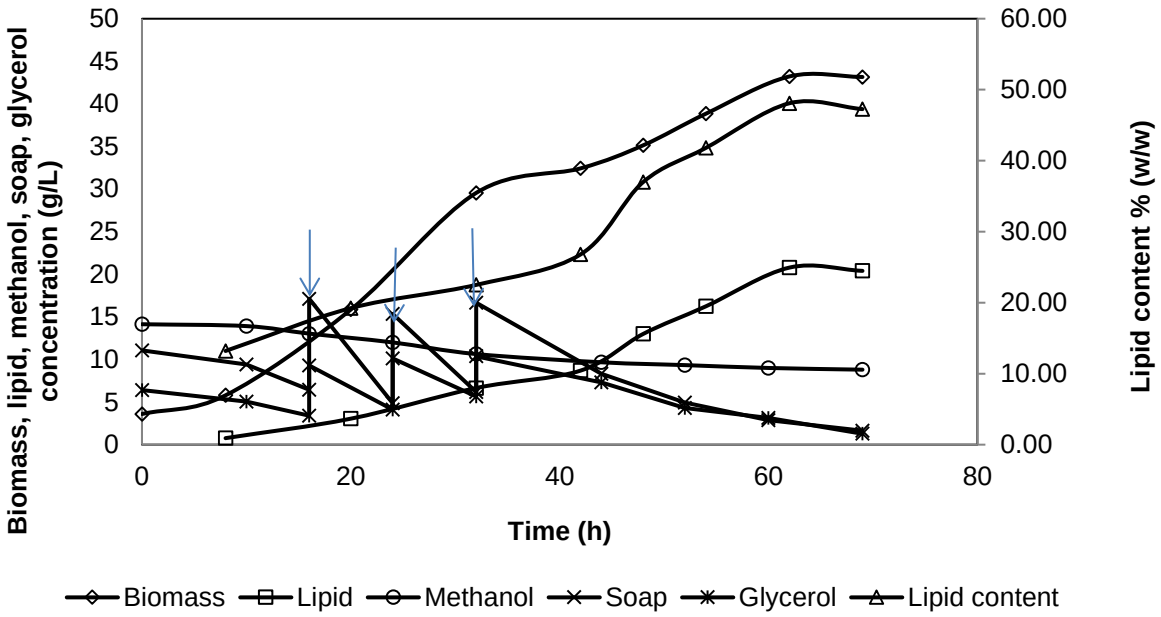


Figure 5. 3 Fed-batch fermentation with initial methanol concentration of 1.4% (w/v)

The pH-based fed-batch for lipid production from *Trichosporon oleaginosus* with crude glycerol

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6 THE PH-BASED FED-BATCH FOR LIPID PRODUCTION FROM *TRICHOSPORON OLEAGINOSUS* WITH CRUDE GLYCEROL

6.1 Résumé

Dans cette étude, il a été constaté que le pH optimal pour la croissance de *Trichosporon oleaginosus* était lié au milieu de fermentation. Une condition de pH acide faible ou neutre était optimale pour la croissance de *Trichosporon oleaginosus* dans le milieu extrait-peptone-dextrose et boues d'épuration. Une inhibition significative a été observée à pH neutre dans les boues d'épuration + de glycérol brut en raison de la teneur élevée en savon du glycérol brut. En convertissant le savon en acide gras libre à pH 5, l'inhibition du savon pourrait être évitée. La fermentation fed-batch basé sur le pH et l'alimentation contrôlée automatiquement par une solution de glycerol brut a été utilisée pour produire des lipides à partir de *Trichosporon oleaginosus*. Une concentration de biomasse (65,63 g/L) et une concentration de lipides (35,79 g/L), qui sont remarquablement élevées, ont été atteintes à partir de la fermentation fed-batch.

Mots clés : la production de lipides; pH optimal; le glycérol brut; fermentation fed-batch.

6.2 Abstract

In this study, it was found that the optimal pH for the growth of *Trichosporon oleaginosus* was related to the fermentation medium. A neutral or weak acid pH condition was optimal for the growth of *Trichosporon oleaginosus* in the extract-peptone-dextrose and wastewater sludge medium. Significant inhibition was observed at neutral pH in the wastewater sludge + crude glycerol medium due to the high soap content of the crude glycerol. By converting the soap to free fatty acid (FFA) at pH 5, the soap inhibition could be prevented. Fed-batch fermentation was employed to produce lipid from *Trichosporon oleaginosus* at pH 5 controlled by feeding crude glycerol. A remarkably high biomass (65.63 g/L) and lipid (35.79 g/L) concentration were achieved from the pH-based fed-batch fermentation in this study.

Keywords: Lipid production; suitable pH; crude glycerol; fed-batch fermentation.

6.3 Introduction

For nearly a century, researchers have been exploring the opportunities to produce oil using microorganisms. The microorganisms, which contain more than 20% oil of the total dry biomass weight, are defined as oleaginous microorganisms (Liang & Jiang, 2013).

Oleaginous yeasts, such as *Trichosporon oleaginosus* (previously named as *Cryptococcus curvatus*), *Lipomyces starkeyi* and *Yarrowia lipolytica*, are widely studied to produce microbial oil (lipid), due to their capacity to accumulate high lipid content (Sitepu et al., 2014; Zhang et al., 2014a). Nowadays, the lipid production cost is high which is mainly from the utilization of the high-grade fermentation substrate (Cho & Park, 2018). In order to reduce the lipid production cost, organic wastes (low or free of cost), such as crude glycerol and wastewater sludge, are investigated to replace high-cost substrate for oleaginous yeast fermentation (Zhang et al., 2014a; Cortes & de Carvalho, 2015). Even though utilization of organic wastes as raw materials can reduce the lipid production cost, inhibition of impurities on the growth of oleaginous yeast and lipid production was observed (Xu et al., 2015; Yen & Chang, 2015; Yen et al., 2015). To enhance the cell growth and lipid production of oleaginous yeast from organic wastes, the optimization of fermentation parameters was generally performed (Sitepu et al., 2013; Tanimura et al., 2014; Chang et al., 2015).

The fermentation parameters, which affect the cell growth and lipid production, are C/N ratio, temperature, and pH (Zhang & Jahng, 2012; Nagano et al., 2013; Espinosa-Gonzalez et al., 2014). Among all, pH was normally controlled in an optimal range during the fermentation because either lower or higher pH caused a remarkable decrease in lipid production (Cappai et al., 2014; Nguyen et al., 2014; Alfè et al., 2015; Fumasoli et al., 2015). However, it is found that the optimal pH for a lipid-producing strain (such as oleaginous yeast *Trichosporon Oleaginosus*) varied with the change of the fermentation medium (Zhu et al., 2008; Cui et al., 2012; Yu et al., 2014).

For high lipid production, fed-batch fermentation is proved to be superior to the batch fermentation. So far, the highest biomass concentration of 185 (g/L), the highest lipid content of 76% (w/w) and the highest lipid productivity of 1 (g/L/h) were obtained in fed-

batch fermentations (Koutinas et al., 2014). Fed-batch fermentation is a biotechnological process that substrates are fed to the reactor through multiple steps. To achieve a good performance of fed-batch fermentation, the feeding strategy is essential and important. The feeding strategy can be based on time, pH, DO (dissolved oxygen) or limiting substrate's dilution rate (Zhang et al., 2011; Chen et al., 2017; Farinha et al., 2017). Our previous research shows that fed-batch based on time could highly increase the lipid production (Chen et al., 2017). However, the operation of the time-based fed-batch fermentation was complicating and the substrate for the feed was not accurate (Chen et al., 2017). Therefore, an easier but more reliable feeding strategy should be investigated.

In this study, the optimal pH for oleaginous yeast *T. oleaginousus* was investigated and discussed with respect to different media. A pH-based fed-batch fermentation was designed and operated to enhance lipid production.

6.4 Materials and methods

6.4.1 Strain

Trichosporon oleaginosus (ATCC 20905) was employed as the lipid producing strain in this study.

6.4.2 Medium

6.4.2.1 The pre-culture and YPD medium

The pre-culture medium was prepared from the yeast extract-peptone-dextrose (YPD) (50 g/L). The optimal pH was investigated with YPD medium as well.

6.4.2.2 Wastewater sludge medium

Studies have revealed that municipal secondary wastewater sludge was better for *T. oleaginosus* cultivation compared to the primary and mixed sludge (Zhang et al., 2014a). Hence, secondary wastewater sludge collected from a municipal wastewater treatment plant Communauté Urbaie de Québec (CUQ), Québec, Canada was utilized in this study. The characteristics of the secondary wastewater sludge were presented in the previous study accomplished in our lab (Zhang et al., 2014b). After collection, the sludge was

allowed to settle at 4 ºC for 24 h. The supernatant was withdrawn. The suspended solids (SS) concentration of the resulting sludge solution was around 20 g/L. Previous studies in our lab found that SS concentration of 30 g/L was optimal for *T. oleaginosus* cultivation (Zhang et al., 2014a). To obtain SS concentration of 30 g/L, part of the resulting sludge was centrifuged to concentrate the sludge. The concentrated sludge was then mixed with the resulting sludge to obtain the SS concentration of 33 g/L. The sludge (SS 30 g/L) after sterilization at 121 °C for 30 min was cooled down to the room temperature and inoculated with 10% of pre-culture to make the final SS of 30 g/L.

6.4.2.3 Wastewater sludge and crude glycerol medium

The crude glycerol solution used in this study was obtained from a biodiesel production industry in Quebec, Canada. The characteristic of the crude glycerol was done following the methods presented in our previous study (Chen et al., 2017). The crude glycerol solution contained 31.14 ±1.22 (% w/v) of methanol, 26.80±1.35 (% w/w) of soap, 15.05±0.39 (% w/w) of glycerol and other minor components.

The 200 mL pre-concentrated sludge with SS concentration of 37.5 g/L (same method as above) was mixed with 25 mL methanol free crude glycerol solution (methanol evaporated) to prepare 225 mL sludge and crude glycerol medium. After sterilization and cooling down to the room temperature, 25 mL pre-culture was inoculated into the sludge and crude glycerol medium. After inoculation, the concentration of SS, glycerol and soap of the medium was 30 g/L, 20.50 g/L and 36.50 g/L, respectively.

6.4.2.4 Crude glycerol synthetic medium

To prepare one-liter synthetic medium, the chemicals of 5.4 g KH_2PO_4, 1.9g Na_2HPO_4, 3.232 g NH_4Cl, 0.1g EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.8 g peptone, 0.04g $CaCl_2 \cdot 2H_2O$, 0.0055g $FeSO_4 \cdot 7H_2O$, 0.001g $ZnSO_4 \cdot 7H_2O$, and 0.00076g $MnSO_4 \cdot H_2O$ were used. The amount of crude glycerol solution varied according to the required carbon concentration. The detailed information was described below.

6.4.3 Methods

6.4.3.1 The primary screening of the optimal pH of *T. oleaginosus* growth

Nine shake flasks (each volume of 1 L) were filled with 250 mL YPD medium. After sterilizing and cooling, the pH was adjusted to 3.5, 4.5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0 with 4 M H_2SO_4 solution or 4 M NaOH solution, respectively, in the laminar hood under aseptic condition. After pH adjustment, they were inoculated with a loopful of *T. oleaginosus* culture and then incubated at 30°C and 180 rpm for 24 h. The Colony-Forming Units (CFU) of *T. oleaginosus* was determined after the incubation.

6.4.3.2 Verification of the optimal pH range for the cell growth of *T. oleaginosus* in wastewater sludge medium

Five shake flasks (each volume of 1 L) were filled with 225 mL of sludge with SS of 33 g/L. They were sterilized at 121 °C for 15 minutes. After cooling, the pH of the sludge mediums was adjusted to 5.0, 5.5, 6.0, 6.5 and 7.0 with 4 M H_2SO_4 solution or 4 M NaOH solution in the laminar hood under aseptic condition. The 25 mL of *T. oleaginosus* pre-culture (produced in YPD medium) with its corresponding pH was inoculated to sterilized sludge which was pre-adjusted to the pH value as that inoculum followed by incubation at 30 °C and 180 rpm, for instance, The pre-culture cultivated at pH 5.0 was inoculated to the sludge medium with pH 5. Samples were taken at every 4 h, and the CFU of *T. oleaginosus* was determined.

6.4.3.3 Verification of the optimal pH range for the cell growth of *T. oleaginosus* in wastewater sludge fortified with crude glycerol medium

The wastewater sludge was pre-treated using the method described above to obtain the required SS concentration of 37.5 g/L. Three flasks with the capacity of 1 L were filled with 200 mL sludge (SS=37.5 g/L) and 25 mL methanol free crude glycerol solution (glycerol concentration=20.5 g/L, soap concentration=36.5 g/L). After being sterilized and cooling, the pH of the sludge and crude glycerol media was adjusted to 5.0, 6.0, and 7.0 in a laminar hood under aseptic condition. The other operation steps in the wastewater sludge and crude glycerol media were similar to those in the sludge media. At an interval of 4 h, samples were taken for the determination of CFU and pH.

6.4.3.4 The pH based fed-batch fermentation using crude glycerol synthetic medium

The fed-batch fermentation was operated in a 15 L fermenter with a working volume of 10 L. 54 g KH_2PO_4, 19g Na_2HPO_4, 32.32 g NH_4Cl, 1g EDTA, 2 g $MgSO_4 \cdot 7H_2O$, 8 g peptone, 0.4 g $CaCl_2 \cdot 2H_2O$, 0.055 g $FeSO_4 \cdot 7H_2O$, 0.01g $ZnSO_4 \cdot 7H_2O$, 0.0076 g $MnSO_4 \cdot H_2O$ and 450 mL of crude glycerol solution were dissolved with tap water to prepare 9 L crude glycerol synthetic medium. After being transferred to the fermenter, the pH of the crude glycerol synthetic medium was adjusted to pH 5 with 4 M H_2SO_4 solution. 1 L of the *T. oleaginosus* pre-culture cultivated in YPD medium was added to the fermenter and the pH was adjusted 5 again. Then, the fermentation started. During the fermentation, pH was controlled at 5±0.1 by a prepared crude glycerol solution (pH around 11).

6.4.4 Analytical techniques

The methods of the determination of CFU, biomass concentration, lipid concentration, lipid content, glycerol concentration, and soap concentration were similar as utilized in our previous research (Chen et al., 2017).

CFU was determined by serial dilution and plate counting method (Ziegler & Halvorson, 1935).

Biomass concentration was measured by centrifuging 10 mL sample at 8000 rpm for 15 min. After dumping the supernatant, the resulting solids were washed and centrifuged twice with distilled water. Thereafter, the solid residue was collected and transferred to a pre-weighed aluminum cup. The aluminum cup with residue solid was dried at 105 °C until a constant weight and then weighed. The weight difference of aluminum cup between in empty and containing dry solids was used to calculate the biomass concentration. The biomass concentration was the weight of solid residue (g) divided by the volume of the sample (0.01 L).

To determine the lipid concentration and lipid content, biomass from 30 mL of fermentation broth was dried by lyophilization after washing. The dried biomass was added to 30 mL mixture of chloroform and methanol (2:1 v/v) and 6 mL Zirconia beads (1 mm diameter) and subjected to a continuous shaker for 12 h and then was centrifuged at

6000 rpm for 15 min. The solution was filtered and the filtrate was collected. The filtrate was transferred to a pre-weighed glass tube and underwent nitrogen gas evaporation. The weight of the glass tube was measured when it was constant. The lipid concentration was the weight difference of the glass tube with and without filtrate divided by the sample volume (0.03 L). The lipid content (% w/w) was calculated by dividing lipid concentration with biomass concentration.

Glycerol concentration was determined based on the method presented by Bondioli and Bella (Bondioli & Della Bella, 2005).

For the determination of soap concentration, the 50 mL of fermentation broth was adjusted to pH 1 with 4 M H_2SO_4 to convert soap to FFA. Then, 10 mL chloroform was added to the pH adjusted fermentation broth to extract the FFA. After centrifugation at 5000 rpm for 20 minutes, the bottom layer was collected and transferred to a pre-weighed glass tube followed by the evaporation of chloroform. The FFA amount of 50 mL fermentation broth was the weight difference between the tube with FFA and the empty tube. The FFA concentration was FFA amount divided by 0.05 L. The soap concentration was calculated based on the equation: 304×FFA concentration/282 (304 was the average soap molar mass and 282 was the average FFA molar mass).

The experiments were performed in duplicates. The results presented in this article were the mean values.

6.5 Results and discussions

6.5.1 Primary screening on pH

The primary screening of the optimal pH for oleaginous yeast *T. oleaginosus* was conducted in the pH range from pH 3.5 to pH 9 with an interval of 0.5 around neutral pH or interval of 1 in strong acid and alkaline condition (Almeida, 1950). It was found that the neutral or weak acid pH conditions (pH 5.5, 6.5 and 7) achieved higher CFU concentration than the other pH conditions at 24 h and were advantageous for the cell growth of *T. oleaginosus*. The similar results were reported by other researchers (Capusoni et al., 2017; Lee et al., 2017; Meo et al., 2017). In order to precise the optimal pH and verify its

suitability in different media, the pH at neutral and weak acid conditions were tested for the cell growth of *T. oleaginosus* in wastewater sludge medium and in wastewater sludge and crude glycerol medium.

6.5.2 The precision and verification of the optimal pH in wastewater sludge medium

Since the optimal pH was narrowed to the neutral or weak acid condition, the pH range 5 to 7, the interval of 0.5, was investigated.

Wastewater sludge is cost-free and widely available. Previous studies in our lab showed that wastewater sludge was a good basic medium for *T. oleaginosus* after certain pre-treatments (Zhang et al., 2014b). Thus, the suitability of the optimal pH range for *T. oleaginosus* was verified in wastewater sludge medium.

The experiments conducted in wastewater sludge medium showed that the maximum CFU of *T. oleaginosus* was observed at pH 7 which was 3.88E+10 CFU/mL. For pH 5.0, 5.5, 6.0 and 6.5, the highest CFU concentration was 2.20E+10 CFU/mL, 2.41E+10 CFU/mL, 2.78E+10 CFU/mL, 3.40E+10 CFU/mL, respectively (Fig. 6.1a and 6.1b). The maximum CFU at different pH conditions was comparable.

The CFU data was then employed to calculate the specific growth rate according to Eq. 1.

$$\mu = \frac{\ln \frac{CFU_2}{CFU_1}}{t2 - t1} \quad (1)$$

Where CFU1 and CFU2 were the CFU of *T. oleaginosus* at time t1 and t2, respectively.

It was found that the maximum specific growth rate of *T. oleaginosus* was 0.37 (h^{-1}) at pH 5, 0.43 (h^{-1}) at pH 5.5, 0.38 (h^{-1}) at pH 6, 0.41 (h^{-1}) at pH 6.5 and 0.43 (h^{-1}) at pH 7. The comparable maximum specific growth rate of *T. oleaginosus* indicated that the pH range (pH 5.0 to 7.0) was suitable for *T. oleaginosus*. However, it should be mentioned that a remarkable lag phase was observed at pH 5.0 and 5.5, which was not obvious at pH 6.5 and 7.0 (Fig. 6.1a). Thus, the time to achieve the maximum CFU was longer at pH 5.0 and 5.5 compared to pH 6.5 and 7.0 (Fig. 1b). Wastewater sludge was mainly composed of complex organic compounds, which must be decomposed to small molecules before

being used by *T. oleaginosus* (Zhang et al., 2014b; Deeba et al., 2016). In order to improve the biodegradability, studies have employed pre-treatment to assist the breakdown of the sludge and achieved better results than the one without (Zhang et al., 2014b; Selvakumar & Sivashanmugam, 2017). In this study, pre-treatment was not performed; hence it required *T. oleaginosus* to secrete relative enzymes for breaking down sludge. It was reported that the enzymes, for breaking down the complex organic matter, preferred neutral pH condition. For instance, the optimal pH for the enzyme polyphenol oxidase was 7.0; its activity was negatively affected at acid pH (de Oliveira Carvalho & Orlanda, 2017). Thus, the lag phase occurring at acid pH condition might be due to the low enzyme activity.

6.5.3 The verification of the optimal pH in wastewater sludge and crude glycerol medium

Even though wastewater sludge was proved to be a good alternative of YPD medium for *T. oleaginosus* cultivation, wastewater sludge was poor in carbon source. The carbon to nitrogen ratio (C/N) of wastewater sludge was around 5 to 12 (Zhang et al., 2014b). However, the high lipid production is generally achieved in carbon-rich and nitrogen-poor medium (high C/N ratio). Thus, the lipid production was low when wastewater sludge was solely applied for *T. oleaginosus* cultivation (Zhang et al., 2014a; Zhang et al., 2014b). To enhance lipid production, the addition of substrates to enrich the wastewater sludge could be a great option (Cho & Park, 2018). The addition substrate brought more available carbon to the system and hence increased the lipid accumulation. In this study, a crude glycerol solution from a local biodiesel company was studied to fortify the wastewater sludge medium. Thereafter, the optimal pH for *T. oleaginosus* was investigated in the wastewater sludge and crude glycerol medium. The pH 5, 6 and 7 were studied. In addition to the CFU, the pH variation during the cultivation was recorded.

In the sludge fortified with crude glycerol medium, the maximum CFU of *T. oleaginosus* was 1.88E+10 CFU/mL at pH 5, 9.84E+09 CFU/mL at pH 6 and 7.80E+08 CFU/mL at pH 7 (Fig. 6.2). It was obvious that the cell production of *T. oleaginosus* was better at pH 5 than pH 6 and 7 in sludge and crude glycerol medium. By comparing the maximum CFU at the same pH in sludge with and without crude glycerol, it was found that the addition

of crude glycerol did not highly affect the cell production at pH 5. However, the cell production was significantly affected at pH 6 and 7 with the addition of crude glycerol (Table. 6.1). Thus, it was predicted that some components from crude glycerol affected the cell production of *T. oleaginosus* at pH 6 and 7 but not at pH 5. It was found that the biomass production and lipid production were similar in sludge medium at pH 5, pH 6 and pH 7 (Table. 6.1). However, the biomass and lipid production were highly affected by the pH. At pH 5, the biomass and lipid concentration reached 36.88 g/L and 10.75 g/L, respectively. However, the biomass and lipid concentrations were only 29.62 and 2.98 g/L at pH 6 and 30.21 and 2.20 g/L 7, respectively. Hence, it was concluded that crude glycerol inhibited the cell production and lipid production of *T. oleaginosus* at pH 6 and pH 7. However, no significant effect was observed at pH 5.

By investing the components of the crude glycerol solution, the two major components contributing as carbon sources were soap and glycerol because methanol was already evaporated from crude glycerol before being used to prepare the medium. Glycerol was stable with pH variation and glycerol didn't inhibit the cell growth of the microorganism (Ma et al., 2016). Comparatively, soap could be affected by the pH variation. Under high pH condition, it was formed as soap, which was highly soluble in the medium. In low pH conditions, soap was converted to free fatty acid (FFA), which was less soluble in the medium (0.34 g/L at 25°C) (Khuwijitjaru et al., 2002). The form of soap is determined by pH which can be described by Eq. 2

$$C_{17}H_{35}COO^{-}\ (soap) + H_2O \longleftrightarrow C_{17}H_{35}COOH\ (FFA) + OH^{-} \quad (2)$$

Accordingly, the soap concentration at different pH conditions could be calculated based on the Eq. 3

$$Kb = [C_{17}H_{35}COOH][OH^{-}]/[C_{17}H_{35}COO^{-}] \quad (3)$$

Where Kb was the equilibrium constant (Kb=7.69E-10); $[C_{17}H_{35}COOH]$ was the concentration of FFA (0.34 g/L at 25°C); $[OH^{-}]$ was the concentration of OH^{-} ($[OH^{-}]=10^{(pH-14)}$); $[C_{17}H_{35}COO^{-}]$ was the concentration of soap.

In the wastewater sludge and crude glycerol medium, the initial soap concentration was 36.5 g/L. According to Eq. 3, the soap concentration at pH 5, 6 and 7 was 0.474 g/L, 4.74

g/L and 36.5 g/L, respectively. Furthermore, the FFA concentration at pH 5, 6 and 7 was 36.03 g/L, 31.76 g/L and 0 g/L, respectively. It meant that 98.71% of soap was converted to FFA at pH 5 (36.03/36.5=98.71%), which was 87.01% at pH 6 and 0.00% at pH 7.

Soap (or detergent) was an inhibitor of the microorganisms in the natural water system. Soap could highly affect the microorganisms' growth by decreasing their motility parameters, impairing their orientation and transforming their morphology (Kosmela et al., 2017; Rahman et al., 2017). Once soap was converted to FFA, FFA inhibited the growth of the microorganisms depending on its concentration and the inoculum to FFA ratio (Rinzema et al., 1994; Ma et al., 2015). In this study, the free fatty acid (mainly oleate acid) had a low solubility and *T. oleaginosus* concentration from inoculum was high. In addition, it was found that oleate acid inhibition was minor (Angelidaki & Ahring, 1992). Thus, a low pH which converted soap to FFA could avoid the inhibition when crude glycerol contained high soap content was used for *T. oleaginosus* cultivation. In this study, pH 5, which convert most of the soap to FFA, was found optimal when crude glycerol containing high soap was employed as a fermentation medium.

By investigating the pH variation during the fermentation, it was found that pH continuously decreased at all conditions (pH 5, 6 and 7) (Fig. 6.2a, 6.2b and 6.2c). There might be two factors, which caused the decrease in pH. Firstly, as an aerobic microorganism, oleaginous yeast *T. oleaginosus* oxidized the substrates to generate energy for the cell growth. Along with the cell production, certain necessary organic acids (such as amino acids) were generated and the protons inside the cells were pumped out which led the decrease of the medium's pH (Kumari, 2018). Secondly, soap (or FFA) was a long chain organic compound, which should be biodegraded before being utilized by *T. oleaginosus*. Short chain organic acids (such as acetic acid and propanoic acid) were generated from soap biodegradation, which also contributed to the decrease in pH (Scott & Jones, 2000).

6.5.4 The pH based fed-batch fermentation

Fed-batch fermentation was superior for the lipid production from *T. oleaginosus* compared to batch fermentation (Chen et al., 2017). For a good performance of fed-batch

fermentation, the feeding strategy was essential (Mears et al., 2017). Feeding should follow the pace of consumption of substrate and nutrients and thus to provide the maximum efficiency of their utilization. In this study, it was observed that pH was a very important parameter for the cell growth of *T. oleaginosus* when the fermentation medium contained high soap. It was supposed that the feeding strategy might be accomplished with the control of pH during the fermentation. Thus, pH-based fed-batch fermentation was proposed and conducted. It was previously observed that the pH continuously decreased during the fermentation. Thus, to control the pH during the fermentation, the only alkaline solution was needed.

Our previous research proved that methanol in crude glycerol solution was able to assist *T. oleaginosus* to overcome the contaminants and produce lipid in non-sterile condition at a concentration of 1.4 % (w/v) (Chen et al. 2018). Thus, 450 mL crude glycerol, which gave a methanol concentration of 1.4 % (w/v), was used to prepare the basic 10 L fed-batch medium. In order to prevent the overdose of methanol which could cause the inhibition, methanol was evaporated from another 2 L crude glycerol (pH=8.98) before being used for pH control and feed. After removing the methanol from crude glycerol, the pH of the solution was 10.98 in strong alkaline. Thus, the methanol free crude glycerol solution was used to control the fermentation pH at 5±0.1. Once the fermentation pH decreased, the methanol free crude glycerol (pH 10.98) was pumped into the fermenter which was automatically controlled by the control system.

In the fed-batch fermentation, soap existed in the form of FFA because the fermentation pH was 5. The fermentation started with the FFA concentration of 10.08 g/L and the glycerol concentration of 6.22 g/L (Fig. 6.3). It was found that the FFA concentration rapidly decreased to 2.5 g/L at 24 h but the biomass concentration didn’t increase a lot. Thus, the decrease of FFA was not due to the biomass production. It might indicate that the FFA was biodegraded to another form (Fig. 3). Different from soap concentration, glycerol concentration increased to 9.26 g/L at 24 h from 6.22 g/L at 0 h (Fig. 6.3). As been discussed above, short chain organic acids from FFA biodegradation decreased the fermentation pH, thus, crude glycerol solution was automatically pumped into the fermenter to control the fermentation pH. The crude glycerol solution used for pH control

was a mixture of soap and glycerol. Thus, glycerol was added to the fermenter along with the pH control, which led to an increase of glycerol concentration.

From 24 h to 48 h, the FFA concentration increased from 2.5 g/L to 6.2 g/L and the glycerol concentration increased from 9.26 g/L to 16.16 g/L (Fig. 3). It was observed that the total biomass increased fast from 24 h to 48 h, which indicated a fast FFA biodegradation rate as it depended on the metabolic activity of *T. oleaginosus* and proton pumping rate from the newly generated cells (Fig. 6.3) (Munawar et al., 2016). Thus, the crude glycerol solution (pH =10.98) had to be rapidly pumped into the fermenter in order to maintain the pH. It was observed that the FFA and glycerol concentration varied between 3.8 g/L and 6.5 g/L and 15.44 g/L and 17.75 g/L, respectively, during 48 h to 70 h (Fig. 6.3).

It was due to the addition of crude glycerol to maintain the pH. Our previous research found that glycerol and FFA utilization were highly associated with the biomass production (Chen et al., 2017). However, the glycerol and FFA concentration decreased fast from 70 h to 86 h which were from 17.75 g/L to 11.84 g/L and from 6.5 g/L to 2.3 g/L, respectively, though the biomass concentration increase was not significant (Fig. 6.3). At 70 h, the biomass concentration was 57.97 g/L which was rather high; thus high energy would be demanded to maintain the cell activities which led to the remarkable decrease of glycerol and FFA concentration but limit biomass concentration increase was observed.

After 86 h, the biomass and lipid production might still occur; however, the fermentation stopped at 86 h because it was found very difficult to maintain the dissolved oxygen concentration (DO) at the desired level (DO>30% v/v). The high biomass concentration at 86 h meant high cell concentration, which indicated high oxygen demand. In addition, the high biomass concentration increased the medium's viscosity, which reduced the oxygen transferring rate (two-film theory) (Lewis & Whitman, 1924; Barreto et al., 2017; Rodriguez et al., 2018). The high oxygen demand and low oxygen transfer rate made the DO maintenance difficult. A biomass concentration of 65.63 g/L, lipid concentration of 35.79 g/L and lipid content of 54.53% (w/w) were achieved at 86 h (Fig. 6.3). It should be mentioned that the constant decrease of methanol during the fermentation was due to evaporation (Chen et al. 2018).

In this study, the pH-based fed-batch fermentation achieved high biomass production, lipid production and lipid content. The concentration of glycerol and FFA was maintained at a high level (glycerol concentration between 6.22 g/L and 17.75 g/L, FFA concentration between 2.3 g/L and 10.08 g/L) but not too high to cause substrate inhibition throughout the fermentation (Fig. 6.3). The pH-based fed-batch fermentation could be widely applied in most of the situations with a minor adjustment of the solution's pH (solution for pH control) and/or the control precision. For instance, a similar method could be used to prepare an acid crude glycerol solution and control the fermentation pH when the pH increased continuously during fermentation; or an acid crude glycerol solution with an alkaline crude glycerol solution could control the fermentation pH when it went up and down. If the substrates (FFA and glycerol) were overdosed and caused substrate inhibition during the pH control, the pH of the crude glycerol solution could be increased to avoid the overdose of the substrate. For example, increasing the pH of the methanol free crude glycerol from pH 11 to pH 12, the addition of the substrates (glycerol and FFA) for pH control would become one-tenth. Thus, the overdose of substrates could be avoided. If a substrate shock was preferable (substrate concentration varied in a wide range), it could be realized by increasing the control precision from ±0.1 to a higher level. The exact control precision could be calculated. For example, the control precision in this study was ±0.1. Each time, to increase the fermentation from pH 4.9 to pH 5.0, 2.59 mL of methanol free crude glycerol solution (pH=10.98) was needed for per liter of fermentation medium. The 2.59 mL of crude glycerol solution (pH=10.98) contained 0.98 g FFA and 0.55 g glycerol, thus, 0.98 g FFA and 0.55 g glycerol were added to per liter of fermentation medium for pH adjustment from 4.9 to 5.0. Similarly, increasing the control precision from ±0.1 to ±0.2, 1.55 g FFA and 0.87 g glycerol were needed for per liter of fermentation medium to adjust the pH from 4.8 to 5.0. Hence, a higher substrate shock was achieved by increasing the control precision from ±0.1 to ±0.2. In all, the pH-based fed-batch fermentation was widely applicable by modifying the pH of the pH control solution and/or the control precision.

6.6 Conclusions

In this study, the optimal pH for the lipid production using oleaginous yeast *T. oleaginosus* was related to the substrate. When crude glycerol solution containing soap was employed as the fermentation medium, pH 5 converting soap to FFA was considered optimal in order to avoid the soap inhibition. A pH-based fed-batch fermentation was designed and operated. High biomass production, lipid production and biomass lipid content were achieved simultaneously from the fed-batch fermentation.

6.7 Acknowledgments

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6.8 References

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Table 6. 1 Comparison of the pH effect on *T. oleaginosus* in sludge medium and in sludge and crude glycerol medium

	Maximum CFU/mL		Biomass concentration (g/L)		Lipid concentration (g/L)	
pH	**sludge**	**sludge+crude glycerol**	**sludge**	**sludge+crude glycerol**	**sludge**	**sludge+crude glycerol**
5.0	2.20E+10	1.88E+10	29.64	36.88	6.31	10.75
6.0	2.78E+10	9.84E+09	30.02	29.62	6.66	2.98
7.0	3.88E+10	7.80E+08	28.70	30.21	5.89	2.20

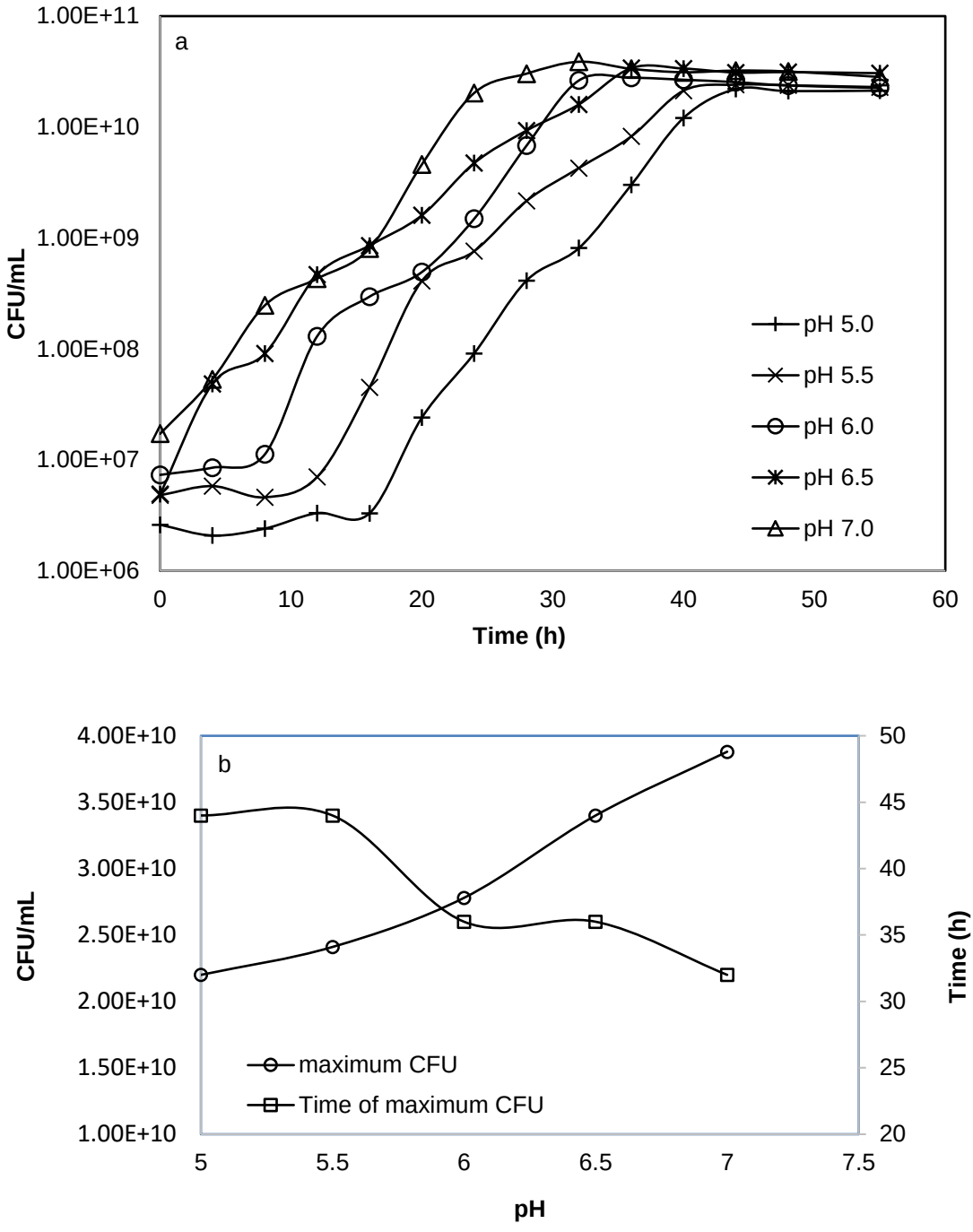
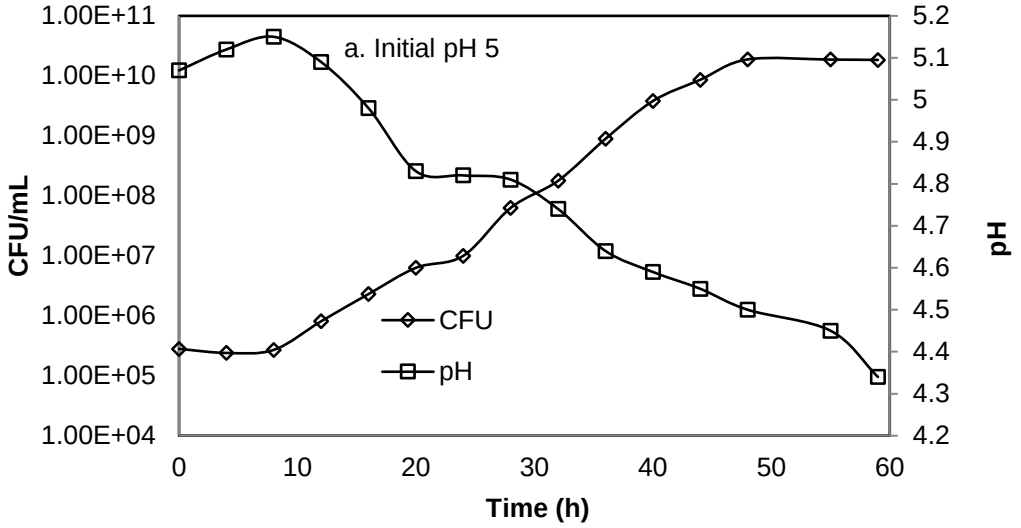
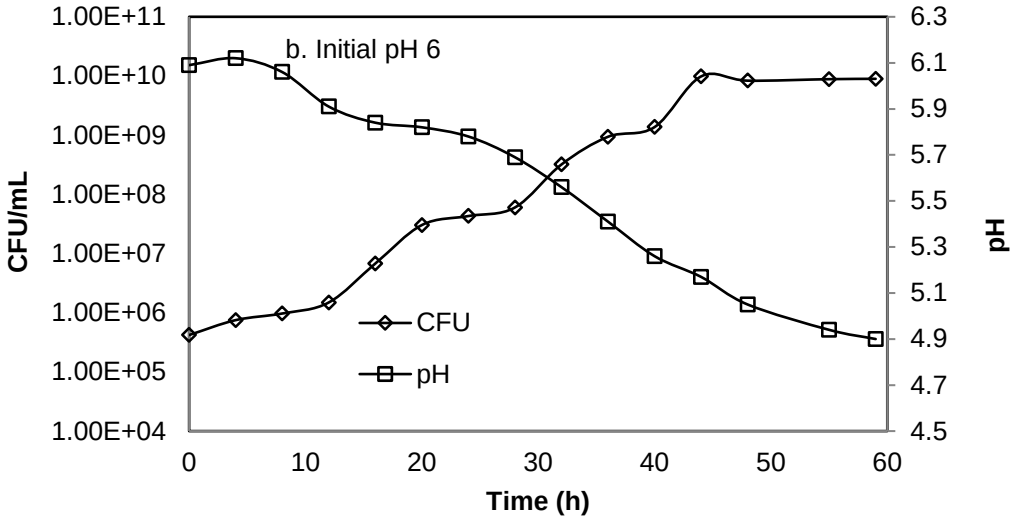


Figure 6. 1 The cell growth of *T. oleaginosus* at different pH conditions


a. Initial pH 5
CFU/mL
pH
Time (h)
CFU
pH
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
5.2
5.1
5
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
0
10
20
30
40
50
60


b. Initial pH 6
CFU/mL
pH
Time (h)
CFU
pH
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
6.3
6.1
5.9
5.7
5.5
5.3
5.1
4.9
4.7
4.5
0
10
20
30
40
50
60

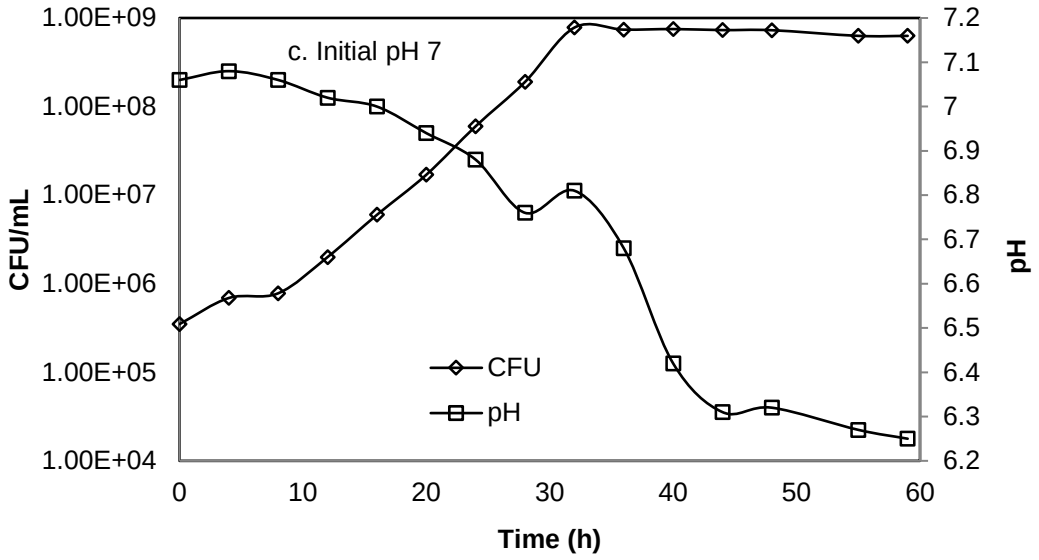


Figure 6. 2 **The cell growth of *T. oleaginosus* and pH variation in sludge with crude glycerol medium**

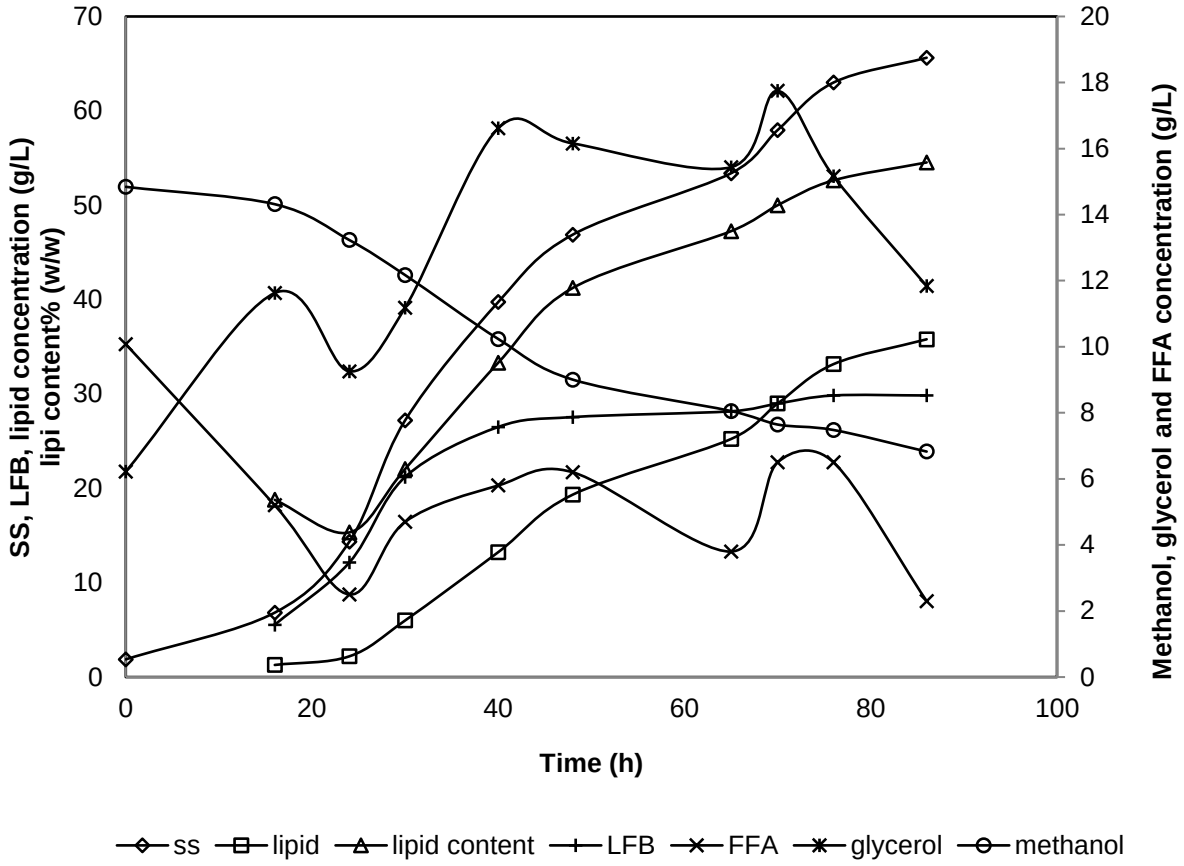


Figure 6. 3 pH based fed-batch fermentation

A thermodynamic model of lipid accumulation *Trichosporon oleaginosus*

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7 A THERMODYNAMIC MODEL OF LIPID ACCUMULATION *TRICHOSPORON OLEAGINOSUS*

7.1 Résumé

L'étude aime à révéler la corrélation entre l'utilisation du substrat et la croissance cellulaire (production de biomasse sans lipides) et l'accumulation de lipides. Entouré de biomembrane et séparé du milieu environnant, chaque cellule oléagineuse peut être considérée comme un système isolé. Un modèle thermodynamique de l'accumulation de lipides dans les cellules oléagineuses peut être établi sur la base de la loi de conservation de l'énergie. Une nouvelle équation mathématique est proposée basée sur le bilan énergétique. Le modèle et l'équation sont vérifiés en employant la levure oléagineuse *Trichosporon oleaginosus* cultivée dans du milieu synthétique de glycérol brut. Il est constaté que les substrats étaient essentiels pour soutenir l'énergie, qui est utilisé pour la maintenance cellulaire, la croissance cellulaire, la production de lipides, etc. La relation entre la production de biomasse et la production de lipides est la compétition pour l'énergie générée par l'utilisation du substrat. En raison de la teneur élevée en énergie du substrat principal (acide gras libre ou AGL), le rendement en lipides atteint dans cette étude est aussi élevé que 0,39 (g/g).

Mots clés: Modèle thermodynamique; système isolé; la loi de conservation de l'énergie; corrélation; la production de lipides.

7.2 Abstract

The study was aimed to reveal the correlation between the substrate utilization and cell growth (lipid-free biomass production) & lipid accumulation. Surrounded by biomembrane and separated from the surrounding environment, each oleaginous cell could be considered as an isolated system. A thermodynamic model of lipid accumulation in the oleaginous cells could be established based on the law of energy conservation. A new mathematical equation was proposed based on the energy balance. The model and equation were verified by employing oleaginous yeast *Trichosporon oleaginosus* cultivated in crude glycerol synthetic medium. It was found that substrates were essential to support energy for cell maintenance, cell growth, lipid production, etc. The relation between biomass production and lipid production was the competition for the energy which was generated by substrate utilization. Due to the high energy content of the main substrate (free fatty acid or FFA), the lipid yield achieved in this study was as high as 0.39 (g/g).

Keywords: Thermodynamic model; isolated system; the law of energy conservation; correlation; lipid production.

7.3 Introduction

Lipids (or microbial oils), which are accumulated in oleaginous microorganisms, showed great potential to replace plant seed oils for biodiesel production [1-5]. Great interest has been paid to biodiesel production from lipids [6]. The obstacle, preventing the industrial practice of biodiesel production from lipids, is the high lipid production cost [7]. In addition to the high lipid production cost of the microbial process, there is great need of the understanding on the correlation of cell growth (biomass production), lipid production and substrate consumption to provide knowledge on how to enhance the lipid production and efficiently convert substrate to lipid [8, 9]. Currently, kinetic study and mechanism of lipid production are performed in order to reveal the correlation [10-12]. Even though kinetic and mechanism studies are important and useful to understand the lipid production pattern and to find the route to enhance the lipid production, it cannot provide the strategy on how to efficiently convert substrate to lipid because the relation of substrate utilization and lipid production is not well understood [10, 13-15].

The reason, why the correlation is still unclear, is that the kinetic study and mechanism investigation are based on the existing structured models; however, none of these models can fully represent the correlation [16-18]. For instance, kinetic study to investigate the cell growth is mainly based on Monod equation or Logistic equation. According to Monod equation [19], the specific growth rate is determined by the limiting substrate concentration and the environmental conditions; however, Logistic equation considers that mainly the biological and geometrical parameters in spite of the limiting substrate concentration determines the cell growth [20]. Obviously, neither Monod model nor logistic model can fully represent the cell growth of an oleaginous microorganism, which may be simultaneously affected by the limiting substrate concentration, the environmental conditions, and the biological and geometrical parameters. Not only cell growth but also lipid production, the current model employed for the kinetic study has its limitation. The Luedking–Piret model is generally employed to describe the lipid production rate [21]. The Luedking–Piret model considers both growths associated and non-growth associated product formation. In other words, the product is produced either along with the generation of the new cells or the existing cells and the product is not considered to be

reusable (by the same microorganisms) once it is formed [21, 22]. However, the lipid is stored in the cells as an energy source and can be reused under external substrate limiting condition or fast cell growth phase [10]. Thus, the Luedking–Piret model may not be suitable to describe lipid production in an oleaginous microorganism.

Overall, the current models can't fully describe the cell growth or the lipid production. Hence, kinetic study and mechanism investment of lipid production based on the current models are not able to reveal the correlation between substrate utilization, biomass production and lipid production. The modification of the current models or a new model is demanded to describe the substrate consumption, cell growth (lipid-free biomass production) and lipid production, as well as their correlation.

First of all, the substrate is the energy and carbon source for cell growth and lipid accumulation [18, 23]. It indicates that energy generated from the substrate consumption may be the essential parameter, which determines cell growth (biomass production), lipid production and cell maintenance energy.

Living cells are surrounded by the cell membrane (and cell wall), which restrains the cell content and separates the cell from the surrounding medium. A substrate may diffuse into the cells by passive transport or be transported into the cells by membrane proteins depending on its solubility and molecular size [24-26]. Normally, the cells tend to maintain the substrate concentration inside the cell at a certain level regardless its concentration in the medium [27]. It all indicates that oleaginous cells are isolated from the surrounding environment by the cell membrane. Thus, each oleaginous cell can be considered as an isolated system. According to the law of energy conservation, the energy remains constant and can only be transformed from one form to another in an isolated system. Hence, once the substrate is diffused or transferred into the oleaginous cell and consumed to generate energy, the energy can be transformed into new cells, lipid, etc. but not destroyed.

In this study, a model to present the correlation between substrate utilization, cell growth, and lipid production was developed according to the law of conservation of energy. Oleaginous yeast *T. oleaginosus* was cultivated in crude glycerol synthetic medium in batch fermentations and employed to verify the model developed.

7.4 Materials and methods

7.4.1 Materials

7.4.1.1 The strain

Oleaginous yeast *Trichosporon oleaginosus* (ATCC 20905) was employed to investigate the correlation between lipid accumulation, lipid-free biomass production, and substrate utilization.

7.4.1.2 Crude glycerol characterization

The crude glycerol characterization was performed with the similar method presented in our previous study [10]. The crude glycerol solution used in this study mainly contained methanol, soap and glycerol. Most of the methanol was evaporated from crude glycerol solution before being used as fermentation medium. Thus, the carbon sources were from soap and glycerol.

7.4.1.3 Pre-culture, fermentation medium and fermentation

The pre-culture was prepared by inoculating a loop full of *T. oleaginosus* into 100 mL sterilized YPD medium and incubated for 24 h at 30 °C, 180 rpm for 24 h in an incubating shaker; then transferred to another 900 mL sterilized YPD medium (20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract) and incubated for another 24 h.

The fermentation medium was prepared using crude glycerol synthetic medium. The crude glycerol contained 31.14±2.02% (w/v) of methanol, 23.58±1.53% (w/v) of soap and 13.24±0.63% of glycerol. Methanol was evaporated from crude glycerol solution at 60 °C for 15 min and not counted as a carbon source [10]. In per L of fermentation medium, it contained 2.7 g KH_2PO_4, 0.95 g Na_2HPO_4, 1.616 g NH_4Cl, 0.1g EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g peptone, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.0055 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4 \cdot 7H_2O$, 0.00076 g $MnSO_4 \cdot H_2O$ [28], and 67.5 mL methanol evaporated crude glycerol solution. The fermentation initial C/N ratio was 45.

The fermentation was carried out in a 15 L fermenter with a working volume of 10 L. During the fermentation, the pH was controlled at pH 5 and the DO (dissolved oxygen) was maintained above 30% (v/v) [10].

7.4.2 Methods

7.4.2.1 Cell counting

Samples (each of 0.1 mL) were diluted with Isoton II electrolyte (Beckman, USA) and transferred into cuvettes (25 ml×25 mL) with a volume of 10 mL. In the counting volume of 0.05 ml, the cell counts were 10,000 to 100,000. Cell counting was carried out with an electronic particle counter (Multisizer™ 3 Coulter Counter with a 70 μm aperture; Beckman, USA) using a 2 to 10 μm orifice. The experimental results were analyzed by the software Multisizer 3 for the cell counts and the mean size of the cells [29].

7.4.2.2 Biomass, lipid, soap (or free fatty acid) and glycerol concentration analysis

Biomass concentration was determined by centrifuging 30 mL sample and washing the pallet twice, followed by drying and weighing the residue solid. The lipid concentration was then determined by extracting lipid from the solids residue using methanol and chloroform. Soap concentration was measured by converting soap to free fatty acid (FFA) and extracting FFA using chloroform. After evaporating the chloroform at 60 °C overnight, the residue liquid (FFA) was weighted. Glycerol concentration was measured according to Bondioli and Bella [30]. The details were described in our previous study [10].

7.4.3 Establishment of the model

Substrate consumption serves two items: 1) carbon source to generate new cells and 2) as energy source, which is used for cell growth, lipid production and cell maintenance. Since energy derived from substrate consumption was considered as the driving force for both cell growth (lipid free biomass production) and lipid production, the establishment of the model was based on the energy flow, which started from the substrate transportation and consumption and ending to cell growth or lipid production. In addition, as each oleaginous cell was recognized to be an isolated system, energy from substrate consumption would be transformed to other forms but not eliminated in the cell. In all, the model based on the consideration of oleaginous cell as an isolated system could be established and all the involving parameters during the lipid production in the oleaginous cells were related to energy (Fig. 7.1). Correspondingly, the reactions occurring inside and outside the cells were: substrates transportation (k_1); substrates consumption to

generate energy (k_2); energy (containing the energy in the carbon source which was directly used to generate new cells) used to produce new cells (k_3); energy used to produce lipids (k_4); and energy for maintenance. In addition to these reactions, lipids might be reused to support the cell growth (k_5) in substrate deficient or fast growing condition. Thus, the whole lipid production process could be simply represented by the procedure of the reactions considering all the reactions were aiming to produce energy or driven by the produced energy (Fig. 7.2).

Following the establishment of the model, a new thermodynamic equation could be proposed according to the energy balance between energy generated from substrate consumption and energy consumed for cell growth, lipid production, cell maintenance, etc (Eq. 1):

$$\alpha \cdot dS/dt = \beta \cdot dp/dt + \gamma \cdot dX/dt + \delta \cdot X \quad (1)$$

Where α was the energy content of the substrate (equivalent to ATP/g), dS/dt was the substrate consumption rate (g/L/h), β was the energy content of the lipids (equivalent to ATP/g), dp/dt was the lipid production rate (g/L/h), γ was the energy content of lipid-free biomass (equivalent to ATP/g), dX/dt was the lipid-free biomass production rate (g/L/h), δ was the energy for supporting the basic cell maintenance (equivalent to ATP/g lipid-free biomass/h), X was the dry lipid-free biomass weight (g/L). In this study, the cell growth led to lipid-free biomass production. Thus, lipid-free biomass was used to replace cell count in Eq. 1. The correlation between lipid-free biomass production, lipid production and maintaining the lives of existing cells was the competition for the energy provided by substrate consumption.

7.4.4 Kinetic analysis

Biomass and lipid yield, which revealed the conversion efficiency of the substrate to biomass and lipid, were investigated in this study according to Eq. 2 and 3, respectively.

$$\text{Biomass yield } (Y_{x/s}, \text{g/g}) = (X_2 - X_1)/(S_1 - S_2) \quad (2)$$

$$\text{Lipid yield } (Y_{P/s}, \text{g/g}) = (p_2 - p_1)/(S_1 - S_2) \quad (3)$$

Where X_2 and X_1, p_2 and p_1, S_2 and S_1 were used to present the biomass (X), lipid (p) and carbon (S) (from glycerol and soap in crude glycerol) concentrations (g/L) at time t_2 and t_1, respectively.

7.5 Results and discussion

In this study, the experimental results were employed from our previous research of lipid production using oleaginous yeast *Trichosporon oleaginosus* cultivated with crude glycerol solution from a local biodiesel production company [10]. The carbon sources in the medium prepared from crude glycerol solution for *Trichosporon oleaginosus* cultivation were FFA and glycerol [10].

7.5.1 The correlation corresponding to the growth phase of *T. oleaginousus*

In batch culture, the growth of microorganisms could be generally divided into four different phases which were lag phase, log phase (exponential phase), stationary phase and death phase (decline phase) [31]. Accordingly, the correlation was divided into four situations.

During the lag phase, the growth of the strain was restrained which indicated that K_3 $(dx/dt)\approx 0$ (Fig. 7.2). However, the medium was rich in substrates. The substrates might be constantly and fast transported into the cell, which indicated that K_1 and K_2 maintained in high level (Fig. 7.2). As a result, the energy generated from substrate consumption maintained in high level. Thereafter, the energy should be mainly used for lipid production but not cell growth (Fig. 7.1 and 7.2) during the lag phase. As a result, the lipid content (lipid/dry cell weight) would go up during the lag phase. Even though no obvious lag phase was observed in this study, the cell growth was slow before 16 h (low specific growth rate, Fig. 7.5b). The experiment results showed that a high lipid content of 50.53 % (w/w) was observed at 16 h (Fig. 7.3). The cell count experiment showed that the particle size became bigger at 16 h compared with at 0 h (Fig. 7.4). The mean size of D90 (ninetieth-percentile diameters) at 16 h was 4.02 μm, which was 2.31μm at 0 h (Table 7.1). The size of the cells of *T. oleaginousus* got almost two times bigger at 16 h compared with at 0 h.

It was the common sense that more lipids in the cells, the bigger oleaginous cells were [32].

During the log phase from 16 h to 40 h (high specific growth rate, Fig. 7.5b), the cell grew faster. As long as the substrates were still sufficient in the medium, the substrates could be fast transported into the cell to generate energy (Fig. 7.1 and 7.2). The energy might be mainly used for cell division but not for lipid production during the log phase (Fig. 7.2). It should be mentioned that the lipids stored in the cells might be reused as energy to support the cell division if the energy from substrate consumption was not sufficient to support the high cell growth (Fig. 7.2). Along with the cell growth during the log phase, cells were generating and substrates were consuming in the batch fermentation. The decrease of substrate concentration and the increase of the cells in the medium will slow down the cell growth according to Monod equation and Logistic equation. Since the relation of cell growth and lipid production was the competition of energy from substrate consumption. Thus, the energy was tending to be stored as lipid when the cell growth was slowed down. It was found that there were two active phases of LFB growth: fast growth between 8-24h and slow growth 24-56h. The lipid-free biomass (LFB) concentration increased fast from 8 h to 24 h when the lipid concentration increased slowly (Fig. 7.3). Thus, *T. oleaginousus* tended to produce new cells from 8 h to 24 h and the lipid production was affected. As a result, a low lipid content of 36.99% (g/g) was observed at 24 h. After 24 h, the lipid-free biomass production became slow and the lipid production increased (Fig. 7.3). During the log phase from 16 h (or 8 h) to 40 h (high specific growth rate, Fig. 7.5b), the co-growth of LFB and lipid was observed (both increased) (Fig. 7.3). Maintaining the co-growth and prolonging the log phase would be favorable for both cell growth and lipid production [10, 16]. According to Monod equation, maintaining the substrate concentration at a high level to constantly supply high energy stress might force the cell growth and lipid production keeps on processing. That should be why fed-batch or continuous batch fermentation was advantageous for high lipid production [10, 33, 34]. Our previous research proved that maintaining the substrate concentration by multiple feeds simultaneously enhance and prolong the LFB and lipid production [10]. The particle size distribution showed that the fast LFB production led to a decrease of the mean size of D90 from 16 h to 24 h and then the mean size of D90 kept

increasing from 24 h to 40 h (Fig. 7.4 and Table. 7.1). This indicated that *T. oleaginosous* shifted from preferring cell growth (16 h to 24 h) to prefer lipid production (24 h to 40 h) during the log phase.

During the stationary phase from 40 h to 56 h (specific growth rate was around zero, Fig. 7.5b), the cell growth stopped but the cells were still alive. However, the lipid production kept processing as long as the substrate was still transported into the cells and consumed to provide energy. Nevertheless, the available substrate for lipid production was low (Fig. 7.3) and there was a competition of energy between lipid production and the living cells' maintenance (Eq. 1). Therefore, the lipid production was slow during the stationary phase (Fig. 7.3). It was observed that the LFB concentration was almost constant from 40 h to 56 h, which was the stationary phase (Fig. 7.3). However, the lipid concentration slowly increased from 40 h to 56 h (Fig. 7.3). The mean size of D90 slightly increased which indicated the slight increase of lipid concentration from 40 h to 56 h (Fig. 7.4 and Table. 7.1). As been predicted, there was a competition for substrate between lipid production and cell maintenance; however, the substrates (FFA and glycerol) were in low concentration during the stationary phase (Fig. 7.3). Thus, additional substrates might also be necessary for high lipid production during stationary phase.

During the death phase (from 56 h to 70 h, the specific growth rate was minus zero, Fig. 7.5b), the cell numbers decline. If the decrease of the cell numbers was caused by the deficiency of the substrates, the lipid stored in the cells could be reused to support the cell maintenance. It was favorable to find strategies to reserve the lipid in the cells or to extract the lipids from the cells right after lipid production [35]. From 56 h, the LFB decreased which might indicate the death phase (Fig. 7.3). However, it should be mentioned that the lipid concentration decreased more (from12.14 g/L to 10.54 g/L) than the LFB decrease (from 12.66 g/L to 12.26 g/L) from 56 h to 70 h (Fig. 7.3). Thus, it was supposed that the faster decrease of lipid comparing with LFB was due to the lipid utilization by cells because of the low substrates (FFA and glycerol) concentration in the medium after 56 h (Fig. 7.3) [35].

Since the correlation of substrate consumption with cell growth and lipid production were divided into four situations, the mathematical equation (Eq. 1) proposed in this study could be correspondingly re-described as a piecewise function:

（A）Lag phase: $\alpha \cdot dS/dt = \beta \cdot dp/dt + \delta \cdot X$ ($dX/dt \approx 0$; $dp/dt > 0$)

（B）Log phase: $\alpha \cdot dS/dt = \beta \cdot dp/dt + \gamma \cdot dX/dt + \delta \cdot X$ ($dX/dt > 0$; $dp/dt > 0$ co-growth of cell and lipid; $dX/dt > 0$; $dp/dt < 0$ when the substrate was not sufficient to provide energy for cell growth)

（C）Stationary phase: $\alpha \cdot dS/dt = \beta \cdot dp/dt + \delta \cdot X$ ($dX/dt \approx 0$; $dp/dt > 0$)

（D）Death phase: $\alpha \cdot dS/dt - \beta \cdot dp/dt = \delta \cdot X$ ($dX/dt < 0$; $dp/dt < 0$)

7.5.2 The relation between lipid production and cell growth (LFB production)

Normally, the relation between lipid production and cell growth (biomass production) was represented by the Luedking–Piret model (Eq. 4). The Luedking–Piret model considered that the lipid was either produced by the production of the new cell or the existing cells in the medium [21] (Eq. 4). In all, the Luedking–Piret model considered that lipid production was determined by the biomass production.

$$(dp/dt)/X = \alpha \cdot (dx/dt)/X + \beta \qquad (4)$$

Where $(dp/dt)/X$ was specific lipid production rate, $(dx/dt)/X$ was specific growth rate, α and β were constant parameters. It was obvious that the Luedking–Piret model indicated a linear relationship between specific lipid production rate and specific growth rate. However, no linear phase was observed from the experimental results (Fig. 7.5). It was found that the cell grew faster and faster from 0 h to 16 h and the maximum specific growth rate occurred at 16 h. However, the specific lipid production rate kept low from 0 h to 16 h (Fig. 7.5). The high cell growth rate didn’t lead to a high lipid production rate. From 16 h to 40 h, the cell growth decreased; however, the specific lipid production rate increased (Fig. 7.5). The low cell growth rate didn’t cause low lipid production rate. Thus, it was concluded that the lipid production was not determined by the cell growth. After 40

h, both cell growth and the lipid production rate became slow until finally both started to decline from 56 h (Fig. 7.5). However, the cell concentration (LFB concentration) was high from 40 h to 56 h (Fig. 7.3). Thus the high cell concentration didn't lead to the high lipid production rate either. Therefore, the lipid production rate was neither determined by the cell growth nor determined by the existing cell concentration in the medium. Hence, there was no doubt that the Luedking–Piret model was not suitable for the lipid production using oleaginous yeast *T. oleaginousus*.

In this study, it was revealed that the relation between lipid production and cell growth (LFB production) was controlled by the competition for the energy from substrate consumption (Fig. 7.1 and 7.2). Lipid production and cell growth competed for the energy generated from substrate consumption at each instance. By investigating the specific growth rate and specific lipid production rate (Fig. 7.5), it was found that the specific lipid production rate was low when the specific growth rate was high and the specific lipid production rate was high when the specific growth rate was low. Thus, the opposite trend of specific growth rate and the specific lipid production rate might prove that the competition between the lipid production and cell growth (Fig. 7.5).

7.5.3 The relation between lipid production and substrate utilization

The available energy from substrate consumption was considered to be the only parameter which determined the lipid production in this study. Supposing all the energy contained in the substrate to be used for lipid production, a maximum thermodynamic lipid to substrate yield could be calculated according to Eq. 1. For example, the energy content of glucose was 30 ATP/ one molecule of glucose; the energy content of lipid (stearic acid) was 130 ATP/one molecule of stearic acid. Considering the thermodynamic conversion, 4.33 (moles) glucose (780 g glucose) were demanded to form 1 (mole) stearic acid (lipid) (284 g lipid). Thus, the theoretical thermodynamic lipid yield was 284 g/780 g≈0.36 (g/g) when energy from glucose was completely used for lipid production [23, 36]. The lipid yield from experiments was always less than the theoretical thermodynamic lipid yield owing to the unavoidable energy consumption for the cell maintenance [23].

In this study, a high lipid yield of 0.39 g lipids/g substrates was achieved (substrates were the sum of glycerol and FFA) (Fig. 7.6). The reason for the high lipid yield must be due to the substrates (FFA and glycerol) used in this study. The energy content of glycerol was 15 ATP/glycerol and the energy content of FFA was 130 ATP/g FFA. To compare the energy content of glucose, glycerol and FFA, the energy content of per gram substrate was calculated. For example, the energy content of glucose was 30 ATP/glucose=$6.02\times10^{23}\times30$ ATP/mole glucose=$6.02\times10^{23}\times30$ ATP/(mole glucose×180 g/mole)≈1.00×10^{23} ATP/g glucose. Similarly, the energy content of glycerol was around 1.00×10^{23} ATP/g glycerol, which was same as glucose. The energy content of FFA was around 2.75×10^{23} ATP/g FFA. The crude glycerol solution used in this study had a mass FFA to glycerol ratio of 23.58:13.24. Thus, the energy content of the crude glycerol was 2.12×10^{23} ATP/g. It was obvious that the energy content of the crude glycerol was more than two times higher of pure glucose or pure glycerol (Table. 7.2). Thus, it was found that a high lipid yield of 0.39 g/g was achieved (Fig. 7.6). The theoretical thermodynamic lipid to crude glycerol yield was 2.12/2.75≈0.77 (g/g). However, it should be mentioned that the conversion efficiency of the energy from crude glycerol to lipid was just around 50% (0.39/0.77=0.506) in this study.

7.5.4 Discussion

The substrate, which provided energy for cell growth and lipid production, was proved to be the most important parameter. The ideal substrates should be rapidly transported into the cells and be easily used by the strain to generate the energy (Fig. 7.1). Glucose and glycerol could be diffused very fast into the cell by a non-active facilitated mass transport process. Thus, they were considered and proved to be good substrates for lipid production. The fermentation using glucose and glycerol as substrates normally achieved high biomass and lipid production [37, 38]. However, glucose and glycerol had a low energy content, which caused low lipid yield. Hence, lipid production from glucose and glycerol normally had no economic feasibility due to their high cost and low lipid yield. To make lipid production economically acceptable and achieve high lipid yield, the substrates with high energy content but low price were favorable.

Environmental stresses could either promote the cell growth or restrain the cell growth. As long as the environmental stresses didn't affect the substrate transportation and consumption, the lipid production might not be affected. Thereafter, environmental stresses, which promoted the cell growth, could lead high LFB production but low lipid content. The environmental stresses, which restrained the cell growth, could lead low LFB production but high lipid content. The common expression was that any environmental stresses, which restrained the cell growth, made the oleaginous cells store lipid.

Fed-batch or continuous batch was advantageous for high biomass and lipid production. However, the high LFB and lipid production could not be accomplished simultaneously with high biomass and lipid yield due to the high energy waste for the cell maintenance when the high LFB presented in the medium. To reduce the energy waste for the cell maintenance, biomass should be collected and removed effectively from the medium after lipid production.

7.6 Conclusion

Based on the consideration of energy from substrates consumption as the essential parameter, which determined the biomass and lipid production, a lipid production model was established. A thermodynamic equation of lipid production, different from the common used Luedking–Piret model, was proposed based on the energy balance. It was found that the relation between LFB production and lipid production was the competition for the energy generated from substrate consumption. Lipid yield was mainly determined by the energy content of the substrate used for the cultivation. The high lipid yield achieved in this study (0.39 g/g) was due to the high energy content of the crude glycerol (2.12×10^{23} ATP) which was mainly from FFA.

7.7 Acknowledgments

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Table 7. 1 Mean size of the cells at a different time at C/N ratio 45

Time (h)	Mean size of D90 (µm)
0	2.31
16	4.02
24	3.57
32	3.95
40	4.28
56	4.50

Table 7. 2 Comparison of the energy content of the substrates.

Substrate	Energy content (ATP/g)
Glucose	1.00×10^{23}
Glycerol	1.00×10^{23}
FFA	2.75×10^{23}
Crude glycerol in this study	2.12×10^{23}

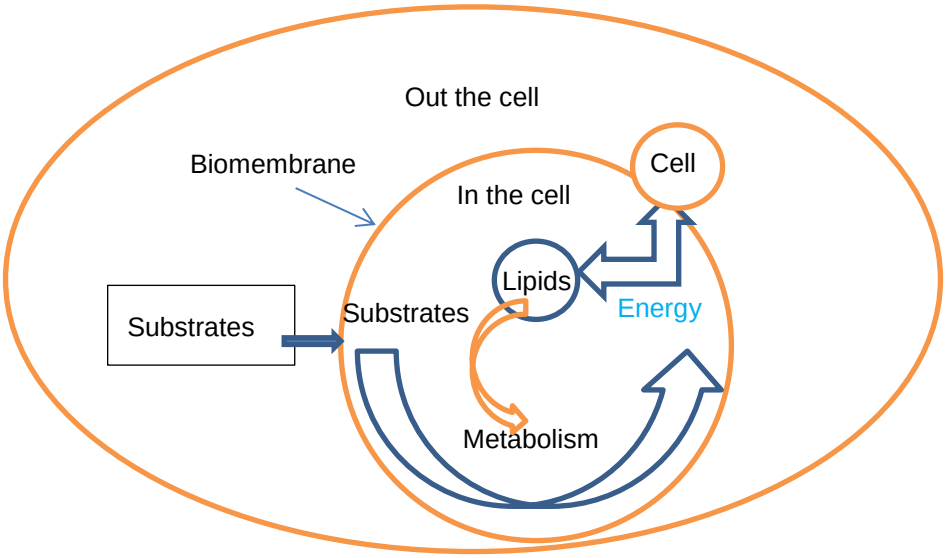

Figure 7. 1 The schematic diagram of the energy flow of the oleaginous microorganisms

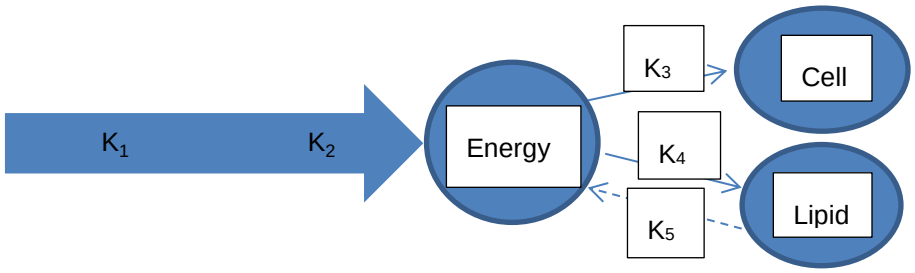

Figure 7. 2 **Lipid production represented by the procedure of the reactions inside and outside the cells**

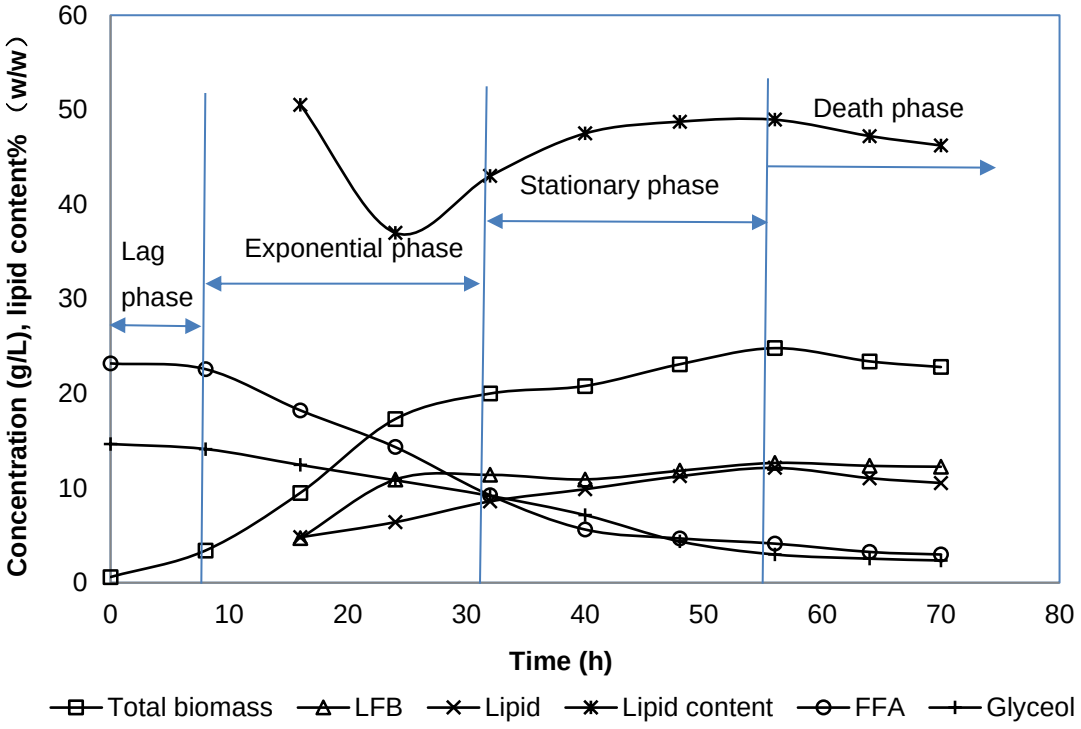

Figure 7. 3 Lipid production from *T. oleaginousus* at C/N ratio 45

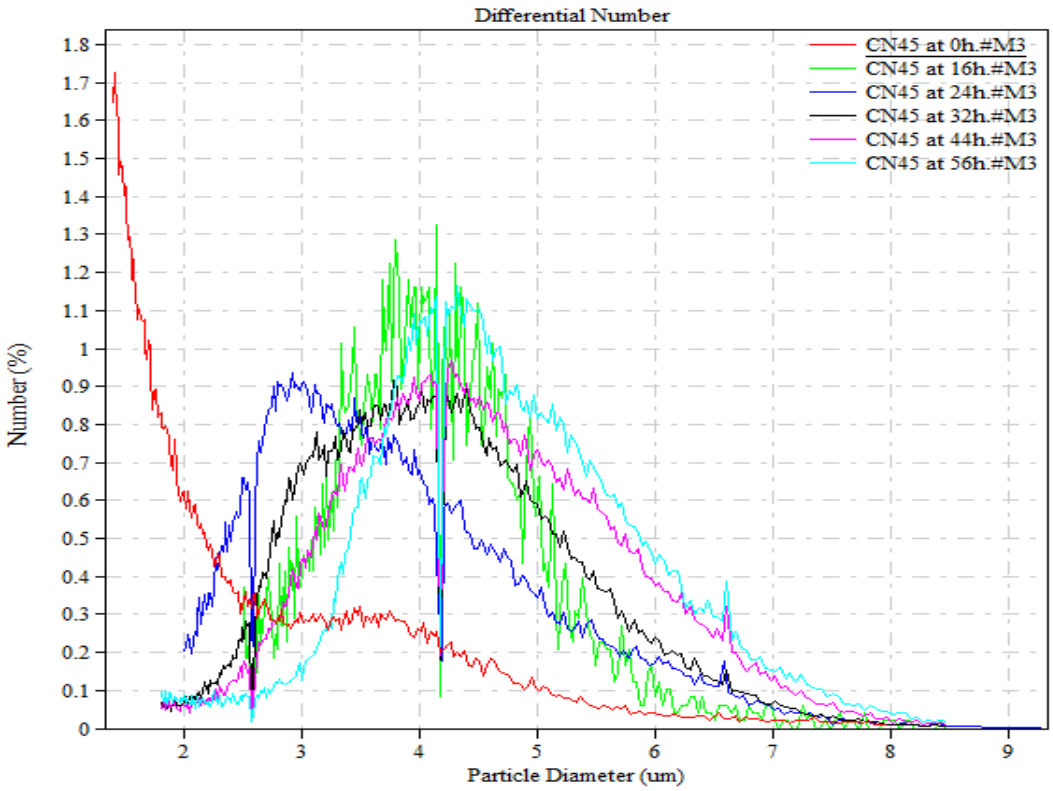

Figure 7. 4 Particle count at different time at C/N ratio 45

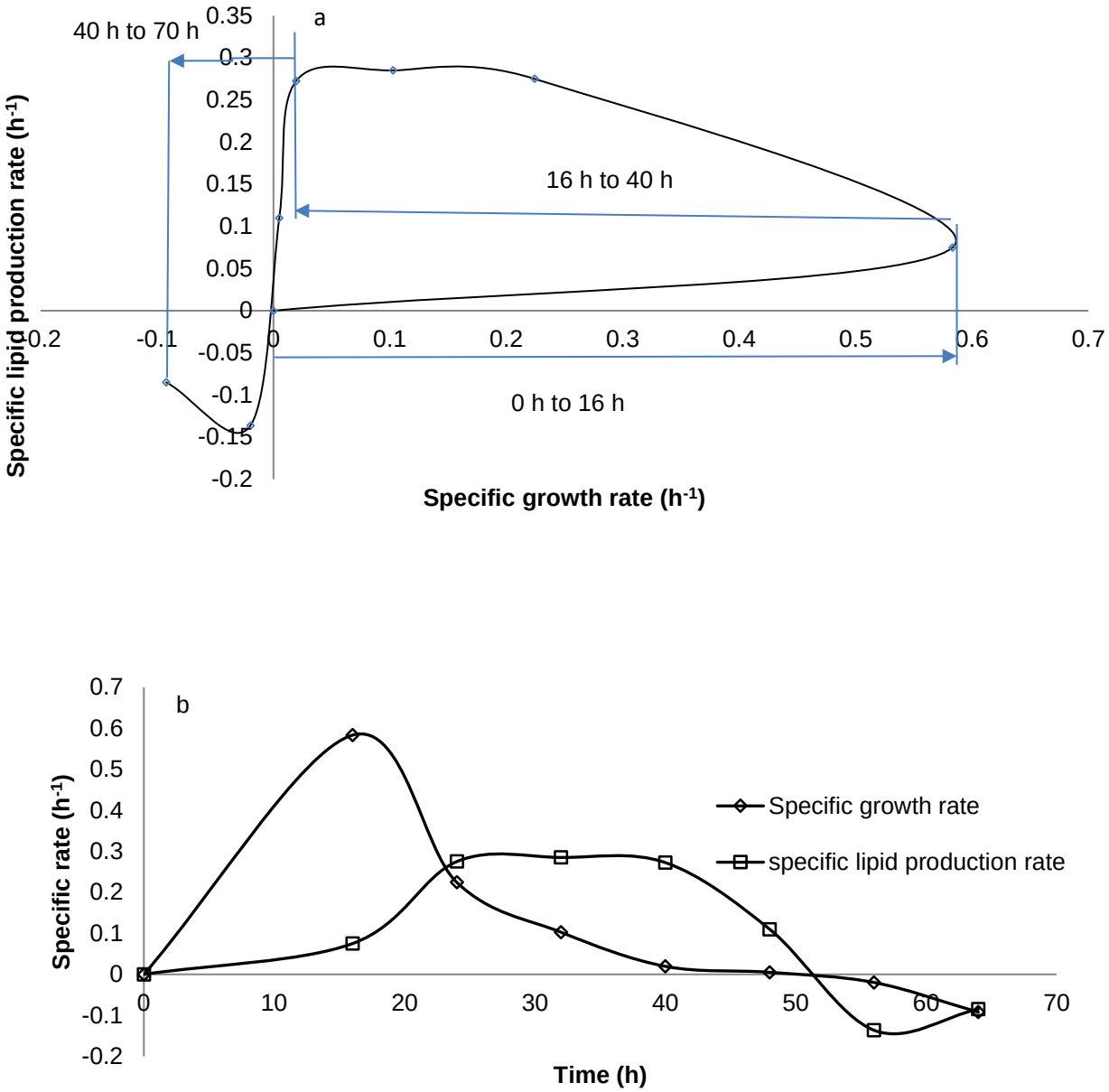

Figure 7. 5 Relation between specific growth rate and specific lipid production rate

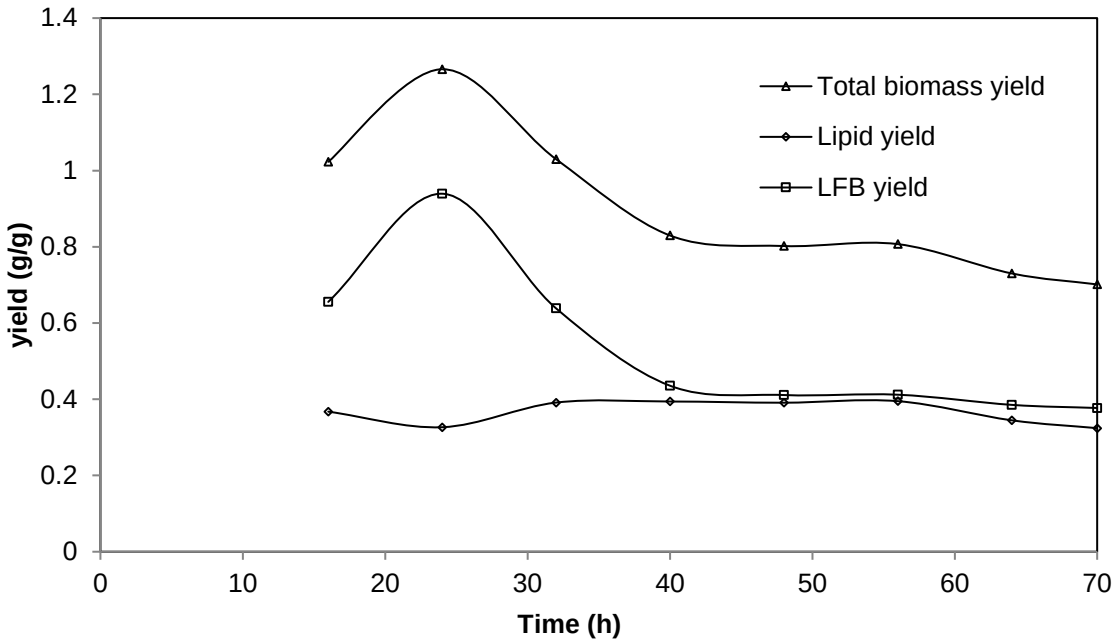

Figure 7. 6 Biomass and lipid yield (g lipid or g biomass generated /g carbon used) (LFB=lipid-free biomass)

Biodiesel production derived from lipid produced from *Trichosporon oleaginosus* cultivated with wastewater sludge and crude glycerol

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8 BIODIESEL PRODUCTION DERIVED FROM LIPID PRODUCED FROM *TRICHOSPORON OLEAGINOSUS* CULTIVATED WITH WASTEWATER SLUDGE AND CRUDE GLYCEROL

8.1 Résumé

Dans cette étude, la levure oléagineuse *Trichosporon oleaginosus* a été cultivée dans un mélange de boues et glycérol brut pour la production de lipides. Des expériences avec ajout de NH_4Cl dans le mélange et sans ajout de NH_4Cl dans le mélange ont été effectuées et comparées pour vérifier l'abondance de l'azote provenant des boues. L'expérience avec ajout de NH_4Cl a montré une meilleure production de biomasse, production de lipides et teneur en lipides qui étaient respectivement de 55,33 g/L, 26,98 g/L et 48,77% (p/p) comparés à 42,66 g/L, 17,88 g/L et 41,91 % (w/w), respectivement, sans ajout de NH_4Cl. Les résultats expérimentaux ont été utilisés pour l'évaluation de la faisabilité industrielle. Le biodiesel produit à partir de lipides avec l'ajout de NH_4Cl dans le mélange de boues et glycérol brut a obtenu un gain énergétique net de 14,26 GJ/tonne de biodiesel avec un rapport d'énergie de 1,53; a reduit de 19,97 tonnes d'émission de CO_2/tonne de biodiesel; avait un coût de production unitaire de 630 $/tonne (prix de détail 900 $/tonne). Il a été prouvé que la production de biodiesel à partir de lipides était faisable pour la pratique industrielle.

Mots clés : Biodiesel; lipide; boue; le glycérol brut; faisabilité industrielle

8.2 Abstract

In this study, oleaginous yeast *Trichosporon oleaginosus* was cultivated in sludge and crude glycerol medium for lipid production. Experiments with and without the addition of NH_4Cl in the medium were conducted and compared to verify the availability of nitrogen from sludge. The experiment with addition of NH_4Cl showed better biomass production, lipid production and lipid content, which were 55.33 g/L, 26.98 g/L and 48.77% (w/w), respectively, comparing with 42.66 g/L, 17.88 g/L and 41.91% (w/w), respectively, without addition of NH_4Cl. The experimental results were employed to evaluate the industrial feasibility of the biodiesel production process. Biodiesel produced from lipid with the addition of NH_4Cl in sludge and crude glycerol medium furnished 14.26 GJ/tonne of biodiesel net energy gain with an energy ratio of 1.53; reduced 19.97 tonnes CO_2 emission/tonne of biodiesel; had the unit production cost of 630 $/tonne (retail price 900 $/tonne). It was proved that biodiesel production from lipid was feasible for industrial practice.

Keywords: Biodiesel; lipid; sludge; crude glycerol; industrial feasibility.

8.3 Introduction

Domestic wastewater sludge (or municipal sludge or sewage sludge), which is a by-product of municipal wastewater treatment, is world widely generated in a great amount on a daily basis. There will be a heavy environmental load if wastewater sludge is not well managed. For a long time, wastewater sludge has been considered to be a waste and its treatments are mainly focused on disposal or reduction. However, neither disposal nor reduction can efficiently or completely eliminate the environmental threat associated with wastewater sludge. Recently, wastewater sludge is recognized as a reusable bioresource due to its high organic content, which is mainly composed of the microbial biomass (Seiple et al., 2017). The most well-known utilization of wastewater sludge as a bioresource is anaerobic digestion. Anaerobic digestion can bioconvert part of the wastewater sludge organic matter to biogas and then biogas can be used as a biofuel to produce energy. Generally, the biogas production and wastewater sludge reduction are limited due to its low biodegradability (Olkiewicz et al., 2015; Tian et al., 2015). Thus, the traditional anaerobic digestion is normally not economical or energy sustainable (Silvestre et al., 2015; Pilli et al., 2016).

Other than biogas, biodiesel is another biofuel, which is now widely studied using wastewater sludge as substrate (Kumar et al., 2016; Capodaglio & Callegari, 2017). Contrary to the biogas production from wastewater sludge, biodiesel production from wastewater sludge is found economically acceptable (Olkiewicz et al., 2016). Biodiesel production from wastewater sludge can be accomplished through two routes. Firstly, wastewater sludge normally contains 5-20% (w/w) lipids and lipids can be directly extracted from wastewater sludge then trans-esterified with methanol to produce biodiesel using strong acid as a catalyst (Chen et al., 2017). Obviously, direct biodiesel production from wastewater sludge is limited to its lipid content (Kumar et al., 2016; Patiño et al., 2018). Secondly, wastewater sludge is rich in the necessary nutrients for the growth of microorganisms (Zhang et al., 2014c). Thus, wastewater sludge can be applied as a raw material for oleaginous microorganisms' cultivation to produce lipids (Zhang et al., 2014b; Zhang et al., 2014c). The lipids accumulated in oleaginous microbial cells can be used to produce biodiesel by the trans-esterification reaction. Even though utilization of

wastewater sludge for oleaginous microorganisms cultivation increases the lipid content of wastewater sludge to around 40% (w/w), there is still a big gap between the lipid content achieved and the maximum lipid content that can be reached by the employed oleaginous microorganisms (Zhang et al., 2014c). It is very well known that oleaginous microorganism accumulates high lipid content in carbon-rich and nitrogen deficient condition (high carbon to nitrogen ratio) (Wiebe et al., 2012; Braunwald et al., 2013; Ma et al., 2016). Wastewater sludge is deficient in carbon source, thus, other substrates, rich in carbon sources such as crude glycerol, are fortified to the wastewater sludge for oleaginous microorganisms' cultivation (Saenge et al., 2011; Polburee et al., 2015). Generally, substrates used to enrich the wastewater sludge may have an important impact on the final cost of biodiesel (Koutinas et al., 2014). Thus, the substrates (carbon sources) with low or free cost are preferable. However, low or free cost substrates normally are difficult to degrade or contain high impurities, which may or may not inhibit the cell growth and lipid production of the oleaginous microorganisms. Pre-treatment to increase the degradability or remove the impurities unavoidably make the whole biodiesel production process more complicating and increase the final biodiesel production cost (Hejna et al., 2016; Kong et al., 2016). Previous research in our lab testes the combination of sludge with crude glycerol for lipid production. Even though the results show that high lipid production can be achieved, the pre-treatments to remove the impurities from sludge and crude glycerol were complicating (unpublished data). Thus, simple but efficient method, combining wastewater sludge with crude glycerol for oleaginous microorganisms' cultivation, needs to be investigated.

In this study, wastewater sludge and crude glycerol were used together for oleaginous yeast *Trichosporon oleaginosus* cultivation. In this research, the fermentation process was simplified by using a new wastewater sludge pre-treatment method; obviating the sterilization of the fermenter and the medium; auto-controlling the substrate feeding during the fermentation. The aim was to achieve high lipid production from a simplified fermentation process using low-cost substrates, so as to reduce the cost. Mass and energy balance, greenhouse gas emission and cost estimation were performed to evaluate the feasibility of the new process for industrial practice.

8.4 Materials and methods

8.4.1 Materials

8.4.1.1 Wastewater sludge

In this study, secondary wastewater sludge from a local wastewater treatment plant (Communauté Urbain de Québec, Québec, Canada) was used. The secondary sludge was characterized using the methods presented in our previous research (Zhang et al., 2014c) and the characteristics were presented in Table. 8.1.

8.4.1.2 Crude glycerol

A crude glycerol solution from a biodiesel production industry in Quebec, Canada was used to fortify the wastewater sludge. The characterization of the crude glycerol was presented in our previous study (Chen et al., 2017) and its characteristics were presented in Table. 8.2.

8.4.1.3 Strain

In this study, the lipid producing strain was oleaginous yeast *Trichosporon oleaginosus (ATCC 20905)*.

8.4.2 Fermentation

8.4.2.1 Pre-culture

A loop full of *T. oleaginosus* colony was firstly inoculated in 100 mL sterile YPD medium (20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract). After 24 h of incubation at 30 °C and 180 rpm, the 100 mL pre-culture was transferred to another 900 mL YPD medium and incubated for another 24 h. The total 1 L YPD pre-culture was used for the 10 L fermenter inoculation.

8.4.2.2 Fermentation medium

Medium (sludge) pre-treatment: After collection of secondary wastewater sludge from the local municipal wastewater treatment plant, the sludge (98% water content) was stored overnight in a 4 °C cold room to let it settle down. After collecting the supernatant, the concentrated sludge was centrifuged. The supernatant was collected and united with

the one collected after gravity settling of the sludge, and then stored for further utilization to dilute sludge medium. The concentration of the sludge resulted from centrifugation was measured which was 196.74 g/L. Two 6 L flasks were used as a container to treat the sludge. In each flask, 1.5 L concentrated sludge (200 g/L) and 450 mL crude glycerol solution were filled. Thereafter, the two flasks with sludge and crude glycerol were covered and stored at room temperature for a day.

Medium preparation: One of the pre-treated medium (sludge in crude glycerol solution) in the 6 L flask was fortified with 32.32 g NH_4Cl, and the other was used as control (no NH_4Cl added), thereafter, the media were transferred into two 15 L fermenters (working volume of 10 L) each. The media in the fermenters were diluted to 9 L with the supernatant of the sludge gravity settling and centrifugation. Then, each fermenter was inoculated by 1 L of pre-culture prior to fermentation.

8.4.2.3 Fermentation operation

Methanol was removed from 4 L of crude glycerol solution by evaporation at 60 °C for 15 minutes. The obtained crude glycerol (methanol free) (pH around 11) was equally divided and transferred into two bottles, which were used to control the fermentation pH at pH 5±0.1. During the fermentation, the dissolved oxygen (DO) was controlled at DO>30% (v/v) by adjusting agitation rate and air flow rate. The temperature was maintained at 30 °C. The anti-foam was used to control the foam.

Samples (70 mL) were drawn at an interval of 12 h for sample analysis.

8.4.3 Analytical techniques

Biomass concentration was determined by centrifuging 30 mL of fermentation broth, discarding the supernatant, washing twice, collecting and drying the residue solids. After weighing the dry solid, the biomass concentration was the weight of the dried solid divided by 0.03 L.

The dried solids were used for lipid extraction with a mixture of chloroform and methanol (2:1 v/v) and 6 mL Zirconia beads (1 mm diameter) at room temperature. After lipid

extraction and chloroform evaporation, the lipid was weighted. Lipid concentration was the weight of lipid divided by 0.03 L.

The lipid content was the weight of lipid divided by the weight of the dried solids.

Glycerol concentration was determined using the method reported by Bondioli and Bella (Bondioli & Della Bella, 2005).

In this study, most of the soap was converted to free fatty acid (FFA) at the fermentation pH 5, thus, FFA concentration was determined instead of soap concentration. FFA was extracted from 50 mL fermentation broth with chloroform. After evaporating the chloroform, FFA was weighted. FFA concentration was the weight of FFA divided by 0.05 L. The details of the analysis techniques were presented in our previous research (Chen et al., 2017).

Since this study aimed to evaluate the feasibility of biodiesel production from lipid produced by *T. oleaginosus* in sludge and crude glycerol medium, but not to investigate how glycerol and FFA were used for lipid production, which has been well determined in our previous research (Chen et al., 2017). Therefore, the glycerol and soap concentrations were just measured at the beginning and the end of the fermentation but not during the fermentation.

The volume of methanol free crude glycerol solution used for pH control during fermentation was measured with a cylinder.

The lipid extracted from biomass was used to produce fatty acid methyl esters (FAMEs) with methanol at a methanol to lipid molar ratio of 6:1 and 1% H_2SO_4 (v/v methanol) as a catalyst at 50 °C for 12 h. The compositions of FAMEs was determined with Gas Chromatography linked to Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500) (Zhang et al., 2014a).

8.4.4 Cost estimation, energy balance and greenhouse gas emission

The biodiesel production facility was supposed to be installed in the local municipal wastewater treatment plant. Therefore, the wastewater sludge transportation was not taken into consideration for cost estimation, energy balance and greenhouse gas

emission. However, crude glycerol solution transportation was taken into account. The cost estimation, energy balance and greenhouse gas emission were calculated based on 80 tonnes dry sludge per day, which was the sludge production of the wastewater treatment plant in Quebec. The fermentation time and lipid production data were adopted from the experimental results of this study. The evaluation was from the collection of raw materials until the pure biodiesel was obtained. The mass balance of the biodiesel production from sludge and crude glycerol through fed-batch fermentation was performed prior to the energy balance and cost estimation.

8.4.4.1 Energy balance

Energy balance was the energy difference between the energy output and net energy input, which was the total energy input after taking out the energy credit. Energy input was from the direct energy (fuels, electricity and steam) input and indirect energy (chemicals) input. The energy contained in the produced biodiesel was considered as the only energy output; energy credit was the energy contained in the by-products of the process.

Total energy input = direct energy input + indirect energy input;

Direct energy input= energy in fuels + energy in electricity+ energy in steam;

Indirect energy input=energy consumed to produce the chemicals utilized in the process;

Energy output= energy contained in the produced biodiesel;

Energy credit= energy content of by-products;

Enet=ΣEin-ΣEcr; where Enet was net energy input; ΣEin was total energy input; ΣEcr was total energy credit

Ebalance = ΣEout – Enet; where Ebalance was the energy balance; ΣEout was the total energy output; Enet was net energy input.

8.4.4.2 Greenhouse gas emission

In this study, the greenhouse gas emission (GHG) of the process was due to GHG emission from the utilization of fuels, electricity, steam, chemicals, as well as the produced biodiesel, which will be combusted instead of diesel afterward. In this study, the

emission factors were from Intergovernmental Panel on Climate Change (IPCC). For instance, the global warming potential of CH_4 21 CO_2-eq which was 310 CO_2-eq for N_2O. GHG emission credit was also considered, which included the elimination of the treatment of sludge and crude glycerol due to that fact that they were used for biodiesel production but not landfilled (Zhang et al., 2013). The net GHG emission of the process was calculated as Net GHG emission= GHG emission - GHG emission credit.

8.4.4.3 Cost estimation

The cost of the biodiesel production from sludge and crude glycerol was estimated with the software SuperPro Designer in this study. The process started with the treatment of the raw materials and ended until the pure biodiesel was produced. The cost of biodiesel production was from the utilization of raw materials and utilities, the depreciation of equipment, labor employment, laboratory quality control/quality assurance (lab/QC/QA), and waste disposal (Chen et al., 2018a). The estimation information was summarized in Table. 8.3.

8.5 Results and discussion

8.5.1 Lipid production using *T. oleaginosus* cultivated with wastewater sludge and crude glycerol medium

In this study, wastewater sludge was pre-treated before being employed as basic fermentation medium. The main purpose of the pre-treatment was to kill most of the bacteria, which might inhibit the growth and lipid production of *T. oleaginosus*. Methanol concentration over 3% (w/v) was found fatal to most of the microorganisms (Chen et al., 1976; Chen et al., 2018b). Our previous research found that methanol concentration of 1.4% (w/v) could be used to control the contaminants and ensure the lipid production from *T. oleaginosus* in non-sterile conditions (Chen et al., 1976; Chen et al., 2018b). Thus, the wastewater sludge was centrifuged and concentrated to around 200 g/L. Then 1.5 L concentrated wastewater sludge (200 g/L) was mixed with 450 mL crude glycerol, which gave a methanol concentration of 7.2% (w/v). After 24 h of pre-treatment with crude glycerol, the mixture was transferred into the fermenter. After dilution and addition of pre-culture, the methanol concentration was 1.4% (w/v) at the beginning of the fermentation.

It was proved that nitrogen concentration of 1.02 g/L from NH_4Cl improved biomass and lipid production in our previous study (Chen et al., 2017). Even though the total nitrogen in the wastewater sludge was more than 1.77 g/L, the dissolved nitrogen was just around 0.2 g/L and the ammonia nitrogen was less than 0.1 g/L (Table. 8.1), thus it was supposed that the available nitrogen from wastewater sludge might not be enough for high biomass and lipid production. Therefore, 3.232 g/L NH_4Cl, which contributed around 1 g/L nitrogen, was added in one of the two fermentations and the experimental results were compared with the other fermentation without the addition of NH_4Cl.

The experimental results showed that the biomass production occurred in fermentations with and without the addition of NH_4Cl (Fig. 8.1). Comparing with the fermentation without the addition of NH_4Cl, biomass production in the fermentation with the addition of NH_4Cl was faster and higher. The highest biomass concentration from the fermentation with NH_4Cl was 55.33 g/L, which was 42.66 g/L in the fermentation without NH_4Cl (Fig. 8.1). The fermentation with NH4Cl also showed better lipid production and lipid content. The highest lipid concentration in the fermentation with NH_4Cl was 26.98 g/L with the lipid content of 48.77% (w/w) which was 17.88 g/L and 41.91% (w/w), respectively, in the fermentation without NH_4Cl (Fig. 8.1). The crude glycerol used for feeding was measured after fermentation. It was found that 811 mL methanol free crude glycerol was consumed for feeding in the fermentation with NH_4Cl which was 683 mL in the fermentation without NH_4Cl. It was found that 18.74% more methanol free crude glycerol was consumed in the fermentation with NH_4Cl which achieved 50.89% higher lipid production (from 17.88 g/L without NH_4Cl to 26.98 g/L with NH_4Cl, (26.98 -17.88)/17.88=50.89%). Thus, *T. oleaginosus* used the methanol free crude glycerol more efficiently in the fermentation with NH_4Cl addition. The lipid productivity in the fermentation with NH_4Cl was 26.98/96=0.28 (g/L/h), which was 17.88/86=0.21 (g/L/h) in the fermentation without NH_4Cl.

By converting the lipid to fatty acid methyl esters (FAMEs) and determining the composition of FAMEs, it was found that the purity of biodiesel was 97.9% (the rest was impurities such as methanol, salts, soaps, heavy metals, and residual fatty acids) with trans-esterification efficiency of 96% in the fermentation with NH_4Cl which was 98.3% and 95%, respectively, in the fermentation without NH_4Cl. The main compositions of FAMEs

were the long-chain fatty acids with 16 and 18 carbon atoms, which were preferable for biodiesel production (Fig. 8.2).

8.5.2 The energy balance of biodiesel produced from lipid accumulated by *T. oleaginosus* in wastewater sludge and crude glycerol medium

The experimental results showed that the lipid production in the fermentation with the addition of NH_4Cl was superior to the one without (Fig. 8.1), but there would be more energy gain without the addition of NH_4Cl as no extra energy required for chemical addition (the energy cost due to NH_4Cl addition was 159.88 GJ/batch) (Table. 8.5). Therefore, the energy balance in both the cases (with and without the addition of NH_4Cl) was evaluated. It was observed that the lipid production became stable after 60 h (Fig. 8.1), hence it was assumed that the fermentation would be stopped at 60 h fermentation. At 60 h, the biomass concentration, lipid content, and lipid concentration were 50.99 g/L, 43.77% (w/w), and 22.32 g/L in the fermentation with the addition of NH_4Cl and 40.66 g/L, 39.36% (w/w), and 16.00 g/L in the fermentation without the addition of NH_4Cl, respectively, and they were used in the energy balance evaluation.

Prior to the energy balance study, the mass balance of the process was performed. The mass balance was based on 80 tonnes dry sludge per day. As the fermentation time was 60 h (2.5 days), which suggested that 200 tonnes sludge (181.82 m^3) was used to cultivate *T. oleaginosus* in each batch, and the initial added crude glycerol in the fermentation was 300 m^3 for each case (with and without NH_4Cl addition), which made the initial total fermentation volume to 6 666.67 m^3 (Table. 8.4). During fermentation, the crude glycerol addition amount in the fermentation with and without NH_4Cl addition was 504.00 m^3 and 423.00 m^3, respectively. The amount of NH_4Cl added was 21.55 tonnes (3.232 g/L) in the fermentation with NH_4Cl addition. With the assumption of lipid extraction efficiency and trans-esterification efficiency of 95% and 95%, respectively, the pure biodiesel produced in the case of with and without NH_4Cl addition were 144.43 tonnes and 102.40 tonnes per batch, respectively. The detailed mass balance was given in Table. 8.4. According to the mass balance, the annual biodiesel production was 21 087.31 and 14 950.25 tonnes in the case of with and without NH_4Cl addition, respectively. It can meet

15% of the biodiesel demand of Quebec according to Natural Resource of Canada, which would be a great contribution in biodiesel production market.

The energy balance was calculated based on the mass balance. The information was given in details in Table. 8.5. The energy consumption was in the process of raw material transportation, fermentation, biomass harvesting, lipid extraction, trans-esterification, and biodiesel purification. For raw material transportation, it was the crude glycerol transporting from the biodiesel production industry (200 km) to wastewater treatment plant where the fermentation takes place as assumed in this study. The energy required for crude glycerol (density: 0.877 tonne/m^3) transportation was 3.47 kJ/kg/km with a cost of 0.3 $/ton/km (Pimentel & Patzek, 2005). Thus, the energy input due to the raw material transportation was 558.0 GJ/batch (or 2.7 GJ/ tonne of dry sludge).

The water required in fermentation to dilute medium was the water containing in the initial sludge collected from wastewater treatment plant, therefore, no energy content was considered in the utilization of the water. The wet sludge was considered no energy content as it was a waste. Pure glycerol has an energy content of 16.71 MJ/kg; however, the energy value of pure glycerol while diluted from 100% to 13.2% purity is reduced approximately by 14.50 MJ/kg (Duque, 2011). Therefore, the energy content of the crude glycerol used in this study was assumed to be 2.21 MJ/kg. In addition, the utilization of NH_4Cl also caused energy input. During fermentation, agitation (0.35 kWh/m^3) and aeration (2 VVM) were performed, and hence there was energy input from the utilization of electricity. The total energy input in the fermentation was 1742.83 GJ/batch.

During biomass harvesting, the energy input was from the pumping and centrifugation. Moreover, there was wastewater (supernatant) generation. It was assumed that the wastewater had a chemical oxygen demand (COD) of 200 mg/L. The energy consumption to treat kg COD was around 2 kWh (Gil-Carrera et al., 2013). Therefore, there was consideration of energy input from the treatment on the wastewater. The total energy input of the biomass harvesting was 267.27 GJ/batch.

The extraction was performed with wet biomass (80% water content), the energy input was from the energy used in mixing, centrifugation, and the mass loss of chloroform and methanol. After extraction, evaporation was employed for solvent recovery. The total

energy input of lipid extraction and recovery was 885.13 GJ/batch (Table. 8.5). The residual biomass was assumed to be transported 20 km away for disposal.

In trans-esterification, methanol was consumed with a ratio of 96 g methanol/kg biodiesel. NaOH as a catalyst was added in a basis of 10 g/kg lipid. Mixing (0.03 kWh/kg biodiesel) and heating (0.24 KJ/kg biodiesel) also contributed the energy input (Batan et al., 2010). The total energy input of trans-esterification was 348.80 GJ/batch.

Water washing was used to purify the biodiesel and the energy consumption 0.49 GJ/batch. In this study, only biodiesel purification was performed and crude glycerol was left as a mixture. The biodiesel (37.8 MJ/kg biodiesel) and crude glycerol produced per batch were 144.43 and 22.99 tonnes. Hence, the energy output (biodiesel generation) was 5459.59 GJ/batch. The crude glycerol (glycerol content of 63%; energy content 10.50 MJ/kg) production was considered as credit. In addition, the sludge was used for lipid production instead of sending to landfill in this study, hence there was energy credit due to the elimination of sludge transport to a landfill. The total energy credit was 310.75 GJ/batch. The net energy was the difference between the total energy input and energy credit, thus it was 3565.90 GJ/batch (200 tonnes dry sludge). Therefore, the energy balance (energy output-net energy input) was 1893.69 GJ/batch, which led an energy ratio of 1.53. It suggested that biodiesel production from sludge and crude glycerol with the addition of NH_4Cl in the way suggested in this study was an energy gain process.

The energy balance of biodiesel production from sludge and crude glycerol without the addition of NH_4Cl was calculated in a similar way as the one with the addition of NH_4Cl, it was found that 102.40 tonnes biodiesel (energy output: 3870.68 GJ/batch) would be produced and the energy balance and energy ratio were 910.85.44 GJ/batch and 1.31, respectively (Annex 2). The study showed that biodiesel production from sludge and crude glycerol with the addition of NH_4Cl had more energy gain (2395.89 GJ/batch) compared to the one without (1362.44 GJ/batch) though there was energy input due to the addition of NH_4Cl. It was due to the higher biodiesel yield with the addition of NH_4Cl compared to the one without. It indicated that it was more favorable to produce biodiesel from sludge and crude glycerol with the addition of nitrogen source in terms of energy balance.

8.5.3 Greenhouse gas emission of biodiesel produced from lipid accumulated by *T. oleaginosus* in wastewater sludge and crude glycerol medium

In this study, CO_2, CH_4, and N_2O were accounted for GHG emissions. They were from the consumed energy and materials, which includes the use of fuels, electricity, and chemicals (Alabi et al., 2009). According to Intergovernmental Panel on Climate Change (IPCC), the global warming potentials of CH_4 and N_2O were 21 CO_2-eq and 310 CO_2-eq, respectively, and employed in this study. The calculation was based on the emission coefficients of materials, electricity, and fuels, which were presented in Table. 8.6. Based on the mass balance results, the GHG emission of the biodiesel production from sludge and crude glycerol with and without NH_4Cl addition was estimated. The result was shown in Table. 8.7.

The GHG emission of crude glycerol employed for lipid production and generated from the biodiesel production with lipid was calculated based on their composition as well as the fraction of each component (Tables 2, 5, and 6). Thus, the crude glycerol used in this study was 5471.39 kg CO_2/tonne crude glycerol and the crude glycerol produced in this study was 8898.29 kg CO_2/tonne. Owing to the reduction of GHG emission from biodiesel, the total GHG emission with NH_4Cl addition was -3355.30 tonnes CO_2/batch (tonnes/year). It suggests that it is a GHG emission sequestration process. Utilization of sludge, crude glycerol, and NH_4Cl for lipid production and then producing biodiesel reduced the carbon dioxide emission.

A similar calculation was conducted to evaluate the GHG emission from the biodiesel production from sludge and crude glycerol without NH_4Cl addition, it was found that the net GHG emission was -3984.09 tonnes CO_2/batch. It shows that the one without NH_4Cl addition provided higher CO_2 sequestration compared to that with NH_4Cl. It was attributed to the less residual biomass production in the one without NH_4Cl addition compared to the one with NH_4Cl addition, and residual biomass has the highest CO_2 coefficient (residual biomass was landfilled).

8.5.4 Cost estimation of biodiesel produced from lipid accumulated by *T. oleaginosus* in wastewater sludge and crude glycerol medium

The cost estimation was accomplished after mass balance and 80 tonnes sludge per day (2.5 days per batch) was used for cost estimation, which was same as energy balance. The process diagram for cost estimation was shown in Fig. 8.3. The cost summary of the process with the addition of NH_4Cl addition was shown in Table. 8.8.

It was found that the unit production cost of biodiesel, produced from lipid with the addition of NH_4Cl, was 0.63 $/kg. The current retail price of biodiesel was 0.90 $/kg. The gross margin of the biodiesel produced from lipid was 30.00 % and the payback time was 4.38 years. Thus, it was concluded that the biodiesel production from lipid accumulated by *T. oleaginosus* in wastewater sludge and crude glycerol with the addition of NH_4Cl was economically feasible for industrial practice. However, the unit production cost of biodiesel, produced from lipid without the addition of NH_4Cl, was 0.86 $/kg, which was very close to the retail price of 0.90 $/kg. The experiment without NH_4Cl addition had almost the same investment and operation cost as that with NH_4Cl addition; however, the biodiesel production from the experiment without NH_4Cl addition was much less than that with NH_4Cl addition and the cost of NH_4Cl addition was negligible compared with the investment and the operation cost (details in ANNEX 4). Hence, the biodiesel production cost, which was estimated by employing the experiment without NH_4Cl, was much higher than with NH_4Cl.

8.6 Conclusion

With the addition of nitrogen from NH_4Cl in the sludge and crude glycerol medium, lipid production using oleaginous yeast *T. oleaginosus* was significantly improved. The experimental results were employed for energy balance, greenhouse gas emission and cost estimation. A large amount of net energy gain, high reduction of greenhouse gas emission and acceptable economic feasibility were found and proved that biodiesel production from lipid accumulated by *T. oleaginosus* in the sludge and crude glycerol medium with the addition of NH_4Cl could be considered for industrial practice.

8.7 Acknowledgments

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Table 8. 1 The characteristics of the secondary wastewater sludge

Characteristics	Concentration
Total solids (TS)	28.43 ± 0.47 g/L
Total suspended solids (TSS)	21.23 ± 0.25 g/L
Volatile solids (VS)	18.76 ± 0.56 g/L
Total carbon (TC)	11.06 ± 0.11 g/L
Dissolved organic carbon (DOC)	1.46 ± 0.08 g/L
Total nitrogen (TN)	1.77 ± 0.14 g/L
Total dissolved nitrogen (TDN)	0.20 ± 0.05 g/L
Dissolved ammonia nitrogen (DAN)	89.78 ± 4.23 mg/L
Total phosphorous (TP)	0.31 ± 0.06 g/L
Total dissolved phosphorous (TDP)	92.20 ± 1.87 mg/L

Table 8. 2 The characteristics of the crude glycerol solution

Characteristics	Concentration
Density	0.877±0.05 (g/mL)
pH	8.32
Methanol	311.4±2.02 g/L
Soap	235.8±1.53 g/L
Glycerol	132.4±0.63 g/L
Water	103.7±0.81 g/L
Others	31.4±0.10 g/L

Table 8. 3 Basic information of the study

Items	Description
Capacity	80 tonnes dry sludge per day
Plant location	In-situ of wastewater sludge treatment
Construction period	2 to 3 months
Project life time	10 years
Production level in the 10 years	100%
Income tax	40%
The plant operation hour	7866 h
Equipment price	Obtained from manufacturer
Chemical price	Built-in mode
Labor	23.00 US $/h
Electricity	0.06 US $/kWh
Steam	12 US $/tonne
Cooling water	0.10 US $/tonne
Chilled water	0.40 US $/tonne

Table 8. 4 Mass balance of biodiesel production from lipid produced with sludge and crude glycerol per batch

Process	Materials	Fermentation with NH_4Cl	Fermentation without NH_4Cl
		Amount (tonne/batch)	**Amount (tonne/batch)**
Fermentation	Sludge (dry basis, tonne/m^3)	200.00/181.82	200.00/181.82
	Crude glycerol (initial, m^3)	300.00	300.00
	Crude glycerol (added during fermentation, m^3)	504.00	423.00
	Water (m^3)	6184.85	6184.85
	NH4Cl (tonne)	21.55	0.00
	Final fermentation broth (m^3)	7170.67	7089.67
Biomass harvesting	Fermentation broth (m3)	7170.67	7089.67
	Biomass concentration (g/L)	50.99	40.66
	Wet biomass (80% water content, tonne)	1828.16	1441.33
	Wastewater	5342.51	5648.34
Lipid extraction	Wet biomass (80% water content, tonne)	1828.16	1441.33
	Chloroform (m3)	2742.24	2161.99
	Methanol (m3)	1828.16	1441.33
	Lipid obtained (95% recovery efficiency, tonne)	152.04	107.79
Solvent recovery	Chloroform loss (m3)	1.37	1.08
	Methanol loss (m3)	0.91	0.72
	Recovered chloroform (m3)	2740.87	2160.91
	Recovered methanol (m3)	1827.25	1440.61
Trans-esterification	Lipids (tonne)	152.04	107.79
	Methanol (tonne)	13.87	9.83
	NaOH	1.52	1.08
	Crude biodiesel (95% trans-esterification efficiency, tonne)	152.04	107.79
	Crude glycerol	22.99	16.30

Biodiesel purification	Water (50 °C)	1.52	1.08
	Pure biodiesel (tonne)	144.43	102.40

Table 8. 5 Energy balance of biodiesel production from lipid produced with sludge, crude glycerol, and NH_4Cl per batch (Ref. (Zhang et al., 2013))

Process	Materials	Mass	Energy content	Energy (GJ/batch)
Raw material transportation	Crude glycerol (m3) Crude glycerol (tonne)	804.00 705.11	3.47 MJ/tonne/km	558.0
Fermentation	Sludge (dry basis, tonne/m^3)	200.00/181.82	0.00 (MJ/tonne)	0.00
	Crude glycerol (initial, m^3)	300.00/263.10 tonne	2.21 (MJ/tonne)	581.45
	Crude glycerol (added during fermentation, m^3)	504.00 m^3/442.01 tonne	2.21 (MJ/tonne)	976.84
	Water (m^3)	6221.75	0.00 (MJ/tonne)	0.00
	NH4Cl (tonne)	21.55	7.42 (MJ/tonne)	159.88
	Agitation	7170.67 m^3	0.35 kWh/m^3 (1 kWh=3600 MJ)	9.04
	Aeration	7170.67 m^3	2 VVM; 4.26 kg air/kWh	15.63
	Total			1742.83
Biomass harvesting	Pumping	7170.67 m^3	32.6 KJ/m^3	233.76
	Centrifugation	7170.67 m^3	1 kWh/m^3	25.81
	Wastewater treatment	5342.51 m^3	2 kWh/kg COD	2.69
	Total			267.27
Lipid extraction and solvent recovery	Mixing	6398.57 m^3	0.35 kWh/m^3	8.06
	Centrifugation	6398.57 m^3	1 kWh/m^3	23.03
	Evaporation	6398.57 m^3	50 kWh/m3	822.67
	Chloroform loss	1.37 m3	7.625 MJ/kg	15.47
	Methanol loss	0.91 m^3	22 MJ/kg	15.89
	Total			885.13
Trans-esterification	Methanol	13.87 tonnes	22 MJ/kg	305.04
	NaOH	1.52 tonnes	18.5 MJ/kg	28.13
	Mixing	144.43 tonnes biodiesel	0.03 kWh/kg biodiesel	15.60
	Heating	144.43 tonnes biodiesel	0.24 kJ/tonne biodiesel	0.03
	Total			348.80

Biodiesel purification	Water required	1.52 m3	0.04 MJ/m3	0.06
	Heating water from 15 °C to 50 °C (tonne)	1.52 m3	1 cal for 1 g water heated for 1 celsius degree	0.22
	Mixing	165.65 m3	0.35 kWh/m^3	0.21
	Total			0.49
Residual biomass transport for disposal	Residual biomass (80% water content)	1068	20 km away, 3.47 MJ/tonne/km	73.82
Total energy input for biodiesel production				3802.51
Energy credit from crude glycerol production		22.99 tonnes	10.50 MJ/kg	241.35
Energy credit from the avoidance of sludge transportation		1000 tonnes	20 km away, 3.47 MJ/tonne/km	69.40
Total energy credit				310.75
Net energy input				3565.88
Energy output	Biodiesel produced	144.43 tonnes	37.80 MJ/kg	5459.59
Energy balance				1893.71
Energy ratio				1.53

Table 8. 6 Emission coefficients of materials, electricity, and fuels (Ref. (Zhang et al., 2013))

Substances	Emission coefficients (kg CO2-equivalent)
NH4Cl (/tonne)	911
Methanol (tonne)	745.54
Glycerol (/tonne)	1.66
Lipid (/tonne)	25560
Diesel vehicle (/km/tonne)	0.11
Electricity (/kWh)	0.0014
Glycerol (/tonne)	1.66
Wastewater (/kg COD)	0.9
Sludge landfill (/tonne)	29400
Biodiesel (/tonne)	2830
Diesel (/tonne)	3750

Table 8. 7 GHG emissions of biodiesel production from sludge and crude glycerol with the addition of NH_4Cl

Items	Quantity	Emission coefficient (kg CO_2-equivalent)	Emission (tonne CO_2/ batch)
Materials			
NH_4Cl	21.55 tonnes	911.03 per tonne	19.63
Methanol	13.87	745.54	10.34
Electricity	320 578.50 kWh	0.0014 per kWh	0.45
Diesel	tonnes 20 km	0.11 per km per tonne	2.02
Biodiesel	144.43 tonne	2830 per tonne	408.75
Crude glycerol generated	22.99 tonnes	8898.29 per tonne	204.55
Residual biomass decomposing	213.60 tonnes	29400 per tonne	6279.75
Wastewater treatment	5342.51 m3, 200 mg COD/L	0.9 kg COD	0.96
Total GHG emission			6926.45
Credit			
Sludge transportation to landfill avoidance	1000 tonnes (80% water content)	0.11 per km per tonne	2.20
Sludge landfilling decomposing avoidance	200	29400 per tonne	5880.00
Crude glycerol utilized for lipid instead of landfill	705.11 tonnes	5471.39 per tonne	3857.92
Biodiesel replacing diesel	144.43 tonne	3750 per tonne	541.63
Total GHG emission credits			10281.75
Net GHG emission			-3355.30

Diesel utilization included crude glycerol transportation from industry to biodiesel production site and residual biomass transportation from biodiesel production site to landfill.

Table 8. 8 Summary of the cost estimation of biodiesel production from sludge and crude glycerol with the addition of NH_4Cl

Total Capital Investment	23,254,000 $
Operating Cost	12,596,000 $/yr
Revenues	18,049,000 $/yr
Cost Basis Annual Rate	20,054,024 kg MP/yr
Net Unit Production Cost	0.63 $/kg
Unit Production Revenue	0.90 $/kg
Gross Margin	30.00 %
Return On Investment	22.82 %
Payback Time	4.38 years

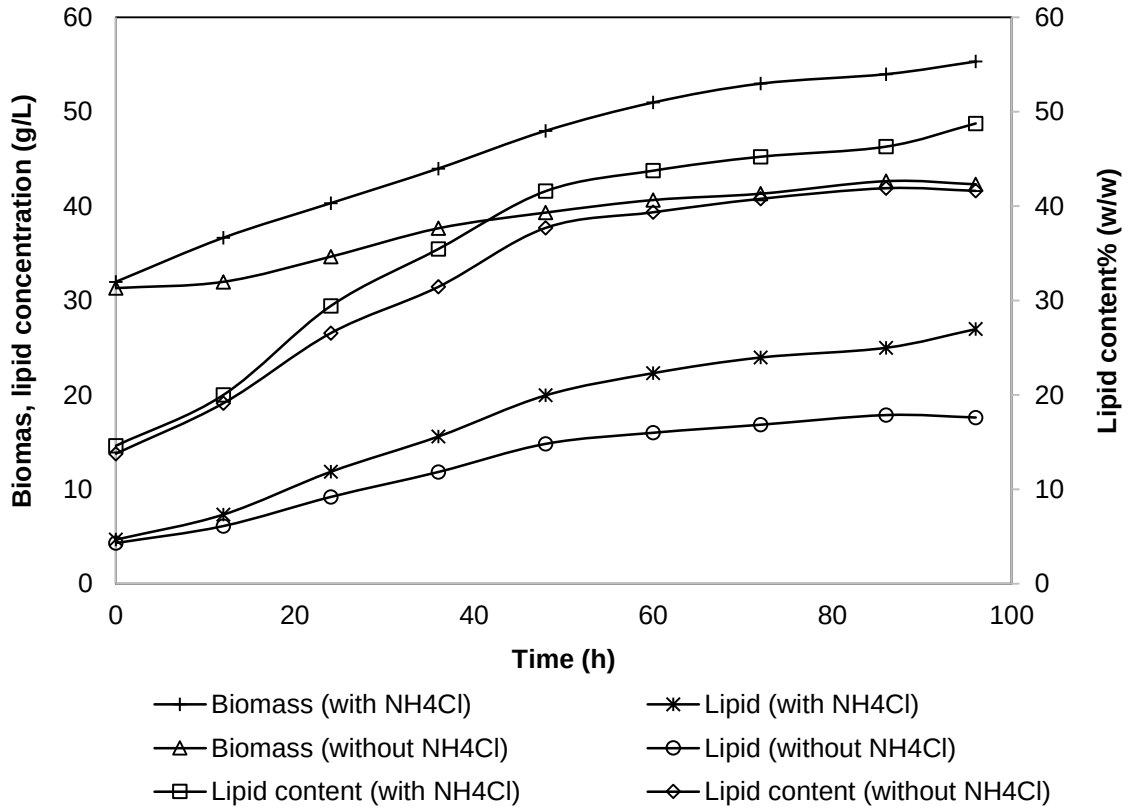

Figure 8. 1 Lipid production from wastewater sludge and crude glycerol using *T. oleaginosus*

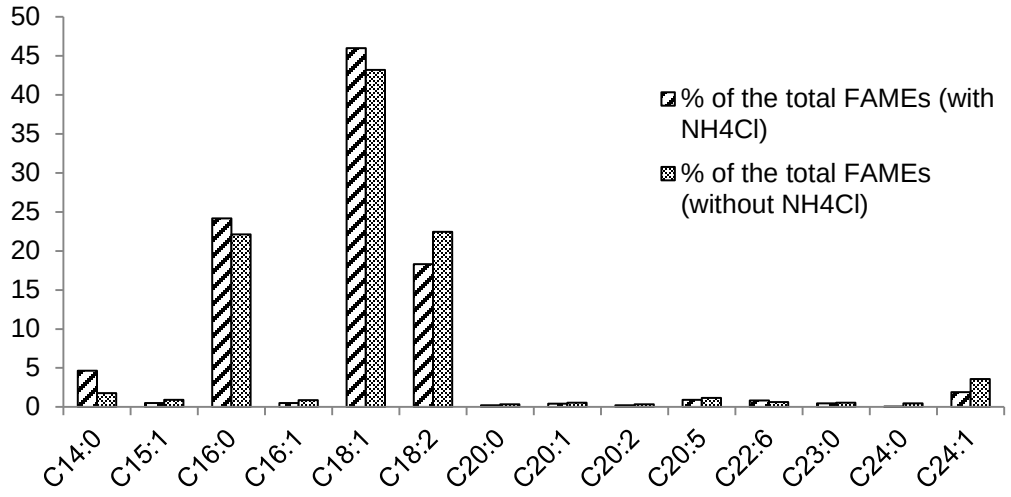

Figure 8. 2 Fatty acid compositions of FAMEs

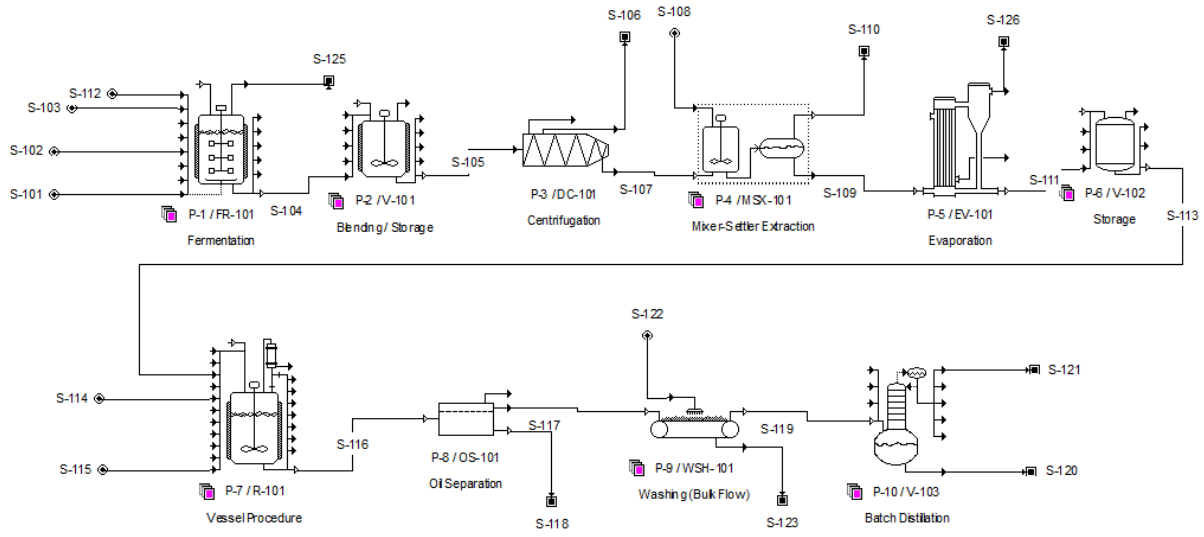

Figure 8. 3 The process diagram of biodiesel production from sludge and crude glycerol

ANNEXES

ANNEXE 1: Mass balance of biodiesel produced from lipid accumulated by *T. oleaginosus* in wastewater sludge and crude glycerol medium

	with addition of NH_4Cl	Without NH_4Cl
sludge （tonne/d）	80.00	80.00
fermentation time (h)	60.00	60.00
fermentation time (d)	2.50	2.50
total sludge per batch	200.00	200.00
sludge con. (g/l）	30.00	30.00
sludge con. (kg/m3）	30.00	30.00
sludge density (1.1 kg/L)	1.10	1.10
sludge volume (m3)	181.82	181.82
fermentation volume (m3)	6666.67	6666.67
Water (m3)	6184.85	6184.85
crude glycerol initial con (L/L fermentation volume)	0.05	0.05
crude glycerol initial volume required (m3)	300.00	300.00
crude glycerol addition during fermentation (L/L fermentation volume)	504.00	423.00
total crude glycerol addition (m3)	804.00	723.00
final fermentation volume (m3)	7170.67	7089.67
NH4CL con. (g/L)	3.23	0.00
total NH4Cl (tonne)	21.55	0.00
Biomass concentration (g/L)	50.99	40.66
wet biomass 80% water (tonne)	1828.16	1441.33
wastewater generated due to centrifugation (m3)	5342.51	5648.34
Total dry biomass (tonne)	365.63	288.27
lipid content (%)	43.77	39.36

lipid (tonne)	160.04	113.46
chloroform required (1.5 times of wet biomass) m3	2742.24	2161.99
methnaol required (1 times of wet biomass)m3	1828.16	1441.33
chloroform loss m3	1.37	1.08
methanol loss m3	0.91	0.72
recovered chloroform (m3)	2740.87	2160.91
recovered methanol(m3)	1827.25	1440.61
lipid extraction efficiency (%)	95.00	95.00
lipid extracted (tonne)	152.04	107.79
Trans-esterification efficiency (%)	95.00	95.00
crude biodiesel (tonne)	152.04	107.79
biodiesel produced (tonne)	144.43	102.40
methonal required in trans-esterification (96 g/kg biodiesel)tonne	13.87	9.83
NaOH 10 g per kg lipid (tonne)	1.52	1.08
crude glycerol (tonne)	22.99	16.30
water for biodiesel washing (m3)	1.52	1.08
biodiesel yield per year (tonne)	21087.31	14950.25

ANNEXE 2: Energy balance of biodiesel produced from lipid accumulated by *T. oleaginosus* in wastewater sludge and crude glycerol medium

	Mass amount		energy content (GJ/tonne)	energy input (GJ)	
	With NH_4Cl	**Without NH_4Cl**		**With NH_4Cl**	**Without NH_4Cl**
transportation					
crude glycerol amount (density 0.877 tonne/m3)tonne	804.00	723.00			
distance of transportation km	200.00	200.00			
energy consumption (3.47 MJ/tonne/km)	3.47	3.47			
Total				558.0	501.8
fermentation					
sludge （tonne/d）	80.00	80.00	0		
fermentation time (h)	60.00	60.00			
fermentation time (d)	2.50	2.50			
total sludge per batch	200.00	200.00	0	0.00	0.00
sludge con. (g/l）	30.00	30.00			
sludge con. (kg/m3）	30.00	30.00			
sludge density (1.1 kg/L)	1.10	1.10			
sludge volume (m3)	181.82	181.82			
fermentation volume (m3)	6666.67	6666.67			
Water (m3)	6221.75	6221.75	0	0.00	0.00
crude glycerol initial con (L/L fermentation	0.05	0.05			

volume)(1000 MJ/tonne)					
crude glycerol initial volume required (m3)(1000 MJ/tonne)	263.10	263.10	2.21	581.45	581.45
crude glycerol addition during fermentation (L/L fermentation volume)(1000 MJ/tonne)	442.01	370.97	2.21	976.84	819.85
total crude glycerol addition (m3)	804.00	723.00			
final fermentation volume (m3)	7170.67	7089.67			
NH4CL con. (g/L)	3.23	0.00			
total NH4Cl (tonne)	21.55	0.00	7.42	159.88	0.00
agitation 0.35 kWh/m3			0.35	9.04	8.93
aeration (2 VVM. 4.26 kg air/kWh, air density 1.29 kg/m3)	18500.32			15.63	15.46
Total energy input from fermentation				1742.83	1425.69
Cell harvestinbg					
pumping (kJ/m3)			32.6	233.76	231.12
centrifugation (1 kWh/m3)			1	25.81	25.52
Total energy from biomass harvesting				267.27	264.78
Biomass concentration (g/L)	50.99	40.66			
Lipid extraction					
total volume	6398.57	5044.65			
mixing (0.35 kwh/m3)			0.35	8.06	6.36
centrifugation (1 kWh/m3)			1	23.03	18.16
wet biomass 80% water (tonne)	1828.16	1441.33			

wastewater generated due to centrifugation (m3)	5342.51	5648.34	2	7.69	8.13
Total dry biomass (tonne)	365.63	288.27			
lipid content (%)	43.77	39.36			
lipid (tonne)	160.04	113.46			
chloroform required (1.5 times of wet biomass) m3	2742.24	2161.99			
methnaol required (1 times of wet biomass)m3	1828.16	1441.33			
chloroform loss m3 (7.625 MJ/KG; density 1.48 kg/L)	1.37	1.08	7.625	15.47	12.20
methanol loss m3 (22 MJ/KG; density 0.79 kg/L)	0.91	0.72	22	15.89	12.53
recovered chloroform (m3)	2740.87	2160.91			
recovered methanol(m3)	1827.25	1440.61			
evaporation (50 kwh/m3)			50	822.67	648.60
Total energy input from extraction and evaporation				885.13	697.84
lipid extraction efficiency (%)	95.00	95.00			
lipid extracted (tonne)	152.04	107.79			
dry residual biomass (tonne)	213.60	180.48			
Trans-esterification					
Trans-esterification efficiency (%)	95.00	95.00			
crude biodiesel (tonne)	152.04	107.79			

biodiesel produced (tonne)	144.43	102.40	37.8	5459.59	3870.68
methonal required in trans-esterification (96 g/kg biodiesel)tonne	13.87	9.83	22	305.04	216.27
NaOH 10 g per kg lipid (tonne)	1.52	1.08	18.5	28.13	19.94
mixing (0.03 kwh/kg biodiesel)				15.60	11.06
Heating (50 C) per kg biodiesel			0.24	0.03	0.02
Total energy from trans-esterification				348.80	247.29
pure glycerol	14.44	10.24			
glycerol concent in crude glycerol	0.63	0.63			
crude glycerol (tonne)	22.99	16.30	10.49900794	241.35	171.11
purification					
water for biodiesel washing (m3)	1.52	1.08	0.04	0.06	0.04
water heating from 15 C to 50 C for biodiesel washing (m3) (1 Mcal will be consumed for 1 tonne of water increasing 1 celsius degree)				0.22	0.16
mixing (0.35 kwh/m3)	165.65	117.44		0.21	0.15
Total energy from purification				0.49	0.35
biodiesel yield per year (tonne)	21087.31	14950.25			
TOTAL ENERGY INPUT				380251	3137.71
Energy output (biodiesel produced)				5459.59	3870.68
total energy credit (crude glycerol+ residual biomass)				241.35	171.11

credit from sludge disposal to transport (water concent 80%)	67.98	97.61	3.47	4.72	(6.77)
net energy input				3565.88	2959.83
energy balance				1893.71	910.85
energy ratio				1.53	1.31
methanol evaporation from crude glycerol(m3)	166.32	139.59			
methanol evaporation from crude glycerol (tonne)	131.39	110.28	22	2890.64	2426.07
evaporation energy input			50	29.94	25.13

ANNEXE 3: Green house gas emission of biodiesel produced from lipid accumulated by *T. oleaginosus* in wastewater sludge and crude glycerol medium

ANNEXE 3.1 Green house gas emission (with NH_4Cl)

energy balance of fermentation with NH_4Cl	Mass amount	energy content (GJ/tonne)	energy input (GJ)	electricity	GHG from diesel (0.11 kg/km/tonne)	GHG from electricity (0.0014 kg/kwh)	material kg CO2 co-efficient/tonne	GHG from materials
transportation								
crude glycerol amount (density 0.877 tonne/m3)tonne	804.00							
distance of transportation km	20.00				1551.23			
energy consumption (3.47 MJ/tonne/km)	3.47							
Total			55.80					
fermentation								
sludge （tonne/d）	80.00	0						
fermentation time (h)	60.00							
fermentation time (d)	2.50							
total sludge per batch	200.00	0	0.00				29400	
sludge con. (g/l）	30.00							
sludge con. (kg/m3）	30.00							

sludge density (1.1 kg/L)	1.10							
sludge volume (m3)	181.82							
fermentation volume (m3)	6666.67							
Water (m3)	6221.75	0	0.00					
crude glycerol initial con (L/L fermentation volume)(1000 MJ/tonne)	0.05							
crude glycerol initial volume required (m3)(1000 MJ/tonne)	263.10	2.21	581.45					
crude glycerol addition during fermentation with NH4Cl case (L/L fermentation volume)(1000 MJ/tonne)	442.01	2.21	976.84					
total crude glycerol addition (m3)	804.00						5471.39	3857920.86
final fermentation volume (m3)	7170.67							
NH4CL con. (g/L)	3.23							
total NH4Cl (tonne)	21.55	7.42	159.88				911.03	19629.65
agitation 0.35 kWh/m3		0.35	9.04	2509.73		3.51		
aeration (2 VVM. 4.26 kg air/kWh, air density 1.29 kg/m3)			15.63	4342.79		6.07		

Total energy input from fermentation			1742.83					
Cell harvestinbg								
pumping (kJ/m3)		32.6	233.76	64934.37		90.90		
centrifugation (1 kWh/m3)		1	25.81	7170.667		10.03		
Total energy from biomass harvesting			267.27					
Biomass concentration (g/L)	50.99							
Lipid extraction								
total volume	6398.57							
mixing (0.35 kwh/m3)		0.35	8.06	2239.49		3.13		
centrifugation (1 kWh/m3)		1	23.03	6398.565		8.95		
wet biomass 80% water (tonne)	1828.16							
wastewater generated due to centrifugation (m3)	5342.51	2	7.69				0.9/kg COD	961.65
Total dry biomass (tonne)	365.63							
lipid content (%)	43.77							
lipid (tonne)	160.04							
chloroform required (1.5 times of wet biomass) m3	2742.24							
methnaol required (1 times of wet biomass)m3	1828.16							

chloroform loss m3 (7.625 MJ/KG; density 1.48 kg/L)	1.37	7.625	15.47					
methanol loss m3 (22 MJ/KG; density 0.79 kg/L)	0.91	22	15.89					
recovered chloroform (m3)	2740.87							
recovered methanol(m3)	1827.25							
evaporation (50 kwh/m3)		50	822.67	228520.2		319.92		
Total energy input from extraction and evaporation			885.13					
lipid extraction efficiency (%)	95.00							
lipid extracted (tonne)	152.04							
dry residual biomass (tonne)	213.60	1067.98			469.91		29400	627974 8.89
					2021.15			
Trans-esterification								
Trans-esterification efficiency (%)	95.00							
crude biodiesel (tonne)	152.04							
biodiesel produced (tonne)	144.43	37.8	5459.59				2830	408747.15
methonal required in trans-	13.87	22	305.04				745.54	10337.38

esterification (96 g/kg biodiesel)tonne								
NaOH 10 g per kg lipid (tonne)	1.52	18.5	28.13				1977.91	
mixing (0.03 kwh/kg biodiesel)			15.60	4333.00		6.06		
Heating (50 C) per kg biodiesel		0.24	0.03	9.62		0.013		
Total energy from trans-esterification			348.80					
pure glycerol	14.44							
glycerol concent in crude glycerol	0.63							
crude glycerol GHG CO2 co-efficiency	8898.29							
crude glycerol (tonne)	22.99	10.49	241.35				8898.29	204551.58
purification								
water for biodiesel washing (m3)	1.52	0.04	0.06					
water heating from 15 C to 50 C for biodiesel washing (m3) (1 Mcal will be consumed for 1 tonne of water increasing 1 celsius degree)			0.22	62.08		0.086		
mixing (0.35 kwh/m3)	165.65		0.21	57.97		0.081		
Total energy from purification			0.49	320578.5		448.80		

biodiesel yield per year (tonne)	21087.31							
TOTAL ENERGY INPUT			3300.33					
Energy output (biodiesel produced)			5459.59					
total energy credit (crude glycerol+ residual biomass)			241.35					
credit from sludge disposal to transport (water concent 80%)	67.98	3.47	4.72					
net energy input			3063.70					
energy balance			2395.89					
energy ratio			1.78					
methanol evaporation from crude glycerol(m3)	166.32							
methanol evaporation from crude glycerol (tonne)	131.39	22	2890.64					
evaporation energy input		50	29.94					
diesel							3750	541626.08
sludge transportation avoidance								
sludge landfill decompsing	200						29400	5880000
Total GHG emisison (kg)	6926446.28							

Total GHG emisison credit (kg)	1028 1746. 94							
net GHG emission (kg)	- 3355 300.6 5							
net GHG emission (tonne)	- 3355. 30							

ANNEXE 3.2 Green house gas emission (without NH_4Cl)

energy balance of fermentation with NH4Cl	**Mass amou nt**	**energy content (GJ/tonn e)**	**energ y input (GJ)**	**elect ricity**	**GHG from diesel (0.11 kg/km/to nne)**	**GHG from electrici ty (0.0014 kg/kwh)**	**material kg CO2 co-efficient /tonne**	**GHG from material s**
transportation								
crude glycerol amount (density 0.877 tonne/m3)tonne	723.00							
distance of transportation km	20.00				1394.95			
energy consumption (3.47 MJ/tonne/km)	3.47							
Total			50.18					
fermentation								
sludge （tonne/d）	80.00	0						
fermentation time (h)	60.00							
fermentation time (d)	2.50							
total sludge per batch	200.00	0	0.00				29400	
sludge con. (g/l）	30.00							
sludge con. (kg/m3）	30.00							
sludge density (1.1 kg/L)	1.10							

sludge volume (m3)	181.82							
fermentation volume (m3)	6666.67							
Water (m3)	6221.75	0	0.00					
crude glycerol initial con (L/L fermentation volume)(1000 MJ/tonne)	0.05							
crude glycerol initial volume required (m3)(1000 MJ/tonne)	263.10	2.21	581.45					
crude glycerol addition during fermentation with NH4Cl case (L/L fermentation volume)(1000 MJ/tonne)	370.97	2.21	819.85					
total crude glycerol addition (m3)	723.00						5471.39	3469249.72
final fermentation volume (m3)	7089.67							
NH4CL con. (g/L)	0.00							
total NH4Cl (tonne)	0.00	7.42	0.00				911.03	0
agitation 0.35 kWh/m3		0.35	8.93	2481.38		3.47		
aeration (2 VVM. 4.26 kg air/kWh, air density 1.29 kg/m3)			15.46	4293.74		6.01		
Total energy input from fermentation			1425.69					
Cell harvestinbg								
pumping (kJ/m3)		32.6	231.12	64200.87		89.88		
centrifugation (1 kWh/m3)		1	25.52	7089.66		9.92		

Total energy from biomass harvesting			264.78					
Biomass concentration (g/L)	40.66							
Lipid extraction								
total volume	5044.65							
mixing (0.35 kwh/m3)		0.35	6.36	1765.62		2.47		
centrifugation (1 kWh/m3)		1	18.16	5044.65		7.06		
wet biomass 80% water (tonne)	1441.33							
wastewater generated due to centrifugation (m3)	5648.34	2	8.13				0.9/kg COD	1016.70
Total dry biomass (tonne)	288.27							
lipid content (%)	39.36							
lipid (tonne)	113.46							
chloroform required (1.5 times of wet biomass) m3	2161.99							
methnaol required (1 times of wet biomass)m3	1441.33							
chloroform loss m3 (7.625 MJ/KG; density 1.48 kg/L)	1.08	7.625	12.20					
methanol loss m3 (22 MJ/KG; density 0.79 kg/L)	0.72	22	12.53					
recovered chloroform (m3)	2160.91							
recovered methanol(m3)	1440.61							
evaporation (50 kwh/m3)		50	648.60	180166.2		252.23		
Total energy input from			697.84					

extraction and evaporation								
lipid extraction efficiency (%)	95.00							
lipid extracted (tonne)	107.79							
dry residual biomass (tonne)	180.48	902.38			397.05		29400	530603 7.95
					1792.00			
Trans-esterification								
Trans-esterification efficiency (%)	95.00							
crude biodiesel (tonne)	107.79							
biodiesel produced (tonne)	102.40	37.8	3870.6 8				2830	289789. 02
methonal required in trans-esterification (96 g/kg biodiesel)tonne	9.83	22	216.27				745.54	7328.88
NaOH 10 g per kg lipid (tonne)	1.08	18.5	19.94				1977.91	
mixing (0.03 kwh/kg biodiesel)			11.06	3071 .96		4.30		
Heating (50 C) per kg biodiesel		0.24	0.02	6.82		0.0095		
Total energy from trans-esterification			247.29					
pure glycerol	10.24							
glycerol concent in crude glycerol	0.63							
crude glycerol GHG CO2 co-efficiency	8898.2 9							
crude glycerol (tonne)	16.30	10.49	171.11				8898.29	145020. 71
purification								
water for biodiesel washing (m3)	1.08	0.04	0.04					

water heating from 15 C to 50 C for biodiesel washing (m3) (1 Mcal will be consumed for 1 tonne of water increasing 1 celsius degree)			0.16	44.01		0.061		
mixing (0.35 kwh/m3)	117.44		0.15	41.10		0.057		
Total energy from purification			0.35	268206		375.48		
biodiesel yield per year (tonne)	14950.25							
TOTAL ENERGY INPUT			2686.12					
Energy output (biodiesel produced)			3870.68					
total energy credit (crude glycerol+ residual biomass)			171.11					
credit from sludge disposal to transport (water concent 80%)	97.61	3.47	6.77					
net energy input			2508.24					
energy balance			1362.44					
energy ratio			1.54					
methanol evaporation from crude glycerol(m3)	139.59							
methanol evaporation from crude glycerol (tonne)	110.28	22	2426.07					
evaporation energy input		50	25.13					
diesel							3750	383996.05

sludge transportation avoidance								
sludge landfill decompsing	200						29400	5880000
Total GHG emisison (kg)	5751360.76							
Total GHG emisison credit (kg)	9735445.78							
net GHG emission (kg)	-3984085.01							
net GHG emission (tonne)	-3984.08							

ANNEXE 4: Cost estimation of biodiesel produced from lipid accumulated by *T. oleaginosus* in wastewater sludge and crude glycerol medium

1. EXECUTIVE SUMMARY (2018 prices)

==========

Total Capital Investment 23,254,000 $

Capital Investment Charged to This Project 23,254,000 $

Operating Cost 12,596,000 $/yr

Credits 0 $/yr

Net Operating Cost 12,595,627 $/yr

Revenues 18,049,000 $/yr

Cost Basis Annual Rate 20,054,024 kg MP/yr

Unit Production Cost 0.63 $/kg MP

Net Unit Production Cost 0.63 $/kg MP

Unit Production Revenue 0.90 $/kg MP

Gross Margin 30.21 %

Return On Investment 22.82 %

Payback Time 4.38 years IRR (After Taxes) 16.48 %

NPV (at 7.0% Interest) 14,967,000 $ MP

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2018 prices)

==========

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
33 / 0 / 0	FR-101	Fermentor	35,000	1,155,000
125 / 0 / 0	V-101	Blending Tank	13,000	1,625,000
1 / 0 / 0	DC-101	Decanter Centrifuge	11,000	11,000
8 / 0 / 0	MSX-101	Mixer-Settler Extractor	42,000	336,000
1 / 0 / 0	EV-101	Evaporator	31,000	31,000
5 / 0 / 0	V-102	Receiver Tank	29,000	145,000

7 / 0 / 0	R-101	Stirred Reactor	71,000	497,000
1 / 0 / 0	OS-101	Oil Separator	12,000	12,000
23 / 0 / 0	WSH-101	Washer (Bulk Flow)	8,000	184,000
6 / 0 / 0	V-103	Batch Distillation Vessel	12,000	72,000
		Unlisted Equipment		1,006,000
			TOTAL	5,028,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2018 prices in $)

==========

3A. Total Plant Direct Cost (TPDC) (physical cost)

1. Equipment Purchase Cost 5,028,000

2. Installation 1,824,000

3. Process Piping 1,760,000

4. Instrumentation 2,011,000

5. Insulation 151,000

6. Electrical 503,000

7. Buildings 503,000

8. Yard Improvement 0

9. Auxiliary Facilities 2,011,000

TPDC 13,791,000

3B. Total Plant Indirect Cost (TPIC)

10. Engineering 3,448,000

11. Construction 1,379,000

TPIC 4,827,000

3C. Total Plant Cost (TPC = TPDC+TPIC)

TPC 18,618,000

3D. Contractor's Fee & Contingency (CFC)

12. Contractor's Fee 931,000

13. Contingency 1,862,000

CFC = 12+13 2,793,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)

DFC 21,410,000

4. LABOR COST - PROCESS SUMMARY

==========

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	23.00	22,660	521,183	100.00

5. MATERIALS COST - PROCESS SUMMARY

==========

Bulk Material	Unit Cost ($)	Annual Amount	Annual Cost ($)	%
sludge	0.000	973,357,810 kg	0	0.00
crude glycerol	0.000	102,948,356 kg	0	0.00
ammonia chlorid	0.080	3,146,379 kg	251,710	6.60
Air	0.000	2,494,541,106 kg	0	0.00
solvent mixutre	0.010	313,837,700 kg	3,075,609	80.65
Methanol	0.240	2,025,071 kg	486,017	12.75
Sodium Hydroxid	0.000	221,926 kg	0	0.00
Water	0.000	221,926 kg	0	0.00
TOTAL			3,813,337	100.00

6. VARIOUS CONSUMABLES COST (2018 prices) - PROCESS SUMMARY

==========

The consumables cost is zero.

7. WASTE TREATMENT/DISPOSAL COST (2018 prices) - PROCESS SUMMARY

==========

The total waste treatment/disposal cost is zero.

8. UTILITIES COST (2018 prices) - PROCESS SUMMARY

==========

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%

Std Power	0.100	25,718,229	kW-h	2,571,823	62.55
Steam	12.000	30,126	MT	361,510	8.79
Chilled Water	0.400	2,945,279	MT	1,178,112	28.65
TOTAL				4,111,444	100.00

9. ANNUAL OPERATING COST (2018 prices) - PROCESS SUMMARY

==========

Cost Item	**$**	**%**
Raw Materials	3,813,000	30.28
Labor-Dependent	521,000	4.14
Facility-Dependent	4,150,000	32.95
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	4,111,000	32.64
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	12,596,000	100.00

10. PROFITABILITY ANALYSIS (2018 prices)

==========

A. Direct Fixed Capital 21,410,000 $

B. Working Capital 773,000 $

C. Startup Cost 1,071,000 $

D. Up-Front R&D 0 $

E. Up-Front Royalties 0 $

F. Total Investment (A+B+C+D+E) 23,254,000 $

G. Investment Charged to This Project 23,254,000 $

H. Revenue/Credit Rates

S-118 (Credit) 3,273,816 kg /yr

S-121 (Main Revenue) 20,054,024 kg /yr

I. Revenue/Credit Price

S-118 (Credit) 0.00 $/1000 kg

S-121 (Main Revenue) 0.90 $/kg

J. Revenues/Credits

S-118 (Credit) 0 $/yr

S-121 (Main Revenue) 18,049,000 $/yr

1 Total Revenues 18,049,000 $/yr

2 Total Credits 0 $/yr

K. Annual Operating Cost (AOC)

1 Actual AOC 12,596,000 $/yr

2 Net AOC (K1-J2) 12,596,000 $/yr

L. Unit Production Cost /Revenue

Unit Production Cost 0.63 $/kg MP

Net Unit Production Cost 0.63 $/kg MP

Unit Production Revenue 0.90 $/kg MP

M. Gross Profit (J1-K2) 5,453,000 $/yr

N. Taxes (40%) 2,181,000 $/yr

O. Net Profit (M-N + Depreciation) 5,306,000 $/yr

Gross Margin 30.21 %

Return On Investment 22.82 %

Payback Time 4.38 years

ANNEXE 5: Materials Report

1. OVERALL PROCESS DATA

==========

Annual Operating Time 7,865.71h

Unit Production Ref. Rate 20,054,024.12kg MP

Batch Size 165,735.74kg MP

Recipe Batch Time 65.01h

Recipe Cycle Time 65.01h

Number of Batches per Year 121.00

2. STARTING MATERIAL REQUIREMENTS (per Section)

==========

Section	Starting Material	Active Product	Amount Needed (kg Sin/kg MP)	Molar Yield (%)	Mass Yield (%)	Gross Mass Yield (%)
Main Section	(none)	(none)	0.00	Unknown	Unknown	Unknown

3. BULK MATERIALS (Entire Process)

==========

Material	kg/yr	kg/batch	kg/kg MP
sludge	973,357,810	8,044,279.420	48.537
crude glycerol	102,948,356	850,812.860	5.134
ammonia chlorid	3,146,379	26,003.130	0.157
Air	2,494,541,106	20,616,042.201	124.391
solvent mixutre	313,837,700	2,593,700.000	15.650
Methanol	2,025,071	16,736.120	0.101
Sodium Hydroxid	221,926	1,834.100	0.011
Water	221,926	1,834.100	0.011
TOTAL	3,890,300,274	32,151,241.931	193.991

4. OVERALL COMPONENT BALANCE (kg/batch)

==========

COMPONENT	INITIAL	INPUT	OUTPUT	FINAL	OUT-IN

ammonia chlorid	0.000	26,003.130	0.000	0.000	- 26,003.130
biodiesel	0.000	0.000	165,735.737	0.000	165,735.737
Biomass	0.000	241,328.383	0.000	0.000	- 241,328.383
Chloroform	0.000	1,919,338.000	1,919,338.000	0.000	0.000
Fats	0.000	200,621.672	0.000	0.000	- 200,621.672
Glycerol	0.000	112,647.623	24,648.644	0.000	- 87,998.979
lipid	0.000	0.000	18,642.614	0.000	18,642.614
Methanol	0.000	956,041.245	675,182.073	0.000	- 280,859.172
Nitrogen	19,830.332	15,814,934.500	15,823,083.082	11,681.750	0.000
Oxygen	6,020.105	4,801,107.701	4,803,581.452	3,546.353	- 0.000
residual biomass	0.000	0.000	248,073.480	0.000	248,073.480
Sodium Hydroxid	0.000	1,834.100	1,834.100	0.000	0.000
Water	0.000	8,077,385.578	6,785,664.928	0.000	- 1,291,720.650
TOTAL	25,850.437	32,151,241.931	30,465,784.111	15,228.103	1,696,080.154