Université du Québec
Institut national de la recherche scientifique
Centre Eau Terre Environnement

L'accumulation de triacylglycérols à partir de déchets organiques : physiologie et performances des bioprocédés

Par

Sarra MAGDOULI

Thèse présentée pour l'obtention du grade de
Philosophiae doctor (Ph.D.) en sciences de l'eau

Jury d'évaluation

Président du jury et Examinateur interne	Prof. Simon Barnabé Université Québec à Trois-Rivières, Canada
Examinateur externe	Prof. Céline Vaneeckhaute Université Laval, Canada
Examinateur externe	Prof. Jean-Michel Lavoie Université Sherbrooke, Canada
Directeur de recherche	Prof. Jean-François Blais INRS-ETE, Canada
Co-directrice de recherche	Prof. Satinder Kaur Brar INRS-ETE, Canada

DÉDICACE

À ma famille

REMERCIEMENTS

L’ensemble des travaux présentés dans ce manuscrit a été réalisé aux laboratoires pour l'innovation scientifique et technologique de l'environnement (LISTE) de l’Institut national de la recherche scientifique à Québec.

Je tiens à remercier le professeur Jean-François Blais de m’avoir accueilli au sein de son équipe. Ces travaux ont été co-encadrés par la professeure Satinder Kaur Brar, codirectrice de thèse. Un grand merci pour leur disponibilité et leur encadrement scientifique offert avec attention et intérêt. Nos conversations ont été une source de richesse incomparable. Je les remercie aussi pour l’honneur qu’ils m’ont fait en acceptant de faire partie de ce jury.

Je remercie également le professeur Simon Barnabé pour m’avoir fait l’honneur de présider le jury de ma thèse, ainsi que d’en être l’examinateur interne.

Un grand merci aux professeurs Céline Vaneeckhaute et Jean-Michel Lavoie d’avoir accepté de juger mon travail et pour leurs remarques pertinentes sur le manuscrit et durant la soutenance.

Un autre grand merci à Mathieu Drouin, professionnel de recherche et responsable du laboratoire de biotechnologies environnementales du LISTE, pour son soutien psychologique et son aide pour démarrer mes fermentations.

Ma reconnaissance particulière aux « fermenteurs ».

De chaleureux remerciements à Pierre Tailleur pour son ouverture d’esprit et sa gentillesse. Mes remerciements vont également à Arnaud De Coninck, Nouha Klai, Tayssir Guedri, Tarek Rouissi et Rama Pulicharla pour l’aide qu’ils m’ont apportée lors de mes travaux de laboratoire. Je tiens à remercier également Mathilde Renaud pour avoir accepté de corriger mon travail.

Nouha, Karama, Leila et Tayssir, notre amitié est éternelle et les jours passés ensemble rendent plus agréable ma condition de thésarde. Merci pour la bonne humeur et la générosité des anciens collègues Manju, Shyam, Xiaolei, Ratul, Saurabh…, des collègues actuels, Tarek et Karima, ainsi que des futurs collègues Tayssir, Merhrdad, Mitra… Bon courage à vous tous! Merci aussi à Lise Rancourt et Stephane Moïse pour leur expertise précieuse en chromatographie et leur aide pour mes analyses.

Toute mon amitié et ma reconnaissance à l’ensemble du personnel de l’INRS et particulièrement à Suzanne Dussault pour son soutien psychologique. Mes dernières pensées, les plus essentielles, vont à mes parents qui ont fait qui je suis et à mon mari d’avoir toujours été présent et d’avoir supporté les nombreux chassés-croisés nuit/jour et, enfin, à ma fille Salma qui m’a donné le courage de poursuivre cette thèse.

RÉSUMÉ

L'épuisement des gisements pétroliers, les variations imprévisibles du coût du baril de pétrole et la prise de conscience environnementale de la nécessité de proposer des options de rechange aux ressources fossiles stimulent la recherche pour développer de nouveaux matériaux renouvelables. Dans ce contexte, la production de lipides constitutifs des triacylglycérols (TAG) à partir de microorganismes hétérotrophes constitue une source renouvelable intéressante à explorer. En outre, l'industrie de fabrication du biodiesel génère sous formes de rejets valorisables une partie de la matière première, le glycérol brut. Or, ce résidu peut être utilisé comme source de carbone par des microorganismes hétérotrophes pour la production de produits à valeur ajoutée, tel que le biodiesel. La présente étude se concentre sur l'accumulation des TAG à partir de glycérol brut par certains microorganismes, à savoir les levures. En fait, il s'agit d'optimiser les conditions de culture afin de maximiser le taux d'accumulation de lipides chez la levure *Yarrowia lipolytica* tout en développant ultérieurement une approche biochimique qui vise encore à augmenter le taux d'accumulation sans recours au génie génétique. La stabilité et la performance de la biogenèse des lipides dépendent de plusieurs facteurs, dont la concentration de glycérol, d'azote et le temps de fermentation.

Des souches de *Yarrowia* lipolytica ont été isolées d'un sol et sélectionnées par culture sur milieu de culture à base de glycérol brut. Un pourcentage de lipides dépassant 76% (en poids sec des cellules) dans du glycérol pur (à raison de 100 g L^{-1}) a été mesuré par culture de la souche la plus performante (souche SM7) en fermenteur. Ensuite, celle-ci a été testée avec différentes concentrations de glycérol brut dans un milieu synthétique. L'optimisation des conditions de culture en présence de glycérol brut a été étudiée tout en variant la concentration du glycérol, la concentration de la source d'azote, ainsi que le temps de fermentation. La croissance optimale de *Y. lipolytica* a été obtenue en présence de 89 g L^{-1} de glycérol brut avec un ratio C:N de 75. Un ajustement de ce ratio permet d'obtenir de plus grandes concentrations de lipides. La souche *Yarrowia lipolytica* SM7 a produit jusqu'à 52% de lipides dans du glycérol brut seul avec un apport d'hydroxide d'ammonium durant la phase de production de biomasse, suivi d'un maintien de la concentration en oxygène à 30% durant la phase de lipogenèse. L'oxygène semble jouer un rôle majeur dans l'accumulation de lipides et dans la détermination de la rhéologie de la culture et de la morphologie de la souche sélectionnée qui a un comportement pseudoplastique lors de

l'accumulation des lipides. Par ailleurs, au cours de ce travail, on a pu constater que l'acide citrique et les biomodulateurs, tels que la biotine et la leucine, jouent un rôle primordial dans la biogenèse des lipides permettant d'atteindre un taux de 63%. Ces paramètres ont été optimisés, ce qui a fait augmenter la concentration de lipides tout en stimulant la production d'acide citrique par le cycle de Krebs, et donc la quantité d'acides organiques impliqués. Ces travaux contribuent à accroître nos connaissances sur la compréhension de la fonction et du rôle métabolique des microorganismes au cours de l'accumulation et du stockage lipidique. Par ailleurs, la souche sélectionnée offre un grand potentiel pour la production de lipases extracellulaires à 38 U mL^{-1} dans du glycérol à 40 g L^{-1} et en présence de déchets, tels que des résidus de crustacés et de différents inducteurs. Ces enzymes pourraient ultérieurement être utilisés pour la dégradation de polluants organiques dans des sites contaminés. Finalement, la réaction de transestérification a été étudiée à l'aide de micro-ondes et la performance du procédé a été comparée avec celle de l'ultrasonication.

PUBLICATIONS DANS CETTE THÈSE

1. **Sarra Magdouli**, Song Yan, Rajeshwar Dayal Tyagi, Rao Y. Surampalli (2014) Heterotrophic microorganisms: a promising source for biodiesel production. *Critical Reviews in Environmental Science and Technology*, 44, 416-453.
2. **Sarra Magdouli**, Satinder Kaur Brar, Jean-Francois Blais, Rajeshwar Dayal Tyagi (2015) How to direct the fatty acid biosynthesis towards polyhydroxyalkanoates production?. *Biomass and Bioenergy*, 74, 268–279.
3. **Sarra Magdouli**, Satinder Kaur Brar, Jean-Francois Blais (2016) Co-culture for lipid production: advances and challenges. *Biomass and Bioenergy*, 92, 20–30.
4. **Sarra Magdouli**, Satinder Kaur Brar, Jean-Francois Blais (2016) Comparative study between microwave and ultrasonication aided in situ transesterification of microbial lipids. *RSC Advances*, 6, 56009-56017.
5. **Sarra Magdouli**, Satinder Kaur Brar, Jean-Francois Blais (2016) Lipid production by *Yarrowia lipolytica* grown on biodiesel-derived crude glycerol: optimization of growth parameters and their effects on the fermentation efficiency. *RSC Advances*, 6, 90547-90558.
6. **Sarra Magdouli**, Satinder Kaur Brar, Jean-Francois Blais (2016) Morphology and rheological behaviour of *Yarrowia lipolytica*: impact of dissolved oxygen level on cell growth and lipid composition. Article soumis à *Energy* (en révision).
7. **Sarra Magdouli**, Satinder Kaur Brar, Jean-Francois Blais (2016) Valorization of raw glycerol and crusteacen waste into value added products by *Yarrowia lipolytica*. Article soumis à *Engineering Life Sciences* (en révision).
8. **Sarra Magdouli**, Satinder Kaur Brar, Jean-Francois Blais (2016) Bio-modulator triggers and citric acid to improve lipid production by *Yarrowia lipolytica* on crude glycerol based media. Article soumis à *Applied and Environmental Microbiology* (en révision).

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES ABRÉVIATIONS

ARA	Arachidonic acid
AGL	Acide gras Libres
ACL	ATP citrate lyase
ACC	Acétyl CoA carboxylase
BDW	Biomass dry weight
BOD	Biological oxygen demand
cAMP	Cyclic AMP
CBP	Consolidated bioprocessing
CFPP	Cold filter plugging point
CN	Cetane number
CO_2	Carbon dioxide
COD	Chemical oxygen demand
DHA	Docohexaenoic acid
DNA	Desoxy-ribonucleic acid
EPA	Eicosapentaenoic acid
EPS	Extracellular polymeric substances
FFA	Free fatty acids
GHG	Greenhouse gases
GLA	ω-linolenic acid
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
IV	Iodine value
NMR	Nuclear magnetic resonance spectroscopy
PHA	Polyhydroxyalkanoates
SCO	Single cell oils
SCP	Single cell protein
SE	Sterol ester
TAG	Triacylglycerols
VAP	Value-added products

1 CHAPITRE 1: SYNTHÈSE

1.1 Partie I : contexte de l'étude et généralités sur la production de biodiesel à partir de glycérol brut – revue de la littérature

1.1.1 Introduction

L'industrie pétrochimique a connu son ampleur suite à l'invention du moteur à explosion (entre 1865 et 1885) et la production de véhicules automobiles. C'est ainsi que le pétrole est devenu la première matière minérale pour la production des polymères, des détergents, des adhésifs, des plastiques et des engrais. L'utilisation intensive de cette matière première, liée principalement à la croissance démographique galopante, a créé une dépendance des pays consommateurs vis-à-vis des pays producteurs qui a pour conséquence l'augmentation du prix du pétrole. Par ailleurs, le pétrole génère des rejets nocifs dans l'atmosphère lors de sa combustion qui ont un effet néfaste sur la santé humaine et l'environnement. Ces facteurs ont incité les instances politiques et les chercheurs à travailler, de façon urgente, à trouver des sources renouvelables alternatives au pétrole. Parmi les énergies non-renouvelables, les biocarburants conventionnels basées sur le charbon et le gaz naturel ont fait l'objet des plusieurs recherches. En 2009, 89% de l'énergie produite au Canada (15 ExaJoule) provient de sources non renouvelables (Statistique Canada, 2011). Le charbon peut être converti par gazéification en gaz de synthèse (CO, H_2) (Naveed et al., 2010), puis en carburant par la réaction de Fischer-Tropsch, tandis que le gaz naturel est converti par des procédés catalytiques (Dry, 1996).

Actuellement, le Canada occupe le cinquième rang mondial des producteurs et le quatrième plus grand exportateur de gaz naturel (Ressources naturelles Canada, 2015). Ces donnés laissent présager un bel anvenir pour le Canda en tant que producteur de gaz naturel et l'un des piliers de l'énérgie mondiale dans les prochaines années.

Toutefois, le retour au biodiesel, utilisé pour la première fois il y a plus d'un siècle par Rudolph Diesel (1858-1913), s'avère aussi l'une des solutions possibles. A l'époque, le diesel utilisait à de l'huile d'arachide comme matiere première pour le carburant (Shay & Griffin, 1993).

Actuellement, les biocarburants sont classifiés selon la provenance dont ceux-ci sont issus à savoir les biocarburants de première, deuxième ou troisième génération.

Les deux principaux carburants de première génération sont le bioéthanol, représentant 85 % du marché mondial des biocarburants et le biodiesel, représentant 15 % du marché (Tramoy, 2008). Généralement, le bioéthanol est produit par la fermentation de plantes riches en sucre et en amidon (maïs et canne à sucre). Il est utilisé comme additif à l'essence jusqu'à des proportions de 20 % ou de 85 % (Gonzales, 2008).

Les biocarburants de 2ème génération sont les biocarburants cellulosiques obtenus à partir de cultures non alimentaires (bois, feuilles, paille, etc.) (Demirbas, 2009).

Les biocarburants de 3ème génération sont des biocarburants produits avec des microorganismes (algues, levures, champignons) (Demirbas, 2009).

Au cours des dernières années, le coût de production de l'éthanol dérivé de la canne à sucre se situait à environ 0,35 à 0,50 dollar US par litre d'équivalent essence, tandis que celui du biodiesel issu des huiles végétales se situait de 0,70 à 1,00 dollar US par litre d'équivalent diesel (OCDE, 2007). Le coût de production du bioéthanol est compétitif à celui du pétrole pour des prix du baril variant de 40 $ à 60 $ (Schenk et al., 2008). Pour que le biodiesel soit compétitif, le prix du baril doit être de 100 $ (Schenk et al., 2008). Cependant, la production de biodiesel à partir d'huiles végétales a pratiquement atteint sa capacité maximale, ce qui ouvre le marché pour les biocarburants de deuxième et troisième génération (Schenk et al., 2008).

Les microalgues (algocarburants) ont reçu actuellement un grand intérêt à cause des avantages qu'elles offrent dont leur croissance extrêmement rapide puisque certaines espèces peuvent doubler leur biomasse de une à trois fois en 24 heures (Khan et al., 2009), l'importante capacité de production de biomasse par hectare, la fréquence de récolte très importante, la diminution de l'utilisation d'eau potable ainsi que celle d'émission de gaz à effet de serre (Chisti, 2007). Par ailleurs, les microalgues ont également la capacité d'absorber les polluants comme les phosphates et les nitrates (Cadoret et al., 2008).

Cependant, plusieurs problémes se posent au niveau de la récolte et l'extraction des lipides issus des microalgues. Pour le moment, les techniques utilisées sont coûteuses et l'efficacité de celles-ci dépend grandement de l'espèce microalgale utilisée. Des efforts de recherche devront être fournis afin d'identifier une technique peu coûteuse et efficace adaptée à une production à l'échelle industrielle. Toutefois, les levures et les champigons representent une alternative incontournable pour permettre de résoudre ses problémes.

Dans le monde, les plus gros producteurs de biodiésel se situent en Europe (Allemagne, France et Italie principalement). En effet, l'Union européenne est responsable de 90 % de la production mondiale de biodiesel qui peut être mélangé au gazole à des teneurs de 5 % à 30 % sans modifications du moteur (Tramoy, 2008).

Le Canada compte quelques usines de production de biodiesel à petite échelle qui ont une capacité de production totale d'environ 100 millions de litres par année. Une usine de grande taille d'une capacité de production de 225 millions de litres est présentement en construction en

Alberta. Cette usine produira du biodiesel à partir d'huile de canola. Au Québec, une seule usine est présentement en activité, soit l'usine de la société Rothsay qui produit 35 millions de litres de biodiesel par année à partir d'huiles de friture et de graisses animales. Une seconde usine appartenant à l'entreprise Bio-Diesel Québec à Saint-Alexis-des-Monts en Mauricie produit également du biodiesel à partir d'huiles usées (Alibeu, 2007). Cette dernière prévoit transformer 1,5 million de litres d'huiles usées (Centre de référence en agriculture et agroalimentaire du Québec, 2008).

En général, le biodiesel est produit par transestérification des huiles (triglycérides ou triacylglycérols, TAG) extraites de matières premières végétales (colza, tournesol, soja, etc.) (Muniyappa et al., 1996). Les TAG peuvent être aussi transestérifiés en esters alkyliques d'acides gras, offrant ainsi des propriétés physico-chimiques similaires à celles du pétrodiesel, qui pourront être utilisés dans un moteur classique sans modification (Knothe, 2010).

Toutefois, le biodiesel offre d'autres avantages et contribue efficacement à la réduction des gaz à effet de serre (GES), en effet, la production mondiale de biodiesel est en augmentation rapide et on prévoit qu'elle atteindra 42 milliards de litres d'ici 2021.

Dans ce contexte, le Canada, important producteur de biodiesel en Amérique du Nord, a mis en place plusieurs démarches environnementales qui visent à soutenir la capacité des producteurs de carburant à utiliser des sources d'énergie renouvelable. Le pays s'est fixé comme objectif la réduction des émissions totales de GES de 17% d'ici 2020 par rapport aux niveaux de 2005 afin d'atténuer les changements climatiques (RNCan, 2016, Règlement modifiant le Règlement sur les carburants renouvelables, de l'environnement canadien loi sur la protection, 1999, ministère de l'Environnement, Canada). Actuellement, le Règlement sur les carburants renouvelable*s*, publié le 1er septembre 2010, dans la *Gazette du Canada,* Partie II, exige des producteurs et importateurs de carburants que l'essence qu'ils produisent ou importent ait une teneur moyenne de 5% de carburant renouvelable (Règlement sur les carburants renouvelables, 2010). Le gouvernement canadien a annoncé, en juillet 2007, un investissement de 1,5 milliards $CAN (le programme écoÉNERGIE pour les biocarburants) sur neuf ans pour stimuler la production de biocarburants au Canada (Ressources Naturelles Canada, 2011a). Par ailleurs, en 2012, l'objectif était d'ajouter 2% de biodiesel dans le diesel (Ressources Naturelles Canada, 2011b). Dans le cas du bioéthanol ou des huiles estérifées, il est possible de les utiliser dans les véhicules routiers en quantités variables sans modification technique majeure des moteurs. Le biodiesel peut être utilisé pur à 100% (B100), contrairement à l'ajout de biodiesel au pétrodiesel qui se fait à des concentrations de 2% (B2), 5% (B5) et 10% (B10). Parmi les voies empruntées pour la production

de biodiesel, l'extraction d'huile de plantes oléagineuses avec ou sans transformation chimique est très commune.

Lors du processus de production du biodiesel, un grand volume de glycérol brut est généré comme sous-produit. Environ 10 kg de glycérol brut sont produits pour 100 kg de biodiesel (Garlapati et al., 2016; Kerr et al., 2007; Yazdani & Gonzalez, 2007) ce qui représente approximativement 10% du poids du biodiesel produit. Le glycérol brut est utilisé dans plusieurs secteurs industriels tels que l'alimentation, la pharmaceutique et les cosmétiques (Guerrero-Pérez et al., 2009).

Le glycérol brut est un substrat osidique riche en carbone, méthanol, eau, savon, sel, alcool, huiles et matières organiques solides. Il contient également des traces de catalyseur non utilisé au cours du processus de transestérification (Chi et al., 2007). L'étape finale de la production de biodiesel comprend une purification par lavage à l'eau chaude afin d'éliminer les résidus de catalyseur et autres impuretés. Toutefois, cette étape de purification est longue et coûteuse (Berrios & Skelton, 2008; Pachauri & He, 2006). Par conséquent, la valorisation de ce produit par un procédé biotechnologique, tel que la fermentation constitue une solution prometteuse pour résoudre les problèmes environnementaux liés au rejet de glycérol brut (da Silva et al., 2009).

Ainsi, l'étape de la purification pourrait être omise lors de la réutilisation et de la valorisation du glycérol brut. Parmi les voies de valorisation proposées, la production de différents produits à haute valeur ajoutée (PHVA), tels que le biodiesel, le 1,3-propanediol, l'éthanol et le biohydrogène, sont les plus cités dans la littérature (Abghari & Chen, 2014; da Silva et al., 2009; Sarma et al., 2012).

L'utilisation de ce sous-produit pour la croissance de microorganismes, dans le cadre d'une approche économique, simple et efficace, s'avère une des solutions les plus intéressantes. Néanmoins, l'accumulation de réserves lipidiques majoritairement constituées d'acides gras se fait principalement chez les cellules eucaryotes.

La production de triacylglycérols (TAG) à partir de glycérol brut à l'aide de microorganismes hétérotrophes dits oléagineux présente des avantages incontestables par rapport aux filières chimiques des huiles végétales. En effet, les microorganismes ont un temps de génération rapide, sont faciles à manipuler et leur culture n'est pas dépendante des conditions climatiques et saisonnières (Chisti, 2007). D'ailleurs, l'analyse de la composition des acides gras dérivant des microorganismes hétérotrophes suggère que les esters méthyliques et éthyliques des acides gras

présentent une composition similaire aux lipides extraits d'huiles végétales (Kraisintu et al., 2010; Li et al., 2007).

La production de lipides à l'aide de ces microorganismes n'est cependant pas une approche nouvelle. Plusieurs études ont été menées sur l'augmentation de la production des TAG ou HOU (huiles d'organismes unicellulaires) en utilisant des outils du génie génétique ou par une modélisation des conditions opératoires (Abghari & Chen, 2014; Blazeck et al., 2014; Kamzolova et al., 2011). Il a été établi que la production de lipides requiert une importante concentration en carbone et une carence nutritionnelle en azote (Chisti, 2007; Meng et al., 2009). Le contenu lipidique intracellulaire est fortement dépendant du rapport molaire initial C:N du milieu de culture utilisé. L'étape clé de l'accumulation lipidique est le clivage de l'acide citrique par l'enzyme ATP citrate lyase.

Compte tenu des contraintes climatiques et géopolitiques, la bioconversion du glycérol brut en TAG constitue une voie prometteuse de réduction de l'utilisation des carburants fossiles diminuant les émissions de GES et augmentant notre indépendance énergétique. Il est donc intéressant de développer un procédé biologique robuste permettant d'augmenter la production d'acides gras par les microorganismes hétérotrophes et de moduler le profil des acides gras en fonction des contraintes d'usage.

Les travaux de cette thèse ont pour objectif l'étude de l'accumulation des TAG chez une espèce de levure nouvellement isolée. Il s'agit de comprendre les mécanismes de synthèse et d'accumulation de lipides chez la levure *Yarrowia lipolytica* YM7 par une approche systémique. Basé sur la connaissance des travaux antérieurs et la mise en place d'un procédé de culture adapté, l'enjeu scientifique de ces travaux réside dans la maîtrise du comportement de la levure et de son métabolisme en condition d'accumulation de lipides à partir de substrats osidiques tel que le glycérol brut. La transposition et l'optimisation du procédé de culture vers la souche *Y. lipolytica* YM7, modèle d'étude privilégié, contribue à élucider le(s) facteur(s) influant sur les vitesses et les rendements de la synthèse et du stockage lipidique en présence de glycérol.

1.1.2 Glycérol : un sous-produit de la production de biodiesel

L'utilisation de biodiesel correspond à approximativement 0,3% de la demande mondiale en carburants pour le transport (Schenk et al., 2008). Le biodiesel est obtenu par un mélange de triglycérides issus d'huiles végétales et de graisses animales en présence d'un alcool et d'un catalyseur sous agitation à températures élevées (Kim et al., 2004). Suite à ce processus, le

mélange réactionnel est séparé en deux phases. La couche supérieure contient l'ester méthylique d'acides gras, soit le biodiesel, et la couche inférieure comprend le glycérol brut (GB) contenant du méthanol n'ayant pas réagi, le sel et les substances solides présentes dans les matières premières. Le glycérol est présent en quantités toujours plus grandes sur le marché mondial, il est utilisé pour la production de HOU (Meesters et al., 1996). Par ailleurs, plusieurs voies sont proposées pour la valorisation de glycérol brût. Cependant, chacune des voies presentent des avantages et des désavantages comme indiqué dans le tableau ci-dessous.

Tableau 1.1 Voies de valorisation de glycérol brut

Voies de valorisation	Avantages	Désavantages
Raffinage	- Réduction de glycérol brût	- Coût -Large volumes des sels inorganiques
Incinération	- Pas de processus en aval -Capacité de stockage minimale - Récupération d'énergie	- Formation de résidus corrosifs et nocifs - Génération des grands volumes des cendres
Décharge environmentale	- Pas de processus en aval	- Plus de temps -Écoulement de drainage de champ (contamination) - Coût de traitement des eaux usées - Demande d'une approbation par les autorités
Conversion métabolique	- Réduction de glycérol brût	- Coût de milieux de cultures - Coût de traitement en aval

1.1.2.1 Transestérification chimique

La transestérification permet la séparation du glycérol des esters d'acides gras par une réaction des triglycérides avec un alcool (éthanol ou méthanol) afin d'obtenir des mono-esters éthyliques ou méthyliques. Les esters méthyliques d'huile végétale (EMHV) et les esters éthyliques d'huile végétale (EEHV) ont une composition similaire aux lipides extraits d'huiles végétales (Kraisintu et al., 2010; Li et al., 2007). La transestérification a été bien étudiée en milieu homogène (Freedman et al., 1984; Schwab et al., 1987). Elle est fortement influencée par plusieurs facteurs tels que le rapport molaire alcool/triglycérides, la nature du catalyseur (acide ou basique), la nature de l'alcool et de l'huile, la température, la présence d'acides libres et d'eau, la vitesse de

l'agitation et le temps de réaction. Il est à noter que la catalyse basique est beaucoup plus rapide que la catalyse acide. En effet, en présence d'un catalyseur acide, le temps de réaction varie de 3 à 48 h, alors qu'il ne dépasse pas une heure pour le catalyseur basique (Freedman et al., 1984). Des températures élevées permettent d'augmenter le temps de réaction (Freedman et al., 1986). Les catalyseurs acides sont cependant rarement utilisés du fait de leur moindre réactivité et des risques élevés de corrosion des installations industrielles. En présence d'une matière grasse avec une forte proportion d'acides libres, le catalyseur basique peut réagir avec ces derniers pour former du savon ce qui augmente la consommation du catalyseur et rend difficile le processus de séparation ayant pour conséquence l'augmentation des coûts de production. Malgré que la catalyse basique soit plus rapide, plusieurs études indiquent que le catalyseur acide est un choix judicieux si l'huile végétale ou la matière grasse utilisée contient une forte proportion d'acides libres (AGL). De plus, la présence d'eau provenant de l'hydrolyse des TAG en diacylglycérol (DAG) a pour effet d'augmenter la teneur en AGL et de retarder le processus de conversion. Il est donc important de bien choisir le type de catalyseur en fonction de l'huile à estérifier.

Par ailleurs, la transestérification peut se dérouler en présence de catalyseurs hétérogènes tels que les résines échangeuses d'ions acides, les oxydes métalliques sulfatés ou les hétéropolyacides (Jitputti et al., 2006; López et al., 2007; Narasimharao et al., 2007). Les catalyseurs hétérogènes acides sont reconnus pour leur faible activité catalytique et leur temps de réaction longs contrairement aux catalyseurs basiques qui génèrent une forte activité catalytique. Les différents types de catalyseurs basiques hétérogènes sont les résines échangeuses d'anions, les oxydes, les oxydes métalliques, les zéolithes et les hydrotalcites (Cantrell et al., 2005; Martyanov & Sayari, 2008; Shibasaki-Kitakawa et al., 2007; Xie et al., 2006). Les principales impuretés résultant de cette réaction sont les acides libres contenus dans l'huile et la teneur en eau des charges (huile et méthanol). Ces impuretés nécessitent une étape supplémentaire de purification qui peut être effectuée par un lavage à l'eau, un séchage ou à l'aide d'une membrane.

D'autres méthodes de transestérification d'huiles végétales sont citées dans la littérature, telles que la transestérification à haute température (Fukuda et al., 2001), à haute pression (Ergün & Panning, 2002) et en présence de méthanol supercritique (Saka & Kusdiana, 2001). La méthanolyse basique de l'huile de colza peut, quant à elle, être effectuée à l'aide de micro-ondes (Azcan & Danisman, 2008) ou sous irradiation ultrasonore (Stavarache et al., 2007).

1.1.2.2 Transestérification enzymatique

La transestérification d'huiles végétales par catalyse enzymatique a connu un grand essor ces dernières années (Ranganathan et al., 2008). La méthanolyse d'huile végétale est possible en présence de lipases qui sont produites sous deux formes (intra et extra) (Adriana et al., 2012). Les souches candidates pour produire des lipases sont *Candida antarctica, Mucor miehei* et *Rhizopus oryzae* (Bako et al., 2002; Ban et al., 2012; Goh & Yeong, 1993). Ces enzymes peuvent être à l'état libre ou immobilisés sur un support (Shibasaki-Kitakawa et al., 2007), afin de permettre leur recyclage pour une utilisation ultérieure (Shimada et al., 2002). L'utilisation d'enzymes dans le processus de transestérification présente plusieurs avantages tels que la sélectivité et la biodégradabilité, ce qui permet d'augmenter le rendement de la réaction tout en diminuant la quantité de sous-produits générés (Shibasaki-Kitakawa et al., 2007).

En outre, le processus enzymatique ne requiert pas des températures et des pressions élevées, comparativement aux autres processus, ce qui diminue les coûts en énergie et équipements. Par ailleurs, les acides libres sont absents lors de la transestérication enzymatique et celle-ci est moins sensible à l'eau, ce qui permet de travailler dans un milieu aqueux. La transestérification enzymatique présente tout de même certains inconvénients tels qu'un coût élevé, une instabilité de l'activité enzymatique au cours du processus et un temps de réaction lent qui peut atteindre 16 h (Shibasaki-Kitakawa et al., 2007). Ceci parce que l'activité enzymatique est inhibée par la présence de glycérol et de méthanol dans le milieu réactionnel. Pour contrer cela, du tert-butanol est ajouté afin de solubiliser le glycérol au fur et à mesure qu'il s'adsorbe sur l'enzyme (Wang et al., 2006), ce qui a pour effet d'améliorer la rentabilité du processus.

1.1.3 Glycérol brut : un substrat pour la production de biodiesel

Le glycérol est un substrat osidique qui peut être utilisé par de nombreux microorganismes pour leur croissance et qui a été amplement étudié pour son potentiel de production de PHVA tels que l'acide propionique, le butanol, l'acide succinique et le dihydroxyacétone (Abghari & Chen, 2014; Asad-ur-Rehman et al., 2008; da Silva et al., 2009; Dabrowski et al., 2012; Sarma et al., 2012) (Figure 1.1).

L'accumulation de lipides est basée principalement sur la présence d'une source importante de carbone. Les levures suivantes citées dans la littérature, *Rhodotorula glutinis, Cryptococcus albidus*, *Lipomyces starkeyi, Candida curvata* et *Yarrowia lipolytica,* sont capables d'utiliser le glycérol brut comme substrat et source d'énergie pour leur croissance.

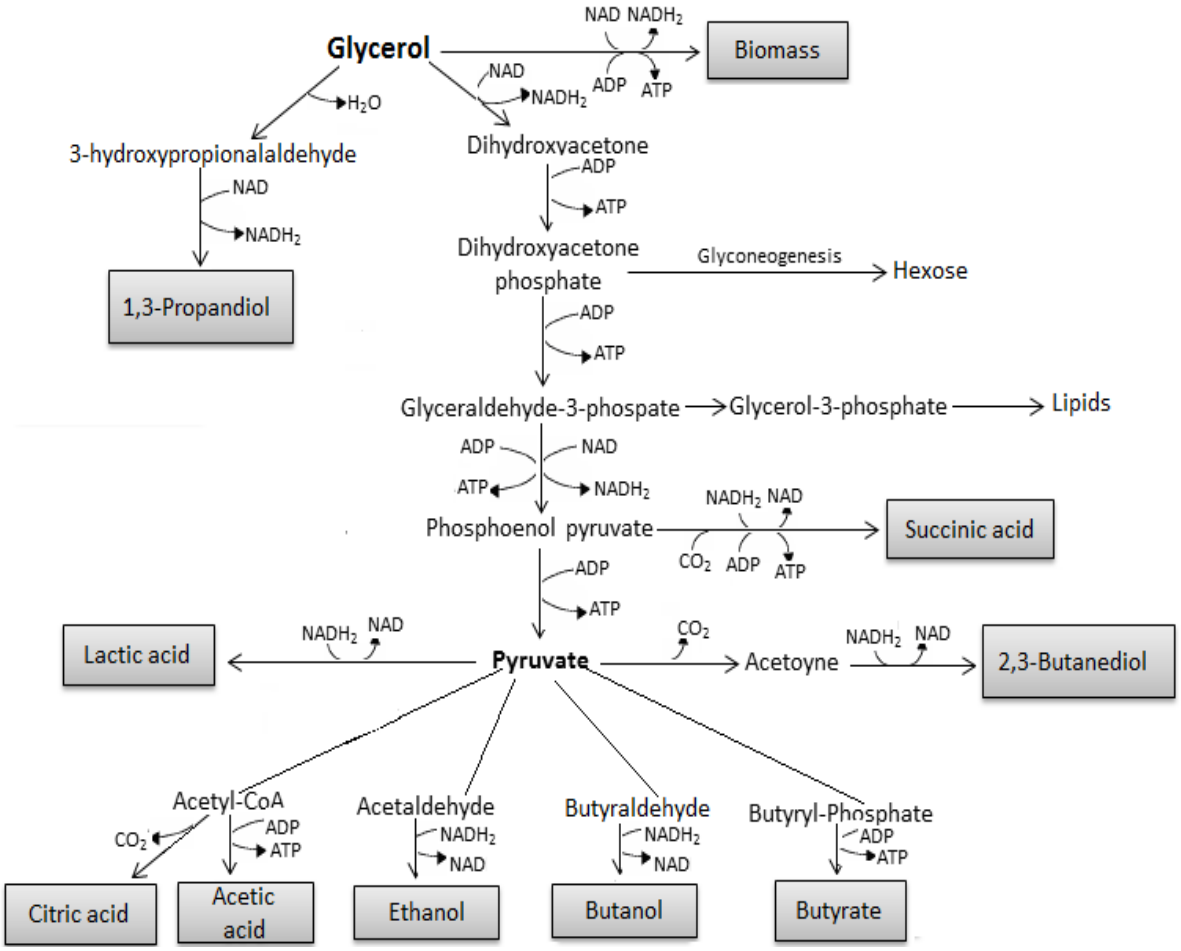


Figure 1.1 Voies métaboliques du glycérol

Dans cette étude, les levures ont constitué la principale voie de production de lipides. L'utilisation de champignons filamenteux a été mise de côté, car lors d'une fermentation à grande échelle, les filaments limitent le transfert d'oxygène causant le colmatage et une pressurisation des sondes, ce qui empêche la conversion de glycérol (Azocar et al., 2010).

Par ailleurs, autres étapes doivent être considérés au cours de la production du biodiesel a l'échelle industrille tels que le transport de la matière première, son prétraitement, la culture du microorganisme, le coût de milieu, l'extraction des lipides, la transestérification etc. Ces facteurs

répresentent le goulot d'étranglement pour permettre une diminution considérable des coûts de production. (Figure 1.2)

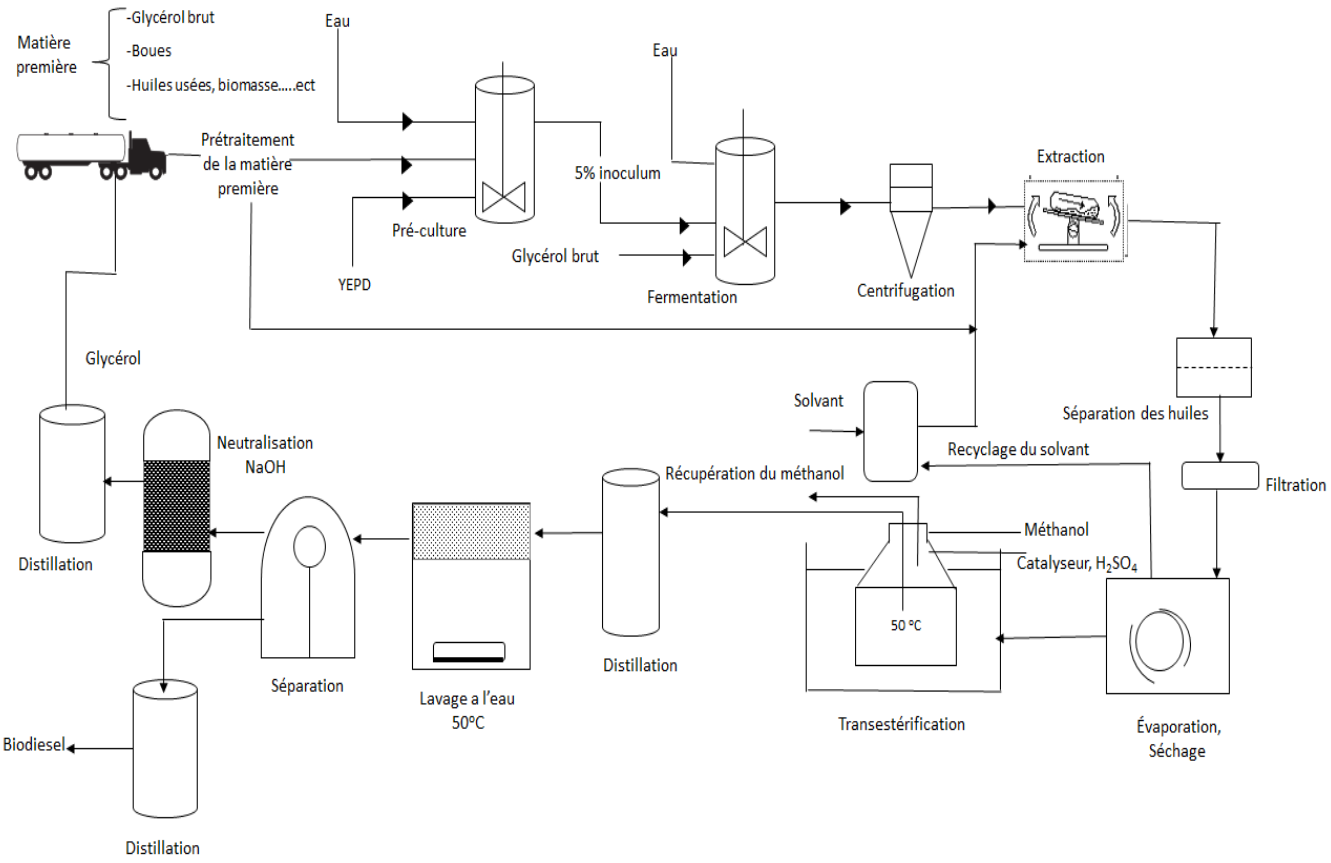


Figure 1.2 Processus de production de biodiesel

Le contenu énergétique du glycérol pur est de 19,0 MJ kg^{-1}, alors qu'il est de 25,3 MJ kg^{-1} pour le glycérol brut en raison de la présence de méthanol et de traces de biodiesel (http://www.esru.strath.ac.uk/EandE/Web_sites/06-07/Biodiesel/experiment.html). Ce contenu élevé en énergie du glycérol brut indique un fort potentiel comme substrat pour la production de TAG. En outre, contrairement à la plupart des matériaux de déchets cellulosiques, il ne nécessite pas de prétraitement pour le rendre disponible pour les microorganismes oléagineux.

1.1.4 Métabolisme du glycérol et de la production de lipides

Le métabolisme du glycérol pour la production de TAG a été amplement étudiée chez *Y. lipolytica* et *Saccharomyces cerevisiae* (Beopoulos et al., 2009; Cescut, 2009). Récemment, des cultures réalisées sur des cosubstrats (glycérol, glucose, oléate, stéarine) ont montré que le glycérol est

consommé préférentiellement aux autres substrats. La stéarine et le glucose peuvent être consommés conjointement, mais l'accumulation de lipides semble moins efficace qu'avec le glycérol et la stéarine (Davies & Holdsworth, 1990).Toutefois, aucune donnée sur les rendements apparents n'est disponible pour ce type de culture sur substrat mixte. Mhairi Workman et al. (2013) ont obtenu des taux de croissance de 0,3 h^{-1} en présence de glycérol dans la culture, 0,24 h^{-1} en présence de glucose seul et 0,38 h^{-1} en présence des deux substrats.

Le glycérol emprunte deux voies de transport, à savoir la diffusion facilitée et le transport actif, afin d'être assimilable par les microorganismes. Une fois que la molécule de glycérol est rendue dans la cellule, elle peut être assimilée par deux voies différentes, la phosphorylation et l'oxydation. Lors de la phosphorylation, le glycérol est transformé en glycérol-3-phosphate à l'aide d'une enzyme glycolytique appelée glycérol kinase. Cette dernière va constituer le squelette de carbone en vue des acylations successives donnant naissance à l'acide phosphatidique et au diacyglycérol (DAG) qui sont deux composés intermédiaires indispensables au métabolisme des lipides (Coleman & Lee, 2004). L'acylation du DAG en TAG utilise l'acyl-CoA, la phosphatidylcholine ou un acide gras comme donneur d'acyle (Sorger & Daum, 2002). En général, les lipides sont accumulés sous forme de TAG, une limitation nutrionnelle en azote et un apport suffisant en carbone déclenchant le processus d'accumulation. Toutefois, la synthèse de TAG et leur accumulation sont des processus distincts. Pour plus de détails concernant la synthèse et l'accumulation des TAG, la revue (Magdouli et al., 2014) présentée au Chapitre 2 de cette thèse peut être consultée. La lipogénicité des microorganismes oléagineux réside dans leur capacité à produire simultanément une grande quantité d'acétyl-CoA avec un pouvoir réducteur élevé. La présence du pool d'acétyl-CoA est attribuable à l'activité de l'ATP citrate lyase (Boulton & Ratledge, 1981). Dans un milieu de culture limité en azote, une surproduction d'acétyl-CoA est observée (Anastassiadis & Rehm, 2005; Boulton & Ratledge, 1981).

Lors de l'oxydation du glycérol, le glycérol-3-phosphate est transformé en dihydroxyacétone phosphate (précurseur du glycérol-3-phosphate) sous l'action du glycérol-3-phosphate déshydrogénase. Le microorganisme emprunte ensuite la voie *de novo* pour la synthèse des TAG. En conclusion, le microorganisme peut accumuler l'acide phosphatidique soit à partir du glycérol-3-phosphate ou du dihydroxyacétone phosphate.

1.1.5 Génie biologique pour la production de TAG

1.1.5.1 Évaluation de différents types de fermentation pour la production de TAG

La majorité des essais effectués dans cette étude pour la surproduction des TAG ont été réalisés en mode *batch*. Durant ce processus, l'accumulation de TAG n'est effective qu'à partir d'un certain niveau de limitation de l'élément nutritionnel, ainsi la phase d'accumulation ne s'effectue pas sur la totalité du temps de culture. Parmi les autres modes de culture rapportés dans la littérature, les modes *fed batch* et continu en réacteur (chémostat) ont pour effet de maintenir les microorganismes dans un état métabolique actif et d'empêcher toute inhibition du substrat utilisé au cours du processus (Saenge et al., 2011a). Pour le mode continu, Papanikolaou and Aggelis (2002) ont trouvé que le maximum de la productivité lipidique était de 0,12 g L^{-1} h^{-1} dans un système bien aéré.

Toutefois, les essais en fiole agitée ne permettent aucun contrôle des conditions expérimentales initiales, comme le rapport des flux de carbone et d'azote, l'apport en oxygène, le contrôle du pH, qui sont définis comme des paramètres clés dans le processus d'accumulation de lipides. Chez *Y. lipolytica*, le type de fermentation le plus utilisé est le mode continu qui permet d'atteindre des taux de conversion se rapprochant de 0,38, 0,34 et 0,16-0,33 C mol L^{-1} en présence de glucose, de glycérol et de glycérol brut, respectivement (Papanikolaou et al., 2002). Chez *R. glutinis*, le mode *fed batch* est préférablement utilisé et permet d'atteindre des valeurs aux alentours de 2,9 C mol L^{-1} en présence de glycérol (Granger et al., 1993). En contrepartie, le mode continu est préféré pour *C. curvata* et *Crytococcus curvatus* et permet d'atteindre un taux de production maximal (Evans & Ratledge, 1983; Hassan et al., 1993).

1.1.5.2 Paramètres importants du procédé et leurs valeurs optimales

La production de biodiesel requiert une source importante de carbone et une concentration en azote ne dépassant pas la valeur critique (10^{-3} mol L^{-1}) (Cescut, 2009). Ces deux principaux paramètres sont potentiellement impliqués dans le processus d'accumulation. La source d'azote organique et inorganique joue un rôle majeur, quant à elle, dans l'augmentation de la production de la biomasse et des huiles d'organismes unicellulaires (HOU) (Evans & Ratledge, 1983; Kalam et al., 2014).

Selon la littérature, le glycérol possède le plus faible rendement de conversion théorique, il est tout de même considéré comme le meilleur substrat pour de nombreux microorganismes. En effet, il a été démontré que les microorganismes utilisent préférentiellement le glycérol lorsque ce dernier est présent en mélange avec le glucose (Workman et al., 2013).

L'utilisation d'autres substrats ou cosubstrats peut modifier le rendement théorique de la production de TAG puisque d'autres voies anaboliques et cataboliques peuvent être empruntées. Plusieurs substrats ou déchets pourraient être convertis efficacement en lipides; les déchets ou substrats suivants on été étudiés : le jus d'artichaut (Hua et al., 2007), les boues d'épuration (Angerbauer et al., 2008), les résidus d'huile de palme (Saenge et al., 2011b), le glutamate monosodique (Xue et al., 2008), les eaux usées, le perméat de lactosérum, la mélasse et des hydrolysats de paille de riz (Li et al., 2007).

D'autre part, certains microorganismes oléagineux peuvent utiliser l'éthanol comme substrat (Fakas et al., 2009; Ratledge, 1988). Le rendement de conversion est estimé à 0,31 g de lipides par gramme d'éthanol. Pour améliorer la productivité, un mélange de substrats organiques est recommandé. Par exemple, *L. starkeyi* a la capacité d'accumuler plus de 70% de lipides, mais il est possible d'augmenter ce taux en utilisant un mélange de glucose et de xylose ainsi que d'autres déchets (Angerbauer et al., 2008).

Plusieurs paramètres abiotiques influencent le rendement du processus d'accumulation de lipides. Par exemple, la concentration initiale en ions hydrogène du milieu de culture influence la croissance microbienne et le contenu lipidique. Les premières études effectuées chez *Rhodotoruia gracilis* et *Candida* sp.107 montrent bien l'effet du pH sur la lipogenèse (Kessell, 1968). Un pH acide entre 5,5 et 6,5 s'est avéré favorable pour la surproduction de lipides chez *Rhodosporidium toruloides* DMKU3-TK16 avec une teneur en lipides atteignant 71,3% (Kraisintu et al., 2010). Le pH varie selon nature du substrat et des microorganismes utilisés. Par exemple, chez *L. starkeyi*, le pH varie entre 4 à 5,5 (Angerbauer et al., 2008; Yamauchi et al., 1983). La température peut être modulée selon le profil de TAG que l'on désire accumuler, la longueur des chaînes d'acides gras, ainsi que leur degré de saturation (Rossi et al., 2009). Par exemple, chez *Mucor circinelloides*, la diminution de la température de 28 à 15°C active la désaturase avec une abondance d'acide oléique de C18:1 et un niveau constant de γ-linolénique (18:3, n-6) (Michinaka et al., 2003). Ce phénomène est régulé par un contrôle transcriptionnel (Laoteng et al., 1999). Pour la concentration en oxygène dissous, plusieurs travaux ont révélé que l'oxygène, en tant qu'accepteur terminal d'électrons, joue un rôle important dans la désaturation des acides gras. Toutefois, l'activité enzymatique peut être affectée par une limitation en oxygène. Aiba et al.

(1973) ont rapporté que le rendement en biomasse peut-être grandement affecté lorsque la concentration en oxygène de la culture n'est pas suffisante. La limite inférieure de la concentration en dioxygène dissous préjudiciable aux rendements de conversion du substrat en lipides se situerait en decà de 2 mg L^{-1}. Des études enzymatiques complémentaires ont abouti à la conclusion qu'une limitation en oxygène pourrait affecter en premier lieu les enzymes du cycle du glyoxylate et l'isocitrate lyase qui sont fortement dépendantes de la présence de fer. Il a été montré que la disponibilité de l'oxygène influe considérablement sur l'expression des désaturases sur le plan transcriptionnel (Wu et al., 2005). La limitation en oxygène induit une forte accumulation de chaîne moyenne des acides gras saturés (C10-C14), par opposition, une culture aérobie conduit à l'accumulation de quantités substantielles de longues chaînes saturées et d'acides gras insaturés (Jeennor et al., 2006). D'autres facteurs semblent aussi affecter le processus de lipogenèse comme la concentration en macroéléments dont le magnésium (Kraisintu et al., 2010).

D'autres études ont révélé que la concentration en ions de Zn^{2+} et Mn^{2+}, et en second lieu Fe^{3+}, Ca^{2+} et K^{+}, peuvent influer sur l'accumulation de lipides (Johnson & Taconi, 2007). Le rôle des agents tensioactifs pour améliorer la production de lipides a également été récemment étudié. Les tensioactifs et les surfactants sont connus pour avoir plusieurs rôles dont l'amélioration de la production de métabolites, la stimulation de la croissance et la modification de la perméabilité des membranes cellulaires (Laoteng et al., 1999). Les trois surfactants les plus couramment utilisés sont le Tween 20, le Tween 80 et la gomme arabique. Il a été prouvé que le Tween 20 augmente la teneur en lipides, ainsi que les caroténoïdes chez *R. glutinis* TISTR (Saenge et al., 2011). Un ajout de Tween 20 dans le milieu de culture a pour effet de stimuler la croissance et l'activation de plusieurs enzymes impliqués dans la biosynthèse des lipides et des caroténoïdes dans des cellules procaryotes et eucaryotes.

1.1.6 Microorganismes oléagineux hétérotrophes utilisés pour la bioconversion du glycérol et la production de biodiesel

Comme le glycérol est utilisé pour fabriquer divers produits, différents microorganismes ont été évalués pour leur capacité de bioconversion du glycérol. Les différents microorganismes actuellement utilisés pour la bioconversion du GB ont été répertoriés aux Tableaux 2.1 et 2.2.

Le métabolisme lipidique est présent chez tous les microorganismes autant procaryotes qu'eucaryotes. Cependant, l'accumulation de réserves lipidiques majoritairement constituées d'acides gras est caractéristique des microorganismes eucaryotes. En général, les lipides sont stockés sous forme d'esters de stérol (SE) et de TAG. Toutefois, l'accumulation de TAG varie

d’une souche à l’autre et seuls quelques acides gras satisfont aux critères pour une utilisation en tant que biocarburants. Pour les bactéries, l’accumulation de TAG semble caractéristique du groupe des Actinomycètes, par exemple *Streptomyces, Nocordia, Rhodoccocus et Mycobacterium.* La teneur en lipides chez les Actinomycètes peut atteindre 70% (Chisti, 2007). Parmi plus de 600 espèces de levures, uniquement 25 sont connues comme oléagineuses. L’accumulation des acides gras polyinsaturés chez ce phylum semble être une réponse à une acclimatation aux conditions environnementales (Libkind et al., 2008). Les champignons dits filamenteux sont également capables d’accumuler des teneurs élevés en TAG et en SE en présence d’une source riche en carbone, parmi ceux-ci notons les Zygomycètes.

Les cultures microbiennes mixtes ont aussi récemment été utilisées pour la bioconversion du GB. Cependant, certaines contraintes concernant la stabilité du processus doivent être considérées et le calcul du rendement de conversion pour chaque espèce semble être difficile à faire.

1.1.7 Génie génétique et métabolique pour l'amélioration de la production de lipides

1.1.7.1 Enzymes impliquées dans la synthèse des TAG

Le génie génétique et métabolique peut être considéré comme un outil potentiel d'amélioration de la production de biodiesel par la bioconversion du GB. Pour cela, un inventaire des enzymes impliqués dans le processus d'accumulation est important. Les enzymes clés pour la synthèse de TAG sont l'ATP citrate lyase (ACL), l'acétyl-CoA carboxylase (ACC) et l'enzyme malique. Les autres enzymes associées sont l'AMP désaminase et l'acyl-CoA oxydase (Boulton & Ratledge, 1981; Coleman & Lee, 2004).

- **L'activité de l'AMP désaminase** s'accroît en cas de carence nutritionnelle, ce qui entraîne une baisse de la concentration en AMP dans le cytosol et la mitochondrie avec également une augmentation de la concentration en ions ammonium. La diminution de la concentration en AMP dans la mitochondrie arrête l'activité de l'isocitrate déshydrogénase, cette enzyme étant strictement dépendante de la présence d'AMP.

- **L'acétyl-CoA carboxylase (ACC)** catalyse la formation de malonyl-CoA à partir d'acétyl-CoA. Les molécules de malonyl-CoA sont ensuite incorporées dans les chaînes d'acides gras.

- **L'enzyme malique** cytoplasmique semble être la principale voie de production de nicotinamide adénine dinucléotide phosphate (NADPH) (Ratledge, 2002) étant incorporée dans un cycle de transhydrogénation du NADH. La provenance du malate cytoplasmique est liée soit à la glycolyse produisant du pyruvate qui est alors décarboxylé en oxaloacétate, soit au cycle des acides tricarboxyliques qui produisent du citrate qui sera scindé en acétyl-CoA et oxaloacétate par l'ATP citrate lyase. Ce dernier sera oxydé en malate.

- **L'ATP citrate lyase** catalyse la formation d'acétyl-CoA à partir de citrate dans les microorganismes oléagineux.

- **L'acide gras synthase** permet l'incorporation du malonyl-CoA dans les chaînes d'acides gras.

- Le malate déshydrogénase permet la conversion de l'acétyl-CoA et de l'oxaloacétate en malate qui sera utilisé ultérieurement par le système du citrate (Evans & Ratledge, 1983).

1.1.7.2 Modification génétique

Les cibles de modifications génétiques sont les enzymes qui sont impliquées dans la voie de synthèse des TAG. L'enzyme malique est suspectée comme étant l'étape limitante de la biosynthèse d'acide gras chez les levures oléagineuses. Des cultures en fioles agitées de *M. circinelloides* surexprimant le gène de l'enzyme malique ont été réalisées. La productivité peut être multipliée par un facteur 1,5 lorsque l'activité enzymatique est multipliée par deux ou trois. Toutefois, à partir d'une certaine teneur en lipides intracellulaires, l'activité de l'enzyme malique auparavant surexprimée devient presque nulle. Les réactions de dégradation des lipides (β-oxydation, hydrolyse des TAG) ont également un impact sur la productivité en lipides. Ainsi, si le cycle futile de β-oxydation était supprimé, il serait possible d'augmenter la productivité en TAG.

Pour une discussion détaillée de la production de biodiesel par les microorganismes oléagineux, la revue (Magdouli et al., 2014) présentée au Chapitre 2 de cette thèse peut être consultée.

1.2 Partie II : problématique

1.2.1 Demande en énergie croissante et augmentation des gaz à effet de serre

Depuis la seconde guerre mondiale, la part du pétrole dans la consommation totale d'énergie n'a cessé de croître suite à une expansion galopante de la demande en énergie. En effet, le pétrole est devenu la matière première minérale couramment utilisée pour la fabrication de plastiques, textiles, résines etc. Au rythme actuel, les prévisions s'accordent sur un début de décroissance de la production mondiale de combustibles fossiles entre 2010 et 2050 et un épuisement des réserves au cours du prochain siècle. L'utilisation des réserves pétrolières a des effets nocifs sur la santé humaine et sur l'environnement puisque la combustion de pétrole rejette des gaz polluants dans l'atmosphère, le CO_2 étant le principal GES (GIEC, 2007). Actuellement, les émissions de GES au Canada sont estimées à 702 mégatonnes d'équivalent dioxyde de carbone (Mt d'éq. CO_2), soit une augmentation d'environ 1 Mt (0,14%) par rapport au niveau de 2010, qui était de 701 Mt. Ce gaz une fois dans l'atmosphère cause divers problème environnementaux : acidification, changements climatiques, etc. En moyenne, chaque citoyen canadien produit 23,6 t de GES par année. Cette production est quatre fois plus que la moyenne mondiale.

Pour faire face à ces enjeux environnementaux, des efforts ont été faits pour diminuer les émissions de GES et une réduction de 36 Mt (4,8%) a été observée au Canada depuis 2005. Cette baisse est attribuable à des améliorations de l'efficacité, à la modernisation des procédés industriels et à des changements structurels dans la composition de l'économie, qui représentent des tendances à long terme ayant un impact croissant sur les émissions depuis la fin des années 1990. Le défi à relever est non seulement en prévision de l'épuisement du pétrole et dans le but de réduire les émissions de GES, mais aussi pour trouver des sources d'énergie de substitution renouvelables afin d'éviter, entre autres, les conséquences de tensions économiques et politiques avec les pays producteurs (comme ce fut le cas lors des chocs pétroliers de 1973 et de 1981).

1.2.2 Pénurie des terres arables et augmentation du coût de production

Les huiles végétales sont une des ressources renouvelables les plus utilisées pour la production de biodiesl, mais on vise de plus en plus à minimiser leur utilisation pour plusieurs raisons. Entre autres, leur culture monopolise d'énormes superficies de terres arables. En effet, simplement pour remplacer 4% de la demande mondiale en carburants du secteur des transports, une superficie de terres arables équivalente à la France et l'Espagne combinés (AIE, 2006) serait nécessaire. Par exemple, les États-Unis consomment annuellement 0,53 milliards de mètres cubes de diesel. Afin de satisfaire à ces besoins, il serait nécessaire de cultiver des palmiers à

huile sur une superficie de 111 millions d'hectares, ce qui équivaut à 61% de toutes les terres agricoles des États-Unis (Chisti, 2007). En Asie, pratiquement 3 millions d'hectares de forêt sont convertis annuellement pour la production d'huile de palme (Gonzales, 2008). D'autre part, le recours à des cultures qui servent à l'alimentation humaine et animale à des fins industrielles pose plusieurs questions de nature éthique. Ainsi, l'augmentation des prix des produits agricoles de 75% observée depuis 2005 dans le monde rend ceux-ci moins accessibles pour tous (Gonzales, 2008). De plus, en favorisant des cultures spécifiques offrant un bon rendement en huile, les monocultures se répandent entraînant des effets néfastes sur les sols comme l'érosion, ainsi que la contamination des eaux de surface et souterraines due à l'augmentation de l'utilisation intensive de fertilisants et de pesticides. En raison des problèmes causés par l'utilisation des huiles végétales pour la production de biodiesel, le recours au biodiesel de deuxième et troisième génération est recommandé. En ce sens, les microalgues offrent un potentiel intéressant de remplacement des carburants conventionnels sans affecter les ressources alimentaires, la consommation d'eau et sans détériorer les sols cultivables (Chisti, 2007). En effet, les microalgues possèdent plusieurs avantages dont une importante capacité de production de biomasse par hectare, une fréquence de récolte élevée, une diminution de l'utilisation d'eau potable, ainsi qu'une plus faible émission de GES. Les lipides accumulés par les microalgues peuvent atteindre 80% de leur poids sec principalement sous forme de TAG. Ces derniers peuvent ensuite être utilisés pour produire du biodiesel. La croissance des microalgues est extrêmement rapide puisque certaines espèces peuvent doubler leur biomasse de une à trois fois en 24 h (Khan et al., 2009). D'autres candidats comme les champignons et les bactéries peuvent être des sources alternatives pour la production de TAG en présentant un temps de génération réduit et une forte adaptabilité aux manipulations génétiques (Meng et al., 2009; Vicente et al., 2004). De plus, leurs conditions de culture sont plus faciles à contrôler que pour les plantes (Widjaja et al., 2009). Ces avantages laissent présager un bel avenir aux microorganismes hétérotrophes pour la production de TAG qui pourront servir à produire du biodiesel répondant aux normes mondiales.

1.2.3 Coût prohibitif de la matière première du biodiesel

Actuellement, la demande annuelle mondiale en biodiesel est en croissance et on prévoit une augmentation de 41 milliards de litres (10,83 milliards de gallons) en 2019 (Annie, 2006). Les coûts reliés aux sources de carbone et d'azote (organique ou inorganique) sont estimés à 40% à 60% du coût total de production du biodiesel (Zhao et al., 2010). Ainsi, la production de biodiesel

est ultimement dépendante des ressources renouvelables dont il est issu, le coût de production pouvant être réduit de 80% selon la ressource utilisée (Haas et al., 2006).

1.2.4 Gestion et valorisation du glycérol brut

Le glycérol brut est un sous-produit de la réaction de transestérification représentant approximativement 10% du poids du biodiesel produit. Singhadhandhu et Tezuka (2010) ont rapporté qu'en Europe les coûts de la purification du glycérol brut sont d'environ 65 millions d'euros (Singhabhandhu & Tezuka, 2010). Par conséquent, la gestion des ces résidus pose problème. Cependant, le glycérol brut peut être utilisé pour fabriquer des produits pharmaceutiques, alimentaires, ainsi que pour la production d'éthanol et de savon (Choi et al., 2011; Johnson & Taconi, 2007). Une autre approche pour la revalorisation de glycérol brut est envisagée dans cette étude étant donné que ce substrat peut être utilisé comme source de carbone et d'énergie.

1.3 Partie III : hypothèses, objectifs et originalité de la recherche

1.3.1 Hypothèses

- Le glycérol brut est un sous-produit de la transestérification, représentant approximativement 10% du poids total de biodiesel produit. En raison de la demande élevée de biodiesel, la gestion de ce résidu pose problème. Néanmoins, c'est un substrat riche en carbone qui peut être utilisé par les microorganismes pour produire du biodiesel. Cependant, les microorganismes oléagineux ne montrent pas tous la même tolérance pour différentes concentrations de glycérol brut. La présente étude veut donc vérifier tout d'abord si *Y. lipolytica*, la levure choisie peut croître en utilisant du glycérol brut comme seul substrat pour produire des TAG.
- Le profil des acides gras joue un rôle important dans la production des biocarburants et plusieurs facteurs peuvent le faire varier leurs propriétés. Concernant ce point, la variation de l'oxygène dissous au cours de la phase de lipogénèse peut influencer le procesus d'accumulation des acides gras, ainsi que leur proprietés. La concentration en oxygène dissous devrait également affecter le comportement rhéologique et morphologique des souches d'*Y. lipolytica* lors de la croisance en fermenteur.
- Le déclenchement de la synthèse des lipides par la voie biochimique est une approche prometteuse. L'hypothèse d'un rôle effectif des cofacteurs biochimiques pour obtenir de très fortes accumulations des TAG peut être émise. On peut ainsi prévoir qu'il y aura de meilleurs rendements lors de l'ajout de cofacteurs, tels que la biotine, la leucine et l'acide citrique.
- Plusieurs inducteurs tels que les huiles et les surfactants sont indispensables à la production des lipases par différentes levures et champignons. Les lipases sont rapportées dégrader les lipides. Par ailleurs, les microorganismes sont rarement rapportés produire simultanément des lipides et des lipases. *Yarrowia lipolytica* est principalement productrice de lipides. Dans ce contexte, la détermination de ses capacités à accumuler des TAG et à excréter des enzymes telles que les lipases, reste une hypothèse à vérifier.
- Diverses méthodes ont été testées pour la transestérification *in situ* de lipides extraits de plantes et de levures. Cependant, aucune étude n'a été menée sur la transestérification *in situ* de lipides levuriens assistée de micro-ondes. Les micro-ondes pourraient aussi avoir un effet sur le rendement de conversion des lipides et présenter la même efficacité que les ultrasons.

- L’approche de co-culture a été recemmet étudiée pour la production des biofuels tels que le biohydrogéne, cette approche pourrait etre exploitée dans la production des lipides tout en utilisant une souche *Aspergillus* productrice d’acide citrique : un clé dans le processus de lipogénèse.

1.3.2 Objectifs

L’objectif principal de la présente étude est d’analyser et de quantifier les mécanismes physiologiques régissant l’accumulation de lipides chez une levure afin d’optimiser la production et ce, en termes de rendement et de productivité dans une perspective de production à grande échelle. Le but ultime étant d’utiliser ces molécules en tant qu’additif ou substitut aux carburants conventionnels.

Dans cette étude, il a été choisi d’évaluer le potentiel de souches de *Yarrowia lipolytica*, une espèce qui a naturellement la capacité d’accumuler des acides gras. Le défi est de maximiser la production de lipides tout en augmentant la concentration de biomasse et en minimisant le temps de fermentation.

Les objectifs secondaires poursuivis sont :

- Optimiser et déterminer la concentration de glycérol, le ratio carbone:azote (C:N) et le temps de fermentation dans le but d’augmenter la concentration de biomasse et l'accumulation de lipides à partir de glycérol brut;
- Développer une approche biochimique d'amélioration de la synthèse des lipides en utilisant la biotine et la leucine pour stimuler l'activité des enzymes lors de la lipogenèse;
- Étudier les autres potentialités de la souche étudiée pour la production simultanée de lipases et de lipides
- Étudier l’efficacité des microndes et des ultrasons dans la conversion des lipides microbiennes
- Examiner la possibilité de co-culture pour maximiser la production de lipides par la levure étudiée.

1.3.3 Originalité

L'originalité de la thése résiderait dans l'isolation et la culture pour la premiere fois d'une souche de levure accumulant des TAG et capable de dégrader le glycérol. Peu d'explications sur les mécanismes et les facteurs impliqés dans l'augmentation ou la diminution des rendements sont fournies dans la littérature. Dans ce contexte, cette thése vise à donner des explications.

1.4 Partie IV : sommaire des différents volets de recherche de cette étude

Les différents volets de recherche réalisés dans cette thèse sont énoncés ci-dessous :

- Production de lipides par des microorganismes hétérotrophes oléagineux (**Chapitre 2**, article revue publié dans *Critical Reviews in Environmental Science and Technology (*2014) 44, 416-453);
- Optimisation de la production de lipides par *Y. lipolytica* et étude de la performance du procédé à l'échelle du fermenteur (**Chapitre 3**, article de recherche publié dans *RSC Advances* (2016) 6, 90547-90558);
- Morphologie et comportement rhéologique de *Y. lipolytica* : effet de la concentration d'oxygène dissous sur la croissance cellulaire et la composition de lipides (**Chapitre 4**, article de recherche soumis à *Energy*);
- Utilisation d'acide citrique et de cofacteurs essentiels afin d'améliorer la production de lipides par *Y. lipolytica* à partir de milieu de culture à base de glycérol brut (**Chapitre 5**, article de recherche soumis à *Applied and Environmental Microbiology*);
- Valorisation du glycérol brut et des déchets de crustacés pour la synthèse de produits à valeur ajoutée par *Y. lipolytica* (**Chapitre 6**, article de recherche soumis à *Engineering Life Sciences*);
- Étude comparative entre les micro-ondes et les ultrasons pour la transestérification *in situ* de lipides microbiens (**Chapitre 7**, article de recherche publié dans *RSC Advances* (2016) 6, 56009-56017);
- Co-culture pour la production de lipides : développements et défis (**Chapitre 8**, article revue publié dans *Biomass and Energy* (2016) 92, 20-30).

1.4.1 Production de lipides par des microorganismes hétérotrophes oléagineux

Cette revue bibliographique a permis de faire le bilan des connaissances sur les lipides levuriens en fonction de leur nature, leurs fonctions et leurs métabolismes de synthèse, d'accumulation et de dégradation. L'analyse a mis en évidence différents facteurs influençant le processus de lipogenèse. Il en ressort les points forts suivants. *Yarrowia lipolytica*, une levure oléagineuse, est un modèle d'étude privilégié et un microorganisme d'intérêt industriel pour ses produits. Les études disponibles se limitent cependant aux substrats glucidiques et à ceux hydrophobes. Rares

sont les travaux relatifs à l'accumulation de lipides par cette souche à partir de glycérol brut, un substrat d'intérêt dans le domaine d'application visé dans cette étude. Le glycérol est un candidat intéressant par sa capacité élevée de réduction et sa structure chimique à la base des TAG. De plus, le glycérol étant un coproduit des voies classiques de production de bioester, son avantage dans les bioprocédés est indéniable.

1.4.2 Optimisation de la production de lipides par *Y. lipolytica* et étude de la performance du procédé à l'échelle du fermenteur

Cette partie de la recherche visait à optimiser les principaux paramètres de production de lipides à partir de glycérol brut en utilisant la levure *Y. lipolytica* SM7. Les paramètres ont été optimisés par une méthode des surfaces de réponses (MSR) en utilisant la technique de Box Behnken. Les paramètres étudiés étaient la concentration de glycérol brut (75 à 100 g L^{-1}), la concentration d'hydroxyde d'ammonium (0,5 à 1,5 g NH_4OH L^{-1}) et le temps de fermentation (36 à 72 h). Les essais ont été réalisés dans un fermenteur de 5 L de capacité avec une température maintenue à 28°C et un pH stabilisé à 6,5 ± 0,5. Une production maximale de 52,7 ± 1,2% (p/p) de lipides, correspondant à une concentration de biomasse de 25,8 ± 1,5 g L^{-1} et une teneur en lipides de 13,6 ± 0,8 g L^{-1}, a été obtenue avec un apport initial de 89 g L^{-1} de glycérol brut, 0,54 g L^{-1} de NH_4OH et un temps de fermentation de 66 h. Les conditions optimales ont ensuite été testées, sans réduction d'efficacité, en utilisant une fermentation en deux étapes, soit en réduisant la teneur en oxygène dissous de 60% à 30% après 18 h de fermentation. Finalement, les lipides produits par *Y. lipolytica* SM7 ont été caractérisés et contiennent principalement des acides oléique, palmitique, linoléique et stéarique, lesquels peuvent servir de précurseurs à la synthèse de biodiesel.

1.4.3 Morphologie et comportement rhéologique de *Y. lipolytica* : effet de la concentration d'oxygène dissous sur la croissance cellulaire et la composition de lipides

La levure aérobie stricte *Y. lipolytica* cultivée en limitation d'azote produit des acides gras de moins en moins insaturés en fonction de la diminution du taux d'oxygène dans le milieu. L'étude des variations de l'oxygène dissous lors de la phase d'accumulation lipidique a été menée après 18 h de fermentation. Toutefois, lors du passage de la phase de croissance à la phase d'accumulation de lipides, la demande en oxygène est moins importante. Trois scénarios de

variation de l'aération ont été testés, 15-20%, 30-35% et 40-60%, en modifiant le coefficient de transfert d'oxygène (70, 80 et 100 h^{-1}, respectivement). Lors de la culture d'*Y. lipolytica* SM7, le coefficient de transfert d'oxygène (k_La) a atteint des valeurs supérieures à 280 h^{-1} en phase de croissance et s'est stabilisé pendant la phase d'accumulation entre 50 et 80 h^{-1}. Une analyse morphologique et rhéologique a été menée montrant un bouleversement de la morphologie cellulaire selon les différentes phases de croissance et d'accumulation des lipides. Un phénomène important de dimorphisme a été observé chez les cellules habituellement ovoïdes, quelques fois allongées de 5 à 6 µm, caractéristiques d'*Y. lipolytica*. Deux populations ont été distinguées, des cellules filamenteuses et les cellules ovoïdes courantes. Les cellules filamenteuses s'enchaînaient en un pseudo-mycéllium linéaire rarement branché et dissocié les uns des autres. Une étude par granulométrie laser a également été réalisée, ce qui a permis d'avoir des mesures de distribution des tailles des différentes cultures soumises aux différents degrés d'oxygénation. Ces analyses de distribution volumique permettent de définir une distribution monomodale. En effet, après 18 h, les cellules produisent des filaments, ce qui confirme le dimorphisme observé chez *Y. lipolytica* et l'effet d'une limitation en oxygène sur la filamentation et l'accumulation de lipides.

1.4.4 Utilisation d'acide citrique et de cofacteurs essentiels afin d'améliorer la production de lipides par *Y. lipolytica* à partir de milieu de culture à base de glycérol brut

L'enzyme clé de la synthèse des acides gras est l'acétyl-CoA carboxylase qui catalyse la formation du malonyl-CoA. Cette enzyme se trouve dans le cytoplasme et elle est biotine dépendante. Elle est considérée par certains auteurs comme l'étape limitante de la synthèse d'acides gras. L'acétyl-CoA provient essentiellement, pour les microorganismes oléagineux, de la production de citrate par le cycle des acides tricarboxyliques. Ce citrate est ensuite exporté vers le cytosol et transformé en acétyl-CoA et en oxaloacétate grâce à l'ATP citrate lyase, l'enzyme clé des microorganismes oléagineux. Par ailleurs, la biotine est responsable de l'activation de β-hydroxy-β-methylglutaryl-CoA, une enzyme intramitochondriale qui stimule l'accumulation des stérols et active la génération d'acétoacétate et d'acétyl-CoA. Cette partie de la thèse a donc été consacrée à l'étude de l'effet d'un ajout de biotine et de leucine sur les teneurs en lipides accumulés par *Y. lipolytica* SM7. La teneur en lipides de *Y. lipolytica* était fortement influencée par l'ajout de biotine, une augmentation de la concentration en biotine de 25 à 200 µg L^{-1} a permis d'augmenter la quantité de lipides à 15 g L^{-1}. Par ailleurs, pour canaliser le

flux métabolique dans la biosynthèse des lipides, un ajout d'acide citrique en tant que précurseur de lipides a conduit à une augmentation de l'activation du catabolisme total et de l'accumulation des lipides pour atteindre environ 63% (p/p). Il ressort donc que cette approche biochimique peut être une voie intéressante pour améliorer l'efficacité de la production de lipides.

1.4.5 Valorisation du glycérol brut et des déchets de crustacés pour la synthèse de produits à valeur ajoutée par *Y. lipolytica*

Dans cette partie de la recherche, différents types de tensioactifs et d'huiles ont été utilisés en complément du glycérol brut afin d'améliorer la production de lipides et de lipases par la levure *Y. lipolytica* SM7. Les ajouts qui ont été testés sont l'huile végétale, l'huile à moteur et certains surfactants (Tween 20, Tween 80, Triton 100) à des concentrations de 5%. Les résultats ont montré qu'un milieu de culture composé de glycérol brut et d'huile d'olive peut augmenter l'activité de la lipase jusqu'à 25 U mL^{-1} et la teneur en lipides jusqu'à 35% (p/p). La fortification du milieu avec des déchets de crustacés a augmenté l'activité de la lipase jusqu'à 38 U mL^{-1}. L'activité hydrolytique des lipases extracellulaires produites dans le milieu était satisfaisante, ceci ouvrant la voie à une utilisation dans d'autres procédés biotechnologiques.

1.4.6 Étude comparative entre les micro-ondes et les ultrasons pour la transestérification *in situ* de lipides microbiens

L'extraction usuelle des lipides à partir des microorganismes oléagineux nécessite de grandes quantités de solvants organiques. Une approche prometteuse afin de contourner ce problème consiste à réaliser l'extraction et la transestérification des lipides en biodiesel en une seule étape. Deux méthodes de transestérification *in situ* (micro-ondes et ultrasonication) ont été comparées sur la base de leurs rendements de conversion et de leur performance. Les paramètres de ces procédés ont été optimisés par une méthode des surfaces de réponses (MSR) en utilisant la technique de Box Behnken. Les paramètres étudiés étaient la température, le temps de réaction, la concentration de catalyseur, et le ratio méthanol/lipides. En utilisant la technique des micro-ondes, des rendements de 99,0 ± 0,5% de conversion des esters méthyliques (lipides convertis/lipides totaux) ont été obtenus en présence d'un ratio molaire de méthanol/lipides de 183:1 et 2% de NaOH pendant 20 min à 100°C.

En comparaison, l'utilisation de la technique des ultrasons a permis des rendements de 95,1 ± 0,2% avec le même ratio méthanol/lipides et 3% de NaOH durant 20 min à 25°C. Le profil final des esters méthyliques était entièrement compatible avec celui du procédé classique à base de chloroforme et de méthanol et l'extraction a nécessité 12 h.

1.4.7 Co-culture pour la production de lipides : développements et défis

L'utilisation de communautés microbiennes pour la production de biocarburants est devenue une approche importante parmi les procédés biochimiques courants. La co-culture a été amplement étudiée dans le but de répondre aux limitations de l'utilisation du substrat par des souches individuelles pour obtenir d'autres bioproduits. Les effets de cette stratégie sur la productivité de lipides ne sont toutefois pas bien connus. Malgré les nombreuses recherches effectuées sur la production de lipides par des microorganismes oléagineux, la stratégie de co-culture a été bien examinée seulement chez certaines algues et la plupart des études se sont attardées sur les différents modes, par exemple hétérotrophe et mixotrophe. La littérature livre peu d'informations sur les stratégies d'amélioration de la production de lipides avec d'autres espèces que les micro-algues. Cette partie de la thèse décrit plusieurs systèmes de co-culture existants pour améliorer la productivité de biomasses et de lipides chez d'autres espèces. Un aperçu des stratégies de culture des micro-algues pour la production de lipides est d'abord présenté. Par la suite, un résumé des autres stratégies rapportées dans la littérature pour d'autres espèces est exposé. Enfin, les avantages et les inconvénients de cette approche, ainsi que les possibilités de surmonter les difficultés sont examinés.

1.5 Conclusions

L'accumulation de triacylglycérols par des espèces levuriennes induite par la limitation en azote en présence de glycérol brut a été optimisée au cours des travaux de cette thèse. La mise en place d'un procédé original de culture en mode *batch* et *fed-batch* a permis de dégager différents points importants :

1. La limitation en azote chez la levure *Yarrowia lipolytica* déclenche une accumulation transitoire d'acide citrique avant le début de la synthèse d'acétyl-CoA et de l'accumulation lipidique et l'oxygène semble jouer un rôle majeur dans le maintien de l'homéostasie et la stabilisation de la production de lipides.
2. L'oxygènation influence non seulement le profil de saturation des acides gras accumulés mais aussi la morphologie et la rhéologie de la culture.
3. Les biomodulateurs semblent jouer un role crucial dans la voie *de novo* de synthèse des acides gras et de très faibles concentrations semblent acccroître la production.
4. Le développement d'une stratégie *fed batch* visant à réduire la production d'acide citrique tout en exerçant une rétroaction d'inhibition basée sur un apport de concentrations maximales d'acide citrique a permis de maintenir une dynamique d'accumulation lipidique optimale sur un substrat osidique sans production concomitante d'acide citrique. Une concentration finale en lipides de réserve de 65% constitue la meilleure performance obtenue à partir de glycérol chez *Y. lipolytica.*
5. La transestérification à l'aide de micro-ondes permet de réduire les besoins en solvant, mais des températures élevées (100°C) sont requises pour atteindre un rendement maximal de conversion. Toutefois, cette méthode peut générer une méthode certifiée d'analyse des lipides à cause de conversion complète des huiles en esters méthyliques.

1.6 Recommandations

Sur la base des résultats obtenus dans cette thèse de doctorat, les recommandations suivantes peuvent être proposées pour la suite des recherches.

1. Pour parvenir à une production optimale de TAG à partir de glycérol brut, les procédés fonctionnant en modes *fed-batch* et continu devraient être considérés.
2. *Yarrowia lipolytica* est une espèce productrice d'acide citrique. L'isolement et la purification de cet acide devraient faire l'objet de recherches plus poussées.
3. Des études complémentaires devraient être réalisées afin de mettre à profit d'autres microorganismes producteurs d'acides organiques.
4. Le potentiel des cultures mixtes pour une production maximale de TAG devrait être exploré. Une approche de co-culture en présence d'un extrait issu d'un organisme potentiellement producteur d'acides organiques tels que *Rhizopus* (acide fumarique) et *Aspergillus* (acide citrique) sera envisagée.
5. Pour atteindre un taux de production de TAG élevé, ainsi qu'une meilleure productivité, le procédé devrait être testé avec une densité initiale élevée de cellules (un plus grand volume d'inoculum).
6. La levure *Y. lipolytica* produit également des lipases dont la production pourrait être optimisée. Ce bio-produit améliorerait le potentiel d'*Y. lipolytica* pour dégrader des résidus à base d'hydrocarbures issus de l'industrie marine.
7. Un réacteur hybride comprenant des micro-ondes et des ultrasons est une option intéressante afin d'optimiser le processus d'extraction et de transestérification en une seule étape.
8. Une étude technico-économique doit être faite afin d'évaluer le coût énérgitique de bioprocédé.

1.7 Références

Abghari, A., Chen, S. 2014. *Yarrowia lipolytica* as an oleaginous cell factory platform for production of fatty acid-based biofuel and bioproducts. *Front Energy Res*, **2**, doi.org/10.3389/fenrg.2014.00021.

Adriana, G., Marius, R., Tos, M., Paizs. C., Irimie F.D. 2012. Biodiesel production using enzymatic transesterification: current state and perspectives. *Renew Energ*, **39** 10-16.

Alibeu, S. 2007. Entreprise Bio-Diesel Québec: l'huile de cuisson transformée en biodiesel dès février 2008. Caradisiac. 31 décembre.

Anastassiadis, S., Rehm, H.J. 2005. Continuous citric acid secretion by a high specific pH dependent active transport system in yeast *Candida oleophila* ATCC 20177. *Electr J Biotechnol,* **8**, 147-162.

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

Annie, D. 2006. Biofuels production, trade and sustainable development: emmerging issues. International Institute for Environment and Development, Londres, Angleterre.

Asad-ur-Rehman, R.G., Wijesekara, S., Nomura, N., Sato, S., Matsumura, M. 2008. Pre-treatment and utilization of raw glycerol from sunflower oil biodiesel for growth and 1,3-propanediol production by *Clostridium butyricum*. *J Chem Technol Biotechnol*, **83**, 1072-1080.

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

Azocar, L., Ciudad, G., Heipieper, H.J., Navia, R. 2010. Biotechnological processes for biodiesel production using alternative oils. *Appl Microbiol Biotechnol*, **88**, 621-636.

Bako, K.B., Kova, F.C.S., Gubicza, L., Hansco, J.K. 2002. Enzymatic biodiesel production from sunflower oil by *Candida antarctica* lipase in a solvent free system. *Biocatal Biotransform*, **20**, 437-439.

Ban, K., Hama, S., Nishizuka, K., Kaieda, M., Matsumoto, T., Kondo, A. 2012. Repeated use of whole cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. *J Mol Catal B Enzym*, **17**, 157-165.

Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.L., Molina-Jouve, C., Nicaud, J.M. 2009. *Yarrowia lipolytica* as a model for bio-oil production. *Prog Lipid Res*, **48**, 375-387.

Berrios, M., Skelton, R.L. 2008. Comparison of purification methods for biodiesel. *Chem Eng J*, **144**, 459-465

Blazeck, J., Hill, A., Liu, L., Knight, R., Miller, J., Pan, A. 2014. Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production. *Nat Commun,* **5**, 3131 doi: 10.1038/ncomms4131.

Boulton, C.A., Ratledge, C. 1981. Correlation of lipid-accumulation in yeasts with possession of ATP-citrate lyase. *J Gen Microb*, **127**, 169-176.

Cadoret, J.P., Bernard, O. 2008. La production de biocarburant lipidique avec des microalgues: promesses et défis. *J Soc Biol*, **202**, 201-211

Cantrell, D.G., Gillie, L.J., Lee, A.F., Wilson, K. 2005. Structure-reactivity correlations in MgAl hydrotalcite catalysts for biodiesel synthesis. *Appl Cat A: Gen*, **287**, 183-190.

Cescut, J. 2009. Accumulation d'acylglycérols par des espèces levuriennes à usage carburant aéronautique: physiologie et performance de procedés. Thèse de doctorat, Université de Toulouse, Tououse, France.

Centre de référence en agriculture et agroalimentaire du Québec, 2008, (En ligne) https://www.craaq.qc.ca/data/DOCUMENTS/EVC031.pdf. (consultée le 15 février 2017).

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

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnol Adv*, **25**, 294-306.

Choi, W.J., Hartono, M.R., Chan, W.H., Yeo, S.S. 2011. Ethanol production from biodiesel-derived crude glycerol by newly isolated *Kluyvera cryocrescens*. *Appl Microbiol Biotechnol*, **89**, 1255-1264.

Coleman, R.A., Lee, D.P. 2004. Enzymes of triacylglycerol synthesis and their regulation. *Prog Lipid Res*, **43**, 134-176.

da Silva, G.P., Mack, M., Contiero, J. 2009. Glycerol: a promising and abundant carbon source for industrial microbiology. *Biotechnol Adv*, **27**, 30-39.

Dabrowski, S., Pietrewicz-Kubicz, D., Zablotna, E., Dlugolecka, A. 2012. 1,3-propanediol production by *Escherichia coli* expressing genes of dha operon from *Clostridium butyricum* 2CR371. 5. *Acta Biochim Pol*, **59**, 357-361.

Davies, R.J., Holdsworth, J.E. 1990. The effect of low oxygen-uptake rate on the fatty-acid profile of the oleaginous yeast *Apiotrichum curvatum*. *Appl Microbiol Biotechnol*, **33**, 569-573.

Dry, M.E. 1996. Practical and theoretical aspects o f the catalytic Fischer-Tropsch process. *Appl Catal A*: General, **38**,319-344

Ergün, N., Panning, P. 2002. Method for producing fatty acid methyl ester and equipment for realizing the same. Brevet américain No. US 6,440,057.

Evans, C.T., Ratledge, C. 1983. A comparison of the oleaginous yeast *Candida curvata*, grown on different carbon sources in continuous and batch culture. *Lipid*, **18**, 623-629.

Fakas, S., Papanikolaou, S., Batsos, A., Galiotou-Panayotou, M., Mallouchos, A., Aggelis, G. 2009. Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*. *Biomass Bioenergy*, **33**, 573-580.

Freedman, B., Butterfield, R.O., Pryde, E.H. 1986. Transesterification kinetics of soybean oil. *JAOCS*, **63**, 1375-1380.

Freedman, B., Pryde, E.H., Mounts, T.L. 1984. Variables affecting the yields of fatty esters from transesterified vegetable oils. *JAOCS*, **61**, 1638-1643.

Fukuda, H., Kondo, A., Noda, H. 2001. Biodiesel fuel production by transesterification of oils. *J Biosci Bioeng*, **92**, 405-416.

Garlapati, V.K., Shankar, U., Budhiraja, A. 2016. Bioconversion technologies of crude glycerol to value added industrial products. *Biotechnol Rep,* **9**, 9-14.

Goh, S.H., Yeong, S.K. 1993. Transesterification of cocoa butter by fungal lipases: effect of solvent on 1,3-specificity. *J Am Oil Chem Soc*, **70**, 567-570.

Gonzales, A.D.C. 2008. Overall stocktaking of biofuel development in Asia-Pacific: benefits and challenges. Background paper for “Policy Dialogue on Biofuels in Asia: Benefits and Challenges,” Beijing, Chine, 24 au 26 september 2008.

Granger, L.M., Perlot, P., Goma, G., Pareilleux, A. 1993. Effect of various nutrient limitations on fatty acid production by *Rhodotorula glutinis*. *Appl Microbiol Biotechnol*, **38**, 784-789.

Guerrero-Pérez, M.O., Rosas, J.M., Bedia, J., Rodríguez-Mirasol, J., Cordero, T. 2009. Recent inventions in glycerol transformations and processing. *Recent Patents Chem Eng*, **2**, 11-21.

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

Hassan M., Blanc P.J., Granger L.-M., Pareilleux A., Goma G. 1993. Lipid production by an unsaturated fatty acid auxotroph of the oleaginous yeast *Apiotrichum curvatum* grown in a single stage continuous culture. *Appl Microbiol Biotechnol*, **40**, 483-488.

Hua, Y., Xin, Z., Jin, Z., Sufang, Z., Zongbao, Z. 2007. Lipid production by *Rhodosporidium toruloides* using Jerusalem artichoke tubers. *Chin Biotechnol*, **27**, 59-63.

Jeennor, S., Laoteng, K., Tanticharoen, M., Cheevadhanarak, S. 2006. Comparative fatty acid profiling of *Mucor rouxii* under different stress conditions. *FEMS Microbiology Letters*, **259**, 60-66.

Jitputti, J., Kitiyanan, B., Rangsunvigit, P., Bunyakiat, K., Attanatho, L., Jenvanitpanjakul, P. 2006. Transesterification of crude palm kernel oil and crude coconut oil by different solid catalysts. *Chem Eng J*, **116**, 61-66.

Johnson, D.T., Taconi, K.A. 2007. The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environ Prog,* **26**, 338-348.

Kalam, A.A., Yousuf, A., Ferdoush, A., Hasan, M.M., Karim, M.R., Jahan, A. 2014. Production of microbial lipids from rice straw hydrolysates by *Lipomyces starkeyi* for biodiesel synthesis, *J Microb Biochem Technol*, S8:008. doi:10.4172/1948-5948.S8-008.

Kamzolova, S.V., Fatykhova, A.R., Dedyukhina, E.G., Anastassiadis, S.G., Golovchenko, N.P., Morgunov, I.G. 2011. Citric acid production by yeast grown on glycerol-containing waste from biodiesel industry. *Food Technol Biotech*, **49**, 65-74.

Keim, G.I. 1943. Treating fats and fatty oils. Brevet américain No. US 2,383,601.

Kerr, B.J., Dozier, W.A., Bregendahl, K. 2007. Nutritional value of crude glycerin for nonruminants. Dans: Proceedings of the 23rd Annual Carolina Swine Nutrition Conference, Raleigh, NC, États-Unis, pp. 6-18.

Kessell, R.H.J. 1968. Fatty acids of *Rhodotorula gracilis*: fat production in submerged culture and particular effect of pH value. *J Appl Bacteriol*, **31**, 220-231.

Khan, S.A., Rashmi, Hussain, M.Z., Prasad, S., Banerjee, U.C. 2009. Prospects of biodiesel production from microalgae in India. *Renew Sustain Energy Rev*, **13**, 2361-2372.

Kim, H.-J., Kang, B.-S., Kim, M.-J., Park, Y.M., Kim, D.-K., Lee, J.-S., Lee, K.-Y. 2004. Transesterification of vegetable oil to biodiesel using heterogeneous base catalyst. *Catal Today,* **93**, 315-320.

Knothe, G. 2010. Biodiesel and renewable diesel: a comparison. *Prog Energy Combust Sci*, **36**, 364-373.

Kraisintu, P., Yongmanitchai, W., Limtong, S. 2010. Selection and optimization for lipid production of a newly isolated oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16. *Kasetsart J Nat Sci*, **44**, 436-445.

Laoteng, K., Anjard, C., Rachadawong, S., Tanticharoen, M., Maresca, B., Cheevadhanarak, S. 1999. *Mucor rouxii* Δ9-desaturase gene is transcriptionally regulated during cell growth and by low temperature. *Mol Cell Biol Res Commun*, **1**, 36-43.

Li, Y.H., Zhao, Z., Bai, F. 2007. High density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme Microb Technol*, **41**, 312-317.

Libkind, D., Arts, M.T., van Broock, M. 2008. Fatty acid composition of cold adapted carotenogenic basidiomycetous yeasts. *Argent Microbiol*, **40**, 193-197.

López, D.E., Goodwin, J.J.G., Bruce, D.A. 2007. Transesterification of triacetin with methanol on Nafion acid resins. *J Catal*, **245**, 381-391.

Martyanov, I.N., Sayari, A. 2008. Comparative study of triglyceride transesterification in the presence of catalytic amounts of sodium, magnesium, and calcium methoxides. *Appl Catal A-Gen*, **339**, 45-52.

Meesters, P.A.E.P., Huijberts, G.N.M., Eggink, G. 1996. High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. *Appl Microbiol Biotechnol*, **45**, 575-579.

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

Michinaka, Y., AKI, T., Shimauchi, T., Nakajima, T., Kawamoto, S., Shigeta, S., Suzuki, O., Ono, K. 2003. Differential response to low temperature of two Ä6 fatty acid desaturases from *Mucor circinelloides*. *Appl Microbiol Biotechnol*, **62**, 362-368.

Muniyappa, P.R., Brammer, S.C., Noureddini, H. 1996. Improved conversion of plant oils and animal fats into biodiesel and co-product. *Bioresour Technol*, **56**, 19-24.

Naveed, S., Ramzan, N., AshraÇ A., Usman, J., Shabbir, H.A.S. 2010. Modelling and simulation of coal gasification plant. *J Pakistan Inst Chem Eng*, **38**, 1-6

Narasimharao, K., Brown, D.R., Lee, A.F., Newman, A.D., Siril, P.F., Tavener, S.J., Wilson, K. 2007. Structure-activity relations in Cs-doped heteropolyacid catalysts for biodiesel production. *J Catal,* **248**, 226-234.

Organisation de Coopération et de Développement Économiques (OCDE) (2007). Biocarburants pour les transports: politiques et possibilités, (En ligne). http://www.oecd.org/dataoecd/2/40/39743323.pdf (Page consultée le 15 Février 2017).

Pachauri, N., He, B. 2006. Value-added utilization of crude glycerol from biodiesel production: a survey of current research activities. Dans: Proceedings of the ASABE Annual International Meeting, Portland, Oregon, États-Unis, 9 au 12 juillet.

Papanikolaou, S., Aggelis, G. 2002. Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. *Bioresour Technol*, **82**, 43-49.

Papanikolaou, S., Chevalot, I., Komaitis, M., Marc, I., Aggelis, G. 2002. Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. *Appl Microbiol Biotechnol,* **58**, 308-312.

Ranganathan, S.V., Narasimhan, S.L., Muthukumar, K. 2008. An overview of enzymatic production of biodiesel. *Bioresour Technol*, **99**, 3975-3981.

Ratledge, C. 1988. Biochemistry, stoichiometry, substrates and economics. Dans: Moreton, R. S. (Ed.), Single Cell Oil. Longman Scientific & Technical, Harlow, Royaume-Uni, pp. 33-70.

Rossi, M., Buzzini, P., Cordisco, L., Amaretti, A., Sala, M., Raimondi, S., Ponzoni, C., Pagnoni, U.M., Matteuzzi, D. 2009. Growth, lipid accumulation and fatty acids composition in obligate psychrophilic facultative psychrophilic and mesophilic yeasts. *FEMS Microbiol Ecol,* **69**, 363-372.

Ressources naturelles Canada, 2015, Le marché Nord-Américain du gaz naturel : Perspectives sur la saison de chauffage 2015-2016. (Consultée le 15 Février 2017).

Saenge, C., Cheirsilp, B., Suksaroge, T.T., Bourtoom, T. 2011. Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochem,* **46**, 210-218.

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

Sarma, S.J., Brar, S.K., Sydney, E.B., Bihan, Y.L., Buelna, G., Soccol, C.R. 2012. Microbial hydrogen production by bioconversion of crude glycerol: A review ?. *Int J Hydrogen Energy*, 37, 6473-6490.

Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B. 2008. Second génération biofuels: high-efficiency microalgae for biodiesel production. *Bioenergy Ressource*, **1**, 20-43.

Schwab, A.W., Bagby, M.O., Freedman, B. 1987. Preparation and properties of diesel fuels from vegetable oils, *Fuel*, **66,** 1372-1378.

Shay, E.G., Griffin, E. 1993. Diesel fuel from vegetable oils: status and opportunities. *Biomass Bioenergy*, **4**, 227-242.

Shibasaki-Kitakawa, N., Honda, H., Kuribayashi, H., Toda T., Fukumura, T., Yonemoto, T. 2007. Biodiesel production using anionic ion-exchange resin as heterogeneous catalyst. *Bioresour Technol,* **98**, 416-421.

Shimada, Y., Watanabe, Y., Sugihara, A., Tominaga, Y. 2002. Enzymatic alcoholysis for biodiesel fuel production and application of the reaction oil processing. *J Mol Catal B: Enzym*, **17**, 133-142.

Singhabhandhu, A., Tezuka, T. 2010. A perspective on incorporation of glycerin purification process in biodiesel plants using waste cooking oil as deedstock. *Energy*, **35**, 2493-2504.

Sorger, D., Daum, G. 2002. Synthesis of triacylglycerols by the acyl-coenzyme A: diacyl-glycerol acyltransferase Dga1p in lipid particles of the yeast *Saccharomyces cerevisiae*. *J Biol Chem*, **184**, 519-524.

Stavarache, C., Vinatoru, M., Maeda, Y. 2007. Aspects of ultrasonically assisted transesterification of various vegetable oils with methanol. *Ultrason Sonochem*, **14**, 380-386.

Statistics Canada, 2011. Energy statistics handbook: First quarter 2011. pp. 1-170, http://www.statcan.gc.ca/pub/57-601 -x/57-601-x2011001-eng.pdf (consultée le 15 Février 2017)

Tramoy, P. 2008. Review on the biofuel market, [En ligne]. http://www.lifescienceonline.com/articles.html?portalPage=Lifescience+Today.Articles&a=1016 (Page consultée le 15 Février 2017).

Vicente, G., Martinez, M., Aracil, J. 2004. Integrated biodiesel production: a comparison of different homogeneous catalysts systems. *Bioresour Technol*, **92**, 297-305.

Wang, L., Du, W., Liu, D.H., Li L.L. 2006. Lipase-catalyzed biodiesel production from soybean oil deodorizer distillate with absorbent present in tert-butanol system. *J Mol Catal B Enzym*, **43**, 29-32.

Widjaja, A., Chien, C.-C., Ju, Y.-H. 2009. Sudy of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. *J Taiwan Inst Chem Engineers*, **40**, 13-20.

Workman, M., Holt, P., Thykaer, J. 2013. Comparing cellular performance of *Yarrowia lipolytica* during growth on glucose and glycerol in submerged cultivations. *AMB Express*, **3**, 1-9.

Wu, S.T., Yu, S.-T., Lin, L.-P. 2005. Effect of culture conditions on docosahexaenoic acid production by *Schizochytrium* sp. S31. *Process Biochem*, **40**, 3103-3108.

Xie, W., Peng, H., Chen, L. 2006. Transesterification of soybean oil catalyzed by potassium loaded on alumina as a solid-base catalyst. *Appl Catal A-Gen*, **300**, 67-74.

Xue, F.Y., Miao, J., Zhang, X., Luo, H., Tan, T. 2008. Studies on lipid production by *Rhodotorula glutinis* fermentation using monosodium glutamate wastewater as culture medium. *Bioresour Technol*, **99**, 5923-5927.

Yamauchi, H., Mori, H., Kobayashi, T. 1983. Mass production of lipids by *Lipomyces starkeyi* in microcomputer-aided fed-batch culture. *J Ferment Technol,* **61**, 275-280.

Yazdani, S.S., Gonzalez, R. 2007. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. *Curr Opin Biotechnol*, **18**, 213-219.

Zhao, X., Wu, S., Hu, C., Wang, Q., Hua, Y., Zhao, Z.K. 2010. Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides* Y4. *J Ind Microbiol Biotechnol*, **37,** 581-585.

2 CHAPITRE 2 : HETEROTROPHIC MICROORGANISMS: A PROMISING SOURCE FOR BIODIESEL PRODUCTION

Heterotrophic microorganisms: a promising source for biodiesel production

Magdouli S.[1], Yan S. [1], Tyagi R. D.[1] and R. Y. Surampalli [2]

[1] Université du Québec, Institut national de la recherche scientifique, Centre Eau, Terre & Environnement, 490 de la Couronne, Québec (QC), G1K 9A9, CANADA

[2] U. S. Environmental Protection Agency, P.O. Box 17-2141, Kansas City, USA, KS 66117

* Corresponding author:

Tel: (418) 654-2617; Fax: (418) 654-2600

E-mail: tyagi@ete.inrs.ca

Critical Reviews in Environmental Science and Technology (2014) 44, 416-453

2.1 Résumé

Dans cet article, la production de biodiesel par des microorganismes hétérotrophes tels que bactéries, levures et moisissures est discuté. Ces microorganismes oléagineux sont reconnus pour leur capacité d'accumulation de triacylglycérols (TAG) qui contiennent des acides gras à longue chaîne. L'ensemble des données suggère que les TAG accumulés sont appropriés pour la production de biodiesel. L'accumulation de lipides requiert une cascade d'enzymes et l'expression de différents gènes et sa régulation est complexe. Les connaissances actuelles sur les espèces oléagineuses hétérotrophes, les acides gras, les TAG, ainsi que la biosynthèse des stérols à l'aide d'approches biochimiques et génétiques sont abordées afin de mieux comprendre cette régulation. De même, l'impact des facteurs abiotiques pouvant contribuer à l'augmentation de l'accumulation de lipides est résumé. Cette revue met également en évidence diverses questions concernant le génie génétique pouvant contribuer à faire avancer les techniques de production de biodiesel à partir de microorganismes.

Mots-clés : microorganismes oléagineux, biodiesel, lipides, régulation, modification génétique

2.2 Abstract

In this paper, occurrence of heterotrophic microbes specifically, bacteria, yeast and molds, in biodiesel production is well discussed. Besides, these oleaginous microorganisms are reported to accumulate triacylglycerols (TAGs) that contain long-chain fatty acids. Most of the evidence suggests that TAGs accumulated are suitable for biodiesel production. This article takes a step in this direction and provides a summary of the actual knowledge on oleaginous species, their fatty acids, TAGs and sterol biosynthesis with a biochemical and genetic approaches to better understand their regulation. Likewise, the impact of abiotic factors that can contribute to higher lipid accumulation is summarized. This review also highlights various issues concerning genetic engineering that may contribute to an advanced path forward for microbial feedstock-based biodiesel.

Keywords: oleaginous microorganisms, biodiesel, lipids, regulation, genetic engineering

2.3 Introduction

Extensive studies have been currently carried out to find new value added products with higher competitiveness and affordable costs in order to replace petroleum-derived diesel. In fact, greenhouse gas (GHG) emissions from fossil fuel and further their disposal are believed to induce global warming (Hill et al., 2009). In order to minimise the use of fossil fuels, renewable resources based on vegetable materials such as palm oil (Darnoko & Cheryan, 2000), canola oil (Kulkarni et al., 2006), straws of rice (Barnwal & Sharma, 2005), cotton seed (Ko¨se et al., 2002), sunflower (Mohamed et al., 2003), rapeseed (Kusdiana & Saka, 2001), sugarcane (bagasse) (Robles-Medina et al., 2009) are commonly used for diesel fuel alternatives (biodiesel). However, plant-based production is insufficient to meet the massive global demand and the sudden increase of raw material's costs (Miao & Wu, 2006).

To cope with these challenges: reducing the environmental impact of GHG emissions, decreasing pressures for the dependence on vegetable oils and maximizing the use of renewable resources, many concerns have been raised to find new feedstocks for biodiesel production. Generally, biodiesel is obtained by transesterification of fatty feedstocks with short-chain alcohols, via methanolysis or ethanolysis, resulting in the conversion of TAGs into mono-alkyl esters of long-chain fatty acids (chain length C14–C22). Oleaginous microorganisms, referred to microorganisms that accumulate more than 20% of their cellular dry weight (CDW) as lipids, may represent a promising alternative source (Ratledge, 2004). Most of the lipids accumulated are triacylglycerols (TAGs) with long chain of fatty acids. Routinely, biodiesel is derived from the transesterification of TAGs yielding to fatty acid methyl esters (FAMEs) and fatty acids-ethyl-esters (FAEEs) (Nomura et al.). Best candidates for TAGs production are microalgae, bacteria, molds and yeast. The focus here will be the biodiesel production by heterotrophic microorganisms. Other reviews that cover aspects of microalgal diesel production have been reported in (Hu et al., 2008; Tabatabaeia et al., 2011). According to Pinzi et al., ideally biodiesel must contain large quantities of monounsaturated fatty acids, small quantities of polyunsaturated fatty acids as well as controlled quantities of saturated fatty acids (Pinzi et al., 2009). Commonly, many FAMEs were usually found in biodiesel such as methyl palmitic, methyl stearate, methyl oleate, methyl linoleate and methy linolenate (Ma & Hanna, 1999). Actually, the analysis of fatty acid composition derived from heterotrophic microorganisms suggested that FAMEs and FAEEs have been identical to those found in petroleum diesel and competitive to conventional vegetable oils (Kraisintu et al., 2010; Li et al., 2007). Therefore, FAEEs, homologue of FAMEs, resulting from ethanolysis are advantageous in term of having high heat capacity and high cetane number

inspite of the highest price of ethanol. Another important advantage in the use of ethanol is that the ethyl esters have cloud and pour points that are lower than FAMEs (Shi & Bao, 2008).

Fatty acids-producing microorganisms accumulate lipids as a stress response when growth becomes limited due to the exhaustion of the nitrogen source or other nutrient but an excess of the carbon source remains (Jakobsen et al., 2008; Murphy, 2001). The variety of fatty acids provided by microbes were stored in specialized compartment in the form of TAGs and steryl esters (SE) that can also store carotenoid pigments especially in red yeast (Davoli et al., 2004). Lipid storage can either result from *de novo* synthesis, implicating the enzymes of the Kennedy pathway (Kennedy, 1956) or the *ex novo* pathway in the presence of hydrophobic substrates. Microbial lipophilic compounds resulted were called single cell oils (SCO). Recent advances have focused on improving fatty acids production and composition either with diverting the pool of acetyl-CoA towards fatty acid biosynthesis or on decreasing consumption of fatty acids. This approach can be adjusted by manipulating key regulators of the biosynthesis of TAGs and fatty acids through genetic and metabolic engineering. Thus, variation of chain length and modifying unsaturation level of fatty acids become feasible in order to get ideal fuel that comply with the existing standards and respect the recommended qualities such as higher oxidative stability and higher heating value. In this review, an overview of the current researches on heterotrophic microorganisms to accumulate fats and lipids, mainly in last decade, is illustrated; major strains are listed and principle tools for their identification including the regulatory mechanism of accumulation is discussed. Also, this paper highlights an understanding of molecular regulation and the influence of environmental factors on the accumulation process. Finally, some critical issues including current perspectives of microbial biodiesel are provided.

2.4 Heterotrophic microorganisms

Heterotrophic microorganisms including bacteria, yeast and fungi are ubiquitous, usually isolated from soil (Kraisintu et al., 2010; Leesing & Baojungharn, 2011; Liu et al., 2010; Pan et al., 2009), plants (in this case called endophytic microorganisms) (Dey et al., 2011; Peng & Chen, 2008), as well as aquatic environment ecosystem (Ivanova et al., 2005; Libkind et al., 2008; Satomi et al., 2003). Thus, screening and identifying other accumulating oil microorganisms from new sources is an attractive area of research. Most reliable methods for identification depends on various biochemical and metabolic tests namely%GC, morphological structure, cell composition, utilization of carbon sources, antibiotic resistance and production of secondary metabolites. This approach was previously crucial as first step to identify some oil-producing bacteria like marine ones, *Shewenella* and *Altermonas sp.* (Ivanova et al., 2005; Satomi et al., 2003). Likewise, the usefulness of chemotaxonomic studies have been proven to be a great informative marker for microbial screening, for instance, proteins or polyamine profiles, isoprenoid or quinone systems and phospholipid fatty acids (PLFAs) (Ivanova et al., 2005). The earliest system for microbial species classification relied on PLFA profile, however, PLFA composition of microorganisms is known to vary with cultivation conditions and cell activity such as carbon and oxygen concentrations, pH and temperature (Haack et al., 1994; Ratledge & Wilkinson, 1989). Consequently, PLFA profile cannot be considered as a reliable method for screening. Due to these limitations, molecular tools are required for identification to give reliable fingerprints of oleaginous bacterial communities and offer more credibility since they are based on the rDNA gene sequence comparison (Woese et al., 1984). The sequencing of the 16S rRNA gene together with the determination of metabolic profile allowed credible phylogenetic classification. For the identification of both yeast and fungi, macromorphological and micromorphological tests are the most updated keys for identification. It contains morphological characteristics such as colony growth, temperature tolerance, presence or absence of aerial mycelium, colony colour, presence of wrinkles and furrows, pigment production, fermentation and assimilation of carbohydrates. Likewise, microscopic study is needed for more screening of fungal spores and mycelial characters. Additionally, molecular analysis to the extent of nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA (rDNA) and the amplification of 5.8S ribosomal gene is performed for identification of newly isolated strains (He et al., 2010; Kraisintu et al., 2010; Pan et al., 2009). Molecular analysis is usually recommended after qualitative analysis with the Sudan Black B staining to identify lipid droplets (Kimura et al., 2004).

Finally, biomass concentration (g/L), lipid yield (g/L), lipid content (% w/w), volumetric lipid production rate (g/L/day), specific substrate consumption rate (g substrates/g cells/day) and growth rate (μ) are more essential kinetic parameters for screening of lipid producing capacities of oleaginous yeast and fungi as well as bacteria. Scaling up the fermentation process and stability of the strains to yield high lipids at large scale is also an essential requirement.

2.4.1 Bacteria

Generally, accumulation of polyhydroxyalkanoates (PHA) such as polyhydroxy-butyrate (PHB) (Alvarez, 2003) for energy storage is widespread among bacterial species, however, the accumulation of TAGs (lipids storage) is found in most eukaryotic microorganisms and there seems to be only few groups of prokaryotic microorganisms belonging to *Actinomycetes* group such as *Streptomyces, Nocordia, Rhodoccocus, Mycobacterium,* and *Gordonia*. During the exponential growth phase, fatty acids are synthesized in need for the biosynthesis of phospholipids which are essential for cell proliferation and membrane composition. *Actinomycetes* exhibited higher lipid content up to 70% (w/w) and accumulate intracellular PHA as well as TAGs under restricted growth conditions (Chisti, 2007). Conversely, for Gram negative strains, *Acinetobacter* was reported as the only genus that was able to accumulate both intracellular and extracellular TAGs, mono- and di-acylgylcerols, free fatty acids and wax esters during cultivation on *n*-alkanes (Alvarez & Steinbu¨chel, 2002). This group is known for its ability to catalyze acyltransfer to make wax esters and triacylglycerols. Additionally, *Pseudomonas* species like *P. aeruginosa* strain 44T1 are able to accumulate TAGs (Andrs et al., 1991), however, its capacity for lipid accumulation is under study.

Actually, gram positive bacteria were assumed to accumulate more lipids compared to gram negative species, and studies have been reported this difference due to the difference in regulatory system and accumulation pathway (Kosa & Ragauskas, 2011). Therefore, further studies are required to confirm these results in bacterial populations and identify the potential mechanisms of interaction. Finally, in marine environment, some aquatic strains have shown their ability to store up to 0.06 g/g (w/w) of eicosapentaenoic acid of polyunsaturated TAGs, for instance: *Alteromonas sp., Shewanella sp., Flexibacter sp., Vibriosp.*(Ivanova et al., 2005; Satomi et al., 2003).

2.4.2 Fungi

Fungi, which include yeast and filamentous fungi, were placed under the common umbrella of oleaginous microorganisms. Yeast, unicellular fungal microorganisms, has been reported to produce higher levels of lipids over 20% CDW. Their duplication time (less than one hour), less sensibility towards environmental conditions and their ease to cultivate compared to microalgae lead them to be potentially crucial for biodiesel production. Among 600 species of known yeast, only 25 of them are known to be oleaginous. Additionally, accumulated lipids present large amount of TAGs up to 90% (Ma, 2006), with a small fraction of steryl ester (SE) and high fraction of polyunsaturated fatty acids (PUFA) (Papanikolaou et al., 2001). Actually, red yeast are known to produce especially high amounts of PUFA (more than 50%) and they mostly belong to *Basidiomycota* phylum and *Sporidiobolales* group. The important yield of PUFA is mainly an indicative of cold adapted metabolism and thermal regulation (Davoli et al., 2004; Libkind et al., 2008). Many species have been investigated in lipid production due to their ability to metabolize pentoses and produce TAGs from lignocellulosic biomass (Papanikolaou et al., 2002; Zhao, 2005) such as, *Rhodotorula graminis, Rhodosporidium toruloides*, *Candida curvatus, Lipomyces starkeyi, Rhodotorula graminis, Trichosporon fermentas, Trichosporon cutaneum* and *Cryptococcus curvatus* (Angerbauer et al., 2008; Papanikolaou & Aggelis, 2003; Ratledge & Wynn, 2002; Wang et al., 2005; Zhu et al., 2008) (Table 2.1). The following lipid content (weight %) has been observed in various species: *Candida curvata* 58%, *Cryptococcus albidus* 65%, (Meng et al., 2009), in *Cryptococcus curvatus* 58% (Ratledge & Wynn, 2002), in *Lipomyces starkeyi* 68% (Angerbauer et al., 2008) and in *Rhodosporidium toruloides* 58% (Liu & Zhao, 2007). Additionally, some oleaginous yeast such as *Rhodotorula glutinis* can accumulate carotenoids rather than lipids (Somashekar & Joseph, 2000).

Moreover, more attention has been given to filamentous fungi. Their broad range of lipid accumulation is attributed to the strong extracellular lignolytic enzymes such as lignin peroxidase, manganese-dependent peroxidase, laccase invertase and xylanase (Schulz et al., 2002). Additionally, they can be grown on a variety of starting materials (substrates) such as waste lignocellulosic materials and production can be scaled in fermentation process to produce more lipid biomass. Fungi can accumulate substantial amounts of TAG and SE when cells are cultivated in the presence of high concentrations of sugars usually glucose and their growth are limited by the deficiency of the nitrogen, phosphorus or sulphur in the growth media. Most fungi accumulate over 25% (w/w) lipid. Among them, the commonly found genera are *Zygomycetes* group that include: *Zygosaccharomyces rouxii, Zygorhynchus moelleri*, *Cunninghamella echinulata,*

Rhizopus stolonifer, Mortierella ramanniana, M. vinacea, M. isabellina, Trichosporan elegans, Pichia membranifaciens, Candida boidinii, C. curvata, C. oleophila, C. pulcherrima, Mucor sp., Rhodotorula sp. (Table 2.2). Kavadia et al. (Kavadia et al., 2001) showed that accumulation of lipid in *Zygomycetes* group started after exhaustion of nitrogen source. However, in *Z. moelleri* 1703, *M. ramanniana, M. isabellina* and *R. stolonifer*, the accumulated oil was used as carbon source after depletion of glucose in the medium, a re-consumption of accumulated oil occurred and synthesis of free cell material was observed (Kavadia et al., 2001). That could be explained as lipid degradation and occurrence of fats as carbon source in oil production (Aggelis & Sourdis, 1997). This also may be considered as a regulatory system for storage lipid degradation in microorganisms growing on fats. Thus, limiting nutrient factors such as nitrogen and magnesium are applied as tools in an attempt to control this mechanism (synthesis of lipids) (Kavadia et al., 2001).

Recently, it is reported that many filamentous fungi are able to accumulate high levels of lipids (on weight basis) *: Humicola lanuginose* up to 75% (Meng et al., 2009), *M. isabellina* up to 86%, *Cuninghamella japonica* up to 60% (Ratledge, 1982), *Asperillus oryzae* up to 57% (Meng et al., 2009), *Aspergillus terreus* up to 57% (Evans & Ratledge, 1983), *Muccor rouxii* up to 30% (Somashekar et al., 2003), *Absidia corymbifera* up to 27% (Ratledge, 1982), *Entomopthora coronate* – up to 43% (Ratledge, 1982), *Aspergillus sp.* up to 23.3% (Subhash & Mohan, 2011). *Mucor circinelloides* up to 19.9% (Ratledge, 2004; Vicente et al., 2009) was used first time for commercial lipid production. Generally, filamentous fungi accumulate high levels of PUFA such linoleic acid (C18:2), oleic acid (C18:1), palmitic acid (C16:0) (Kavadia et al., 2001; Somashekar et al., 2003). The accumulation is enhanced via ATP, citrate lyase action that catalyses cleavage of citrate in the presence of CoA and ATP to acetyl CoA and oxaloacetate. Many filamentous fungi are endophytic such as *Microsphaeropsis sp.,* which is isolated from the oleaginous plant *Sabina chinensis.* These fungi are used to produce biodiesel in solid state fermentation with wheat straw as substrates (Peng & Chen, 2008). Their commercial use could reduce the production cost of lipids. Furthermore, the fungal endophyte *Gliocladium roseum* (NRRL 500072) was obtained from *Eucryphia cordifolia* plant and possesses the ability to produce volatile hydrocarbons (Strobel et al., 2008). Additionally, *Alternaria sp*. (DM09) *and Colletotrichum sp.* (DM06) are two endophytic fungi isolated from plant and could accumulate lipid up to 58.1% and 46.8% respectively (Dey et al., 2011).

Various lipids are implicated in metabolic processes and gene transcription and thus can be divided in two groups; lipids derived from fatty acids and other group from sterols.

2.5 Classification of lipids

Due to the variety of lipids and the difficulty to adopt a universal definition, there is no single classification of lipids. The most adopted classification of lipids is based on their polarities. As their name suggest, polar lipids have a hydrophilic portion that allows them to play a leading role at the interfaces in living organisms.

2.5.1 Fatty acids

Generally, fatty acids are almost entirely straight chain aliphatic carboxylic acids with a skeleton alkane (R) and terminal carboxylic acid (-COOH). They are distinguished from each other by the length of hydrocarbon chain, number of double bonds, degree of saturation, location, configuration (either *cis* or *trans*) and possible modifications like hydroxylation, epoxidation. For their biosynthesis, the chain is built from tow carbon units and cis double bonds are inserted by desaturases enzymes at specific positions relative to the carboxyl group. Most of the fatty acids are amphiphilic, i.e. they possess both hydrophobic and hydrophilic areas causing the formation of surface active compounds. Fatty acids may act as messengers in the process of signal translation and molecular recognition (VanMeer et al., 2008). Saturated fatty acids have a straight hydrocarbon chain and the melting point increases with chain length and decreases with unsaturation (Rossi et al., 2009).

2.5.2 Phospholipids

Phospholipids are composed of a glycerol backbone containing acyl chains linked by an ester bond. These amphiphilic molecules with hydrophobic part interact with aqueous environment to lead to the formation of bilayers that, consequently, form the biological membrane. These membranes perform many functions: allow cells a great separation between internal components and external environment, organelle's differentiation, transport of chemical reactions, modulation of the transport of amino acids. The basic structure of the anchor proteins is a derivative of phosphatidyl inositol. The most represented phospholipids in plasma membrane are phosphatidylcholin (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). This diversity is generated by the large number of possible combinations of different polar heads (choline, ethanolamine, inositol and serine) with different acyl chains. The size of the polar head

determines the state of membranes which is generally liquid-crystalline in most organizations (Zweytick et al., 2000a).

2.5.3 Sphingolipids

They are present in the plasma membrane but at very low concentration. They originate from sphingosine and are formed by binding of carboxylic acid fat on the sphingosine-NH_2. Sphingolipids of yeast contain mainly phytosphingosine. Its structural similarity with the phosphatidylcholine (PC) gives them similar properties.

2.5.4 Glycerides, acylglycerols

They are esters of fatty acids and glycerol including monoglycerides (MAG), diglycerides (DAG) and triglycerides (Nomura et al.). These fats are known as neutral lipids. They are able to form membranes in solution. DAG can be used by the cell via Kennedy pathway (Kennedy, 1956) to give rise to phosphatidyl ethanolamine and phosphatidyl choline. Small quantities of diacyl and monoacylglycerol are presented in yeast and their amounts range from 0 to 5% of total lipids. For TAGs, they represent 0 to 15% of total lipids; they are precursors for biosynthesis of phospholipids, while, MAG and DAG are considered as emulsifiers.

2.5.5 Sterols

They are a family of lipids derived from cholesterol nucleus. Chemically, they are composed by three hexagonal cycles with a pentagonal one that correspond for cyclo-pentanoperhydrophenanthene. They are present mainly in the outer layer of the plasma membrane. Sterols are considered as regulators of member's fluidity, they maintain an almost constant flow across the membrane in a wider temperature range. In yeast, ergosterol, that is about 50% of total sterols, is commonly found in free form and associated with the plasma membrane. Ergosterol also plays important roles in the membrane trafficking, cell signalling, endocytosis, and regulation of enzymatic activities of membranes (Bagnat et al., 2000; Munn & Riezman, 1994).

Their biosynthesis can be summarized in three steps namely **i)** biosynthesis of mevalonate**, ii)** conversion of mevalonate into hydrocarbon chain, namely squalene and finally, **iii)** cyclisation of

squalene into lanosterol. Among principle sterols in yeast, there are squalene; lanosterol and ergosterol.

2.6 Pathway of synthesis

Oleaginous microorganisms synthesize lipid by two pathways either *de novo* or *ex novo* pathways.

2.6.1 De novo pathway

In *de novo* pathway, lipids are formed in the presence of excess carbon source (Daum et al., 1998). This pathway starts with acetyl CoA carboxylase (ACC) which catalyzes the important step of the fatty acid synthesis that consists of biotin-dependent carboxylation of acetyl-CoA to malonyl CoA; the rate-limiting step in fatty acid biosynthesis (Cronan & Waldrop, 2002; Davis et al., 2000). In *E. coli,* ACC contain four enzyme subunits encoded by genes *accAB*CD at different chromosomal locations (Li & Jr, 1993). In yeast and filamentous fungi, ACC contains biotin carboxylase, carboxyltransferase and biotin carboxyl carrier protein domains, and it is involved in the cytosolic fatty acid synthesis. The multifunctional ACC of yeast and fungi catalyzes the carboxylation of acetyl-CoA yielding malonyl-CoA.

They both possess cytosolic and mitochondrial ACC (*Acc1*) (Tehlivets et al., 2007). Once malonyl CoA is synthesized, it is transferred by malonyl CoA: ACP (Acyl Carrier Protein) transacetylase to the ACP of the fatty acid synthase (FAS). In eukaryotic microorganisms; yeast and molds as well as prokaryotes, two FAS systems were found (FASI, FAS II). These cycles are well illustrated in Figure 2.1.

In yeast and molds, FASI is cytosolic composed by two subunits 3α and 3β, arranged in hexamer (α6β6) and encoded by fas2 and *fas1* (Verwoert et al., 1995). In prokaryotes, FASII is an individual enzyme, where subunit β catalyzes the transfer of malonate to ACP on subunit α. The subunit α is implicated in steps of condensation of malonyl-ACP and acyl-ACP, decarboxylation and reduction of β-keto-acyl-ACP (KS). On the other hand, the subunit β is implicated in steps of deshydratation and reduction (Schweizer et al., 1986). Furthermore, yeast needs phosphopantetheine transferase (PPT) to activate ACP on subunit β (E. Schweizer & Hofmann, 2004). Yeast load acetyl CoA to KS through ACP before condensation, after that, FAS catalyzes KS. FAS is reported to be responsible for adding carbons to fatty acids with the generation of

CO_2. Subsequently, β-cetonic group generated from condensation is eliminated after two reductions and one dehydration step. In each cycle, two carbons are added until palmitoyl-ACP is formed for seven cycles. The elongation step depends on intracellular concentration of acetyl CoA and malonyl CoA (Tehlivets et al., 2007). Finally, ACP thioesterases cleaves the acyl chain and liberates the fatty acid.

In bacteria, FAS reaction is catalyzed by 3-ketoacyl-ACP synthase III (KASIII), responsible for the condensation of acetyl CoA and malonyl ACP. Subsequent condensation of malonyl ACP is catalyzed by KAS I and KASII (others subunits of FAS). Fab A isomerizes cis-2-decenoyl-ACP into trans-3-decenoyl-ACP and the latter is elongated by Fab B (White et al., 2005). For the synthesis of unsaturated acyl chains, elongation and desaturation reactions are required. Enzymes involved in these reactions are under study, and researchers turned their attention to explore theses enzymes by overexpressing PUFAs in transgenic plants (Graham et al., 2007; Napier, 2007).

2.6.2 Ex novo pathway

For the *ex novo* pathway, microorganisms utilize hydrophobic substrates such as *n*-alkanes, fatty acids and TAGs.

For fatty acids coming from medium, in order to participate for metabolic reactions, it should be incorporated, transported and activated to generate thiolesters. If substrate exists in TAGs form, subsequent hydrolysis catalysed by lipase is recommended to generate free fatty acids. Mechanism of incorporating free fatty acids is unknown. But it is confirmed that proteolytic and lipolytic modifications are established in order to perform the accessibility of hydrophobic substrates (Barth et al., 2003). *Y. lipolytica* perform these modifications by production of surfactants and emulsifiers in attempt to augment cell-substrate contact and help hydrolysis of TAGs (Mlickova et al., 2004). When substrates are incorporated, their oxidation is achieved via β-oxydation peroxysomal pathway that involved acyl-CoA oxidases encoded by *pox1* to *pox6*. However, in presence of excess of hydrophobic carbon sources, substrates are accumulated in lipid droplets in form of TAG and SE. After that, accumulated lipids are mobilized and transported through plasma membrane with the aid of soluble lipid binding proteins (SLBP), like fatty acid binding proteins (FABP), acyl-CoA binding proteins (ACBP), non-specific lipid transfer protein (nsLTP) and sterol carrier protein, (SCP). In *Y. lipolytica*, FABP and SCP are implicated in the transport of sterol (Ferreyra et al., 2006). In fact, diffusion of free fatty acids is known as ‘flip-flop’

since it occurs rapidly. This phenomena is quite important for preventing toxic effects of free fatty acids in membrane, transduction of signal and transcriptional control (Zimmerman & Veerkamp, 2002).

2.6.3 Regulation of fatty acid synthesis

The enzymes, genes and regulatory mechanisms of fatty acid anabolic pathway were found to be similar in various microorganisms, e.g., in bacteria, fungi and yeast. This variety of encoded genes has chromosomal arrangement. Such arrangement, enzymes, genes and regulatory mechanisms were found to be similar in yeast and bacteria. For no oleaginous bacteria, fatty acids are destined for membrane lipid precursors, and their production is coordinated by growth and macromolecular synthesis (White & al., 2005). Mechanism of synthesis and control of fatty acids synthesis is well reviewed in (Kosa & Ragauskas, 2011). For oleaginous bacteria, β-ketoacyl-ACP synthase, β-ketoacyl-ACP reductase and enoyl-ACP reductase, are under negative feed back control by long chain acyl-ACPs (LCA-ACPs) (White & al., 2005). *E. coli* adjusts FA levels by controlling FA degradation (*fad*) with a bifunctionnal transcription factor FadR. In the absence of acyl-CoA, FadR represses the transcription of *fad* regulon and induces RNA polymerase activity on *fabA* and *fabB* genes. FabR, a repressor, acts against FadR-induced RNA polymerase activity by binding to the promoter of *fabA* and *fabB* adjacent to FadR. This mechanism of binding is unknown. In contrast, for Gram positive bacteria such as *Bacillus subtilis*, FapR, repressor, down regulates transcription for biosynthesis pathway enzymes. When FapR is attached to malonyl CoA, controlled operators are released and the biosynthetic genes are transcribed (KSIII, KR, EAR) and the level of acyl-ACP was increased (White et al., 2005). In fact, FAS activity is regulated with malonyl CoA, ACC activity and availability of cofactor NADPH/H+. Malic enzyme was responsible for forming a complex ACL (ATP citral lyase)-Fas that ensure canalisation of NADPH for fatty acid biosynthesis. Thus, the inhibition of the ACL with cycloheximid represses transcription and consequently accumulation of lipids (Ratledge, 2002).

For yeast and molds, similarly, FASI cycle is regulated with malonyl CoA, ACC activity and availability of cofactor NADPH/H+. The last reductant is gained from the steps of the transhydrogenase cycle (Ratledge & Wynn, 2002). Meanwhile, acetyl CoA is resulted from the extensive production of NADPH and acetyl-CoA. However, in *M. alpine*, FAS activity was inhibited in presence of Tween compounds as a sole carbon source (Wynn & Ratledge, 2000).

More recently it has been reported that a 40% increase of total fatty acid content in *Hansenula polymopha* was achieved by its transformation with the ACC1 gene of *Mucor rouxii* (Ruenwai et al., 2010). Control of *fas1*, *fas2* and *acc1* transcription is coordinated by the presence of precursors like inositol or choline. Haploid mutants in complex of synthase were not viable. Viability is established if medium is supplemented with exogenous acids myristic acid (C14:0), palmitic (C16:0), stearic (C18:0) or oleic (C18:1) (Schweizer & Bolling, 1970). This is due to down-expression of *fas1*, *fas2* and *acc1* which are responsible for biosynthesis regulation. The overexpression of *fas2* increases the expression of *fas1* and *acc1* simultaneously, this may suggest a coordinative regulation of three genes.These consistent findings are of potential importance and overexpression of many precursors such as acetyl CoA, malonyl CoA and $NADPH/H^+$ is required.

2.6.4 TAG synthesis and regulation pathway

TAG, a fatty acyl ester derivative of glycerol, serves to provide reservoirs for membrane formation and maintenance, lipoprotein trafficking, lipid detoxification, evaporation barriers, and fuel in times of stress or nutrient deprivation (Turkish & Sturley, 2009). However, TAG accumulation has been restricted for some bacterial strains (Alvarez & Steinbu¨chel, 2002). Best candidates belong to *Actinomycetes*, whereas the genus *Acinetobacter* is the only Gram-negative oleaginous group that has been extensively studied for its lipid accumulation. This bacterium was known for its high diacylglycerol O-acyltransferase; DGAT activity. DAG is esterified to form TAG through the diacylglycerol O-acyltransferase; DGAT activity (Oelkers & Sturley, 2004). Two main membranes bound DGAT families designated DGAT1 and DGAT2 exist. DGAT1 family exhibit high homology to acyl-CoA: cholesterol acyltransferase (ACAT) of animals and plants (Hobbs et al., 1999). DGAT2 was first characterised in lipid bodies of *M. ramanniana* and revealed 44% sequence homology with *S. cerevisiae* (Lardizabal et al., 2001). DGAT2 has also been cloned from yeast (Sorger & Daum, 2002) and demonstrated to be a major contributing factor to TAG. Nevertheless, DGAT1 and DGAT2 gene families exhibit no significant amino acid sequence homologies to each other and are quite distinctive.

Recently, a third type of DGAT isolated from developing peanut (*Arachis hypogaea*) cotyledons was reported (Saha et al., 2006). However, this DGAT is a cytosolic soluble enzyme and it is possible that the cytosolic DGAT could also be involved in TAG biosynthesis and wax ester synthesis in oil seeds.

For bacteria, as discussed before, complex of WS/DGAT has been responsible for the accumulation of TAG in *A. baylyi ADP1* (*atfA*). Two homologous genes of DGAT; *atf1* and *atf2* have been identified in *R. opacus* PD630 (Alvarez & al., 2008). A system of horizontal transfer between *E.coli* and *R. opacus* has been established (Kalscheuer et al., 1999)

In fungi, de *novo* synthesis of DAG can be accomplished either by glycerol-3-phosphate (G3P) pathway or by dihydroxyacetone phosphate (DHAP) pathway where, DHAP is resulting from glycolysis (Athenstaedt & Daum, 1999). Additionally, DAG can also result from the degradation of phospholipid through phospholipase C, otherwise, via de-acylation of TAG by lipases (Oelkers et al., 2002; Sorger et al., 2004; Sorger & Daum, 2002). Major differences between bacterial species and yeast are in the acyl group acceptor; bacteria used G3P compared to yeast that used both G3P and DHAP (Czabany et al., 2007; Rajakumari et al., 2008).

Acyltransferases (*gat1*, *gat2*) catalyze acyl-CoA esterification into G3P or DHAP. *Gat1* and *Gat2* exhibited differences in substrates specificities, whereas, *gat2* showed preference for palmytoyl-CoA and *Gat1* for acyl-CoA. *Gat2* was responsible for the first acylation of glycerol in position *sn-1*, generating lysophosphatidic acid (LPA), then, second acylation occurred in *sn-2* due to *Slc1* giving rise to phosphatidic acid (PA). Finally, PA is dephosphorylated to produce DAG. DAG can be further acylated by DGAT to form TAG. For dihydroxyacetone phosphate (DHAP) pathway, *Gat2* catalyzes acylation of G3P in *sn1* position to generate l-acyl-DHAP that will be further converted to LPA due to DHAP reductase (*Ayr1*). Subsequent action of *Slc1* gives rise to PA and then DAG. Acyl-CoA is independently catalyzed by phospholipid: diacylglycerol acyltransferase (*Lro1)* which is responsible for the transfer of acyl group of phosphatidylcholine or phosphatidylethanolamine in the position *sn-3* of DAG (Dahlqvist et al., 2000; Oelkers et al., 2002). *Lro1* activity is found in the endoplasmic reticulum (Sorger & Daum, 2002) and most probably in lipid bodies (Athenstaedt & Daum, 1999).

Acyl-CoA depended pathway is summarized in two reactions, one catalysed by diacyl glycerol acyl transferase (*Dga1*) and other by *Lro1*. On the other hand, an acyl-CoA dependant reaction is involved where DAG acylation is catalyzed via acyltransferase *Dga1* (Sandager et al., 2002; Sorger & Daum, 2002). *Lro1* used glycerophospholipids as acyl donors. Meanwhile, *Dga1* catalyzes synthesis of TAG through acyl-CoA and DAG. This enzyme is found to be very sensible to high concentration of K^+ and Mg^+ (Oelkers et al., 2002; Sorger & Daum, 2002). In *S. cerevisiae* and *Y. lipolytica*, *dga1* presented high homology of proteins sequences with DGAT-2. These enzymes are associated with lipid bodies and are showed preferences for oleoyl-CoA and palmitoyl-CoA (Oelkers et al., 2002). Recently, in *S. cerevesiae*, two sterol esters synthases *Are1*

and *Are 2* were identified to catalyze acylation of DAG through acyl-CoA. These two proteins presented large specificities for substrates (Sorger et al., 2004). *Are1* of *Y. lipolytica* exhibited 30% homology with *S. cerevisiae* and *Are1* showed 17% with *Are1/2* of *S. cerevisiae*.

In Gram positive bacteria, Acyl-ACPs are consumed by acyl-ACP:G3P acyltransferase (*PlsX*) and are used first to form acyl-phosphate, then it is transferred onto G3P by (*PlsY)* (*Schujman & Mendoza, 2008*). In contrast, for Gram negative, they only use *PLsB* acyltransferase to load an acyl group directly onto G3P from acyl ACP (Schujman & Mendoza, 2008).

2.6.5 Regulation of accumulation of sterols and steryl esters

Sterols were originally identified as the major membrane components, involved in fluidity regulation, intracellular trafficking and acts as messengers for transduction of signal. Generally, SE was found in lipid bodies; esterification of sterols occurs between hydroxyl group and acyl generated from acyl-CoA or glycerophospholipid. Studies have revealed that this esterification is quite important since their excess or deficit causes significant danger in cells (VanMeer et al., 2008). Thus, SE synthase (ACAT): Acyl-CoA cholesterol acyl transferase has been identified, cloned and characterized. Accordingly, *Are1* and *Are2* are responsible for the synthesis of SE dependent of acyl-CoA.

In yeast, the two representatives of (acyl-CoA: cholesterol acyltransferase); ACAT gene family are *Are1* and *Are2.* In *S. cerevisiae*, these two proteins exhibited 49% of sequence identity between themselves and 24% homology to human ACAT (Athenstaedt & Daum, 2006).These findings with the significant sequence similarities with the human ACAT explain their nomination as ACAT-related enzymes 1 and 2. They are both localized on the endoplasmic reticulum as demonstrated by enzymatic analysis (Zinser et al., 1994) and microscopic visualization of GFP: green fluorescent protein (Sorger et al., 2004; Zweytick et al., 2000a).

Comparison of the concentration of free sterols between single *Are1* or *Are2* and double mutant and wild-type reveal no obvious changes (Yang et al., 1996). It was hypothesized that regulation of sterol biosynthesis in an *are1Δare2Δ* double mutant may account for this observation. Indeed, the rate of sterol synthesis was reduced in *are1Δare2Δ* to 30 to 50% of the wild-type level. This finding may suggest the down regulation of ERG3 expression that encodes sterol C5 desaturase and/or destabilization of the squalene-epoxidase *Erg1* in strains lacking Are-proteins (Sorger et al., 2004). No such regulation, however, was observed in *are1Δ* and *are2Δ* single mutants (Yang et al., 1996). In addition, deletion of both ARE1 and ARE2, reported as regulators of synthesis,

abolish the sterol esterification. Overexpression of *Are1* and *Are2* in *are 2*Δ*are1*Δ double mutant revealed that only *Are2* could restore STE synthase activity (Yang et al., 1996); this suggests that *Are2* activity is the major STE synthase of the yeast. Furthermore, transcriptional regulation of two enzymes including biochemical analysis were studied to reach this conclusion (Jensen-Pergakes et al., 2001), in fact, transcriptional initiation through the ARE1 promoter was significantly lower than ARE2 promoter and the ARE2 mRNA was 12 times longer than that of the ARE1 transcript. Another side of metabolism is studied, their substrate specificities *Are2. Are2* prefers ergosterol as substrate and *Are1* for ergosterol and its precursors mainly lanosterol (Zweytick et al., 2000b) and both of isoenzymes exhibited no preference for different fatty acid. To reach the strength of this study, relationship between esterification of sterols and heme competency has been studied. Thus, it was found that the modulation of role of heme in biosynthesis of sterols (Keesler et al., 1992). SE synthase activity was stimulated in heme auxotroph supplemented with δ-aminolevulinic acid which rescues heme deficiency on either fermentable or non-fermentable carbon sources (Keesler et al., 1992). Consequently, heme competency was proposed to regulate both sterol uptake and SE synthase activity in aerobic cells. Studies in oleaginous yeast and fungi have revealed that several factors, such as ATP: citrate lyase and malic enzyme in the energy-production system (Ratledge & Wynn, 2002), biosynthetic enzymes of TAGs (Lehner & Kuksis, 1996) and modulators of lipid body biogenesis (Murphy, 2001; Zweytick et al., 2000b), play important roles in lipid accumulation.

2.7 Influence of factors on lipid accumulation

Several studies have extended the potential importance of biotic factors in lipid accumulation such as nature of microorganisms (biocatalyst), as well as abiotic factors; temperature, oxygen concentration and carbon sources that can really affect lipid metabolism.

2.7.1 Carbon sources

Many studies address the influence of the carbon source in biodiesel production. In attempt to find new substrates or waste materials which could be efficiently converted into lipids, such as jerusalem artichoke tuber juice (Hua et al., 2007), sewage sludge (Angerbauer et al., 2008), palm oil mill effluent (Saenge et al., 2011), monosodium glutamate wastewater (Xue et al., 2008), whey permeate, sugar cane molasses and rice straw hydrolysate have been investigated (Li et al., 2007). Besides, a wide variety of lignocellulosic materials, other materials such as glycerol,

polysaccharides (e.g. starch and pectin, xylose, as well as sugar-enriched wastes or residues) were also studied for lipid accumulation due to both economic and ecological considerations. Various carbon sources used by different researchers to investigate lipid production by various microbial strains and the yield of lipid accumulation are presented in (Table 1.3). The data of lipid yield on the same carbon source under different experimental conditions cannot be compared; however, it provides an idea of lipid accumulation under experimental conditions employed by different researches.

Efforts were first focused on using glucose as feedstock (Li et al., 2007), and then glycerol and xylose as substrate for biodiesel production. Xylose is abundant and thus can be derived from chemical hydrolysis of lignocellulosic materials. Xylose is metabolized by two pathways: formation of 1 mole of acetyl-CoA per 100 g of xylose utilized via pentose phosphate, and production of 1.2 moles of acetyl-CoA per 100 g of xylose via phosphoketolase pathway (Fakas et al., 2009). Acetyl CoA is the precursor in fatty acid biosynthesis. The maximum theoretical yield of SCO for glycerol was around 0.30 g/g, for glucose was 0.32 g/g and for xylose it was 0.34 g/g. Thus, glycerol possesses lower theoretical conversion yield, however, it is considered as the best substrate for many oil-producing organisms due to its availability as main byproducts of biodiesel. Other sugars such as fructose, lactose and sucrose have been frequently used (Fakas et al., 2009).

Moreover, some oleaginous microorganisms utilise ethanol as substrate. High conversion yield of 0.31 g lipids/g ethanol has been achieved (Fakas et al., 2009; Ratledge, 1988). The final stoichiometric balance for SCO synthesis from ethanol could result in a theoretical yield of 0.54 g of lipid per g of ethanol consumed (Ratledge, 1988). Other substrates explored for SCO production are citric acid (Aggelis, 1996), acetic acid, soluble starch, wheat straw (Wu et al., 2010; Zhu et al., 2008) and other low-molecular weight organic acids (DuPreez et al., 1995; Fei et al., 2011). Additionally, acetic acid can be a great substrate, and due to its hazardous effect on environment, so, its biotransformation in SCO can have ecological interest (Botha et al., 1997). To enhance productivity of lipids with mixture of organic substrates is well studied. For example *L. starkeyi* has the ability to accumulate over 70% of its cell biomass as lipid under defined culture conditions (Angerbauer et al., 2008; Li et al., 2007) , however, lipids production increased by using a mixture of glucose and xylose as well as other wastes (Angerbauer et al., 2008). Finally, crude glycerol is believed as innovative source since it can be converted to 1,3-propanediol (Mu et al., 2008; Zheng et al., 2008) and single cell oil or citric acid (Rymowicz et al., 2006). Glycerol is believed actually as new kind of industrial waste carbon for lipids production (Mu et al., 2008).

2.7.2 Nitrogen sources

The effect of nitrogen to promote lipid accumulation and cell growth is well studied (Li et al., 2007). It is reported that starvation in nitrogen source is required in metabolic pathways of lipid biosynthesis (Ratledge & Wynn, 2002). Its deficiency is responsible of decrease of the activity of nicotinamide adenine dinucleotide isocitrate dehydrogenase (NAD-IDH). Thus, IDH activity is allosterically dependant on intracellular adenosine monophosphate (AMP) levels. Under nitrogen deficient conditions, AMP is converted into inosine 5-monophosphate (IMP), leading to tricarboxylic acid cycle TCA alteration and cessation of IDH activity which is responsible for oxidation of isocitrate to α-ketoglutarate (Ratledge, 2002). So, iso-citric acid is accumulated inside the mitochondrion and equilibrated with citrate due to isocitrate acotinase. Finally, citric acid is cleaved by the ATP-citrate lyase (ATP-CL) into acetyl-CoA and oxaloacetate.

Consequently, Acetyl-CoA generates cellular fatty acids and subsequently triacylglycerols (Cupp & McAlister-Henn, 1992; Cupp & McAlister-Henn, 1991), it is found that yeast lacking the activity of NAD-IDH could not utilize acetic acid as a carbon source but utilize glycerol or lactic acid.

Organic nitrogenous compounds were found good for lipid accumulation, but not for cell growth; on the other hand, inorganic nitrogenous compounds are favourable for cell growth, but not for lipid accumulation (Huang et al., 1998). In *R. toruloides*, yeast extract with ammonium salt yielded higher biomass, hence, lipid production was increased by using peptone with ammonium salt. The highest biomass 8.71 g/L was obtained when combining yeast extract with $(NH_4)_2SO_4$, while a higher cellular lipid content of 53.71 and 53.10% of dry biomass was produced, when peptone was used with NH_4Cl and with $(NH_4)_2SO_4$, respectively (Kraisintu et al., 2010).

This result was confirmed by Saengea et al. (Saengea et al., 2011) that higher values of biomass and lipid content were obtained with a yeast extract (6.33 g/L and 32.63%, respectively) than those with ammonium sulphate (6.29 g/L and 29.15%, respectively), while peptone, ammonium nitrate and ammonium chloride gave poor growth, lower lipid content and carotenoid production. In fact, the explanation might be that, certain essential amino acids could be synthesized from inorganic nitrogen sources. Although the yeast extract gave the highest biomass, lipid content and carotenoid production, its high price was considered to have negative impact for industrial-scale processes. For *R. toruloides* Y4 lipid production reached up to 57% in nitrogen rich medium but in sulphate deprived conditions (Wua et al., 2011), that finding may open new horizon in over

lipid production by using nitrogen rich substrate such as chitin hydrolysate, which contains N-Acetylglucosamine (GlcNAc), highly nitrogenous substrate (Wu et al., 2008).

2.7.3 C/N ratio

The oleaginous potential is critically affected by the carbon-to-nitrogen (C/N) ratio of media. Thus, the increase of cellular lipid content is relatively closed to the increase of the C/N ratio. For instance, the highest cellular lipid content of 62.30% and lipid yield of 4.23 g/L was reached at the C/N ratio of 140; however, the lowest C/N ratio 65 produced the highest biomass 10.52 g/L and the lowest lipid 2.7 g/L (Kraisintu et al., 2010). At a high C/N ratio, when cells run out of nitrogen, they are unable to multiply and excessive carbon substrate is assimilated continuously to produce storage lipids. In *L. starkeyi*, the optimal ratio was reported of 150 with lipid content of 68% and lipid yield of 6.4 g/L (Angerbauer et al., 2008). In *R. toruloides* Y4, lipid content were less than 20% if the initial C/N molar ratio was lower than 100 (Li et al., 2006).

2.7.4 Phosphorus source

Both nitrogen and phosphorus exhaustion shared a common biochemical result, a reduced mitochondrial isocitrate dehydrogenase activity due to depletion of the allosteric activator; adenosine monophosphate (AMP). Under nitrogen limited medium in the presence of excess glucose, cellular AMP level dropped significantly due to the activation of AMP deaminase (Meng et al., 2009; Ratledge & Wynn, 2002). It was suggested that phosphate exhaustion induced a similar event that broke down AMP to release inorganic phosphate for other cellular processes. Phosphate limited conditions have been studied on term of lipid production in various yeast. For example, in *Rhodosporidium toruloides*, lipid content was increased from 21.1% to 62.1% with the increase of C/P molar ratio from 72 to 9552 under C/N molar ratio of 6.1, further increase in lipid content up to 63.3% was achieved by increasing the C/N molar ratio up to 22.3 coupled with C/P molar ratio 9552 (Wu et al., 2010). Phosphorus element was responsible for stimulating microbial lipid production but it has no effect on fatty acid compositional profile. However, it favors the abundance of oleic acid content but lower stearic acid and palmitic contents in *Rhodosporidium toruloides* (Wu et al., 2010). In *C. utilis*, under phosphate-limited condition, intracellular non-polar lipid content was increased, while the content of polar lipid remained relatively constant. However, after phosphate exhaustion, cell number as well as lipid-free biomass increased continually until the carbon source exhausted and lipid content may reach

over 60%. Actually, sulphur exhaustion may induce lipid production (Wua et al., 2011), that is also required in production of citric acid and H_2 (Fouchard et al., 2009).

2.7.5 pH

The initial hydrogen ion concentration in the culture medium greatly influences the microbial growth and lipid content since pH values limit enzymatic activities. This influence was first studied in *Rhodotoruia gracilis and Candida sp.*107, respectively (Hall & Ratledge, 1977; Kessell, 1968). The highest biomass (9.26 g/L) and lipid content (71.30%) could be achieved at the pH 5.5 in *R. toruloides* DMKU3-TK16 (Kraisintu et al., 2010). This result is also approved by Li et al. (2006) that, the maximal accumulation is achieved with pH ranging from 4 to 10 and also depended on carbon sources (Angerbauer et al., 2008). In *L. starkeyi*, the optimal pH 4 is found when ethanol is used as the carbon source (Yamauchi et al., 1983). On the other hand, it is reported that the optimum pH of *L. starkeyi* was at 5.5 with glucose (Ratledge, 1988), while the highest lipid content was obtained when *L. starkeyi* was cultivated at pH 5.0 (Angerbauer et al., 2008). Additionally, in the synthesis of glycolipids by *C. antarctica* and *C. apicola*, a maximal production is enhanced when pH was maintained at 5.5 (Bednarski et al., 2004). This suggests an indirect influence of production and excretion of citric acid which impacts to modify production of lipids. In *Y. lipolytica*, a high lipid production is achieved using stearin with pH values varying from 5.5 to 6.5. Besides, it is reported that pH can induce changes in lipid content and fatty acids (Brown et al., 1990; Hall & Ratledge, 1977) in *Trichoderma reesei* and in *Candida sp.* 107, respectively.

2.7.6 Temperature

Temperature is an important abiotic factor affecting the lipid composition and causing variations in membrane lipids. In oleaginous species, a decrease in temperature causes an acclamatory response and resulted in a higher length and degree of unsaturation of FA (Rossi et al., 2009). This finding was first reported in many organisms and plants that produce higher concentration of unsaturated fatty acids at lower temperature and more saturated fatty acids at higher temperatures in order to keep their membrane fluidity constant and maintenance of membrane's physical properties. Differential responses to temperature changes that depend on microbial strains and a certain range of temperatures have been reported in different strains as all microorganisms don't exhibit the same degree of response (Ratledge, 1982). The increase in unsaturation of fatty acid at low temperature is found to be regulated by transcriptional control

since it was found that fatty acid composition at low temperatures in *M. rouxii* is in correlation with desaturase activity and its gene expression, in which the mRNA level increased after cold induction (Laoteng et al., 1999). In addition, expression of desaturases genes during cold acclimatization in *M. circinelloides* was investigated and the decrease of the culture temperature from 28^{o}C to 15^{o}C led to induction of desaturase activity with the abundance of oleic acid C18:1 and a constant level of γ-linolenic acid (18:3, n-6) GLA (Michinaka et al., 2003). In *Thamnidium elegans*, the expression of desaturase gene was up-regulated by shifting the culture temperature from 30^{o} C to 10^{o} C (Wang et al., 2007). For psychrophilic oleaginous yeast, temperature did not influence the yield coefficients of both biomass and lipid production but it had significant effects on the growth rate and volumetric productivity, so the optimal temperature was found of 15 ^{o}C. Besides, when temperature decreased, both C18 FA and unsaturation degree increased (Rossi et al., 2009). This finding was similar to the studies of Amaretti et al. (2010), in which they found that remarkable amount of linoleic acid (C18:3 w3) were produced at -3^{o}C, accounting for 29% of FA. Consequently, a decrease of temperature may be responsible for higher length and higher degree of unsaturation of FA (Rossi et al., 2009).

2.7.7 Oxygen concentration

Many reports have revealed that dissolved oxygen plays crucial role as a terminal electron acceptor in fatty acid desaturation. Aeration has a profound effect on the growth rate and lipid production since the oxygen transfer becomes a limiting factor when the cell grows and the viscosity of the broth increases. In the aerobic culture, the rate of the dissolved oxygen (DO) supply must at least be equal to the rate of oxygen demand (Wu et al., 2005). Biomass and lipid yield significantly increased when increasing aeration. Evidence shows that oxygen availability considerably influences the expression of desaturases at the transcriptional level or the enzymatic activity (Saengea et al., 2011). Comparative study of *M. rouxii* grown under aerobic and anaerobic conditions showed significant differences in cell morphology and fatty acid profile. Oxygen limitation induced the formation of yeast-like cells of *M. rouxii* accompanied by a high accumulation of medium-chain saturated fatty acids (C10-C14) in contrast to the aerobically grown culture that contained substantial amounts of long-chain saturated and unsaturated fatty acids (Jeennor et al., 2006). Shifting of *M. rouxii* culture from anaerobic to aerobic conditions led to increased biomass and total fatty acid content. Moreover, after the shift, the unsaturated fatty acid content gradually increased with simultaneous decrease of the saturated fatty acids corresponding to the induced expression of desaturase genes (Ruenwai et al., 2009).

2.7.8 Culture processes

Three bioprocesses are commonly used for lipid production: batch, fed-batch and continuous mode. The suitable bioprocesses have to ensure the maximal conversion of the carbon taken up into lipids, minimize by-product (citric acid) production and maximize lipid synthesis and to break them down by β-oxidation.

2.7.8.1 Batch process

Most of the processes are related to batch culture (Evans & Ratledge, 1983; Kim et al., 2000; Papanikolaou et al., 2006). This process is well applied in *R. glutinis* aerated with oxygen-enriched, the cell density obtained was of 185 g/L at 84 h (Pan et al., 1986). In this process, growth remains exponential while nitrogen is not limiting. It is showed that, in *R. glutinis* grown at 30°C in presence of glucose, nitrogen limitation leads to decrease in the rate of substrate consumption by a factor of four with an increase in lipid production rate by a factor of two to three (Granger et al., 1993). Thus, this conversion yield depends mostly on the ratio of biomass constituted during the growth phase to lipids accumulated during the accumulation phase in batch culture. Control of the ratio of carbon consumption to nitrogen consumption is therefore essential to prevent citric acid secretion, hence the importance of monitoring C/N ratio in continuous and fed-batch cultures.

2.7.8.2 Continuous process

In a continuous process, *Y. lipolytica* grown on glycerol accumulated high amounts of lipids at low dilution rates (Papanikolaou & Aggelis, 2002). For low dilution rates, with intermediate C/N ratios, the lipid and biomass concentrations obtained are higher than those obtained for higher dilutions. The optimization of the process therefore requires determining the optimal dilution rate with an optimal intermediate C/N ratio. This fermentation process is applied in *Candida curvata D* growing on glucose and xylose and obtained lipid production rates of 0.16 and 0.27 g/L/h, respectively and a cell density of around 14 g/L (Evans & Ratledge, 1983) and in *Apiotrichum curvatum* using glucose, lipid production rate is 0.42 g/L/h and lipid content of 31.9% (w/w) (Hassan et al., 1996).

2.7.8.3 Fed-batch process

Fed-batch approaches are currently attempted to increase lipid productivity. In this process, nitrogen and carbon flows are monitored to control the specific growth rate and the C/N ratio. Many studies are conducted to reveal this obvious importance. Fed-batch process is applied to improve lipid production up to 48% in *R. toruloides* Y4 to reach 67.5% and overcome inhibitory effect of substrates (Li et al., 2007), in *M. alpina* with a lipid yield up to 1.51 fold compared with those of batch cultures (Zhu et al., 2006) , in *R. glutinis* TISTR 5159 with level up to 60.7% using crude glycerol as a sole carbon source (Saengea et al., 2011) and 52.9% in *C. curvatus* (Liang et al., 2010). The production of lipids by yeast may ensure that lipids are efficiently and reproducibly produced (Dyal & Narine, 2005). If lipid production is controlled, regulation of the environmental variables is required for maximizing the stability of the metabolic state. This stability can be achieved through the precise control of nutrient flow rate. The control of nutrient flow rate is combined with greater control of metabolic state and more optimal production, which is the case for protein production processes. This needs a reliable approach to be used in a biotechnological application for lipid production.

2.7.9 Other parameters

The role of magnesium was investigated by Kraisintu et al. (2010), that supplying medium with $MgSO_4.7H_2O$ was sufficient to obtain higher lipid content up to 69.7% (Kraisintu et al., 2010). However, any further excess of concentration led to decrease in lipid content. Besides, it is reported that ion concentration of Zn^{2+} and Mn^{2+} and to a lesser extent Fe^{3+}, Ca^{2+} and K^{+} may affect lipid accumulation (Johnson & Taconi, 2007). Furthermore, role of surfactants to enhance lipid production is newly investigated (Kim et al., 2006; Saengea et al., 2011). Surfactants are responsible for altering physiological properties of microorganisms, improving metabolites production, stimulating growth and respiration and modifying permeability of cell membranes (Laouar et al., 1996). The mechanism of stimulation of lipid productivity is under study, but surfactants appear to cause different alterations in membrane fluidity. Among three surfactants Tween 20, Tween 80 and gum arabic, only Tween 20 effectively increased lipid content as well as carotenoid production *R. glutinis* TISTR 5159 (Saengea et al., 2011). This finding was proved (Stredanska & Sajbidor, 1993) that supplementation of medium with Tween 20 stimulated the growth, improved the growth rate since several enzymes involved in lipid and carotenoid biosynthesis in prokaryotic and eukaryotic cells such as phytoene desaturase, β-carotene hydroxylase, and lycopene cyclase were stimulated (Kim et al., 1997). Besides, when Tween 20

was used as a carbon source, it increased lipid accumulation, but was not suitable for cell growth found the contradictive result (Saenge et al., 2011).

2.8 Genetic engineering

Microbial fatty acids production capacity depends on many nutritional factors that are already discussed before. Genetic engineering has been considered as a complimentary approach to augment the production of microbial diesel. This has been recognized with bacteria, fungi and higher eukaryotes. Research has mostly focused on *E. coli* due to its well-known genetics, its rapid growth rate and its popularity as a host cell for genetic manipulation. Successful results were obtained for the production of wax esters, FAEEs (Steen et al., 2010), fatty acid butyl esters (FABEs) (Kalscheuer et al., 2006). Additionally, *S.cerevisiae* H1246, expressing waxester synthase/diacylglycerol acyltransferase (WS/DGAT) gene of *A.baylyi* ADP1, has been reported for the production of FAEEs and fatty acid isoamyl esters (FAIEs) (Kalscheuer et al., 2004).

Currently, engineered *E. coli* is used for the production of FAEEs, genes of *Z. mobilis pdc* and *adhB* encoding for pyruvate decarboxylase and alcohol dehydrogenase, respectively, were introduced in *E. coli*, and FAEES level reached up to 674 mg/L (Steen et al., 2010). Besides, biodiesel production from ethanol is enhanced via expression of genes encoding for endoxylanase catalytic domain from *Clostridium stercorarium* and a xylanase from *Bacteroides ovatus*, after that the concentration of biodiesel reached 11.6 mg/L (Steen et al., 2010). Actually, fatty-acid-derived hydrocarbons especially alkanes is considered as good fuel in internal combustion engine. For this attempt, an alkane-producing *E. coli* was developed by the introduction of the alkane operon from *Synechococcus elongatus* PCC7942 (Schirmer et al., 2010). The resulting metabolically engineered *E. coli* strain produced a mixture of alkanes (~ 0.3 g/L). Actually, further advances for the production of *n*-alkanes are proceeded in *Vibrio furnissii* M1 (Park, 2005).

Actually, FAEEs showed better performances in term of biodiesel production more than FAMEs. Accordingly, they showed higher viscosity, lower cloud points and better lubrication performance. Production of FAEEs occurred after expression of WS/DGAT gene (*atfA*) and 12.8 g/L of FAEEs was reached after 72 h in presence of exogenous fatty acids esters. However, the production of FAEEs by microbes needs to be further improved for viable industrial application.

The metabolically engineered *E.coli* was manipulated to be able to produce fatty acids and their derivatives. The production abilities were accomplished through the overexpression of several

genes encoding various enzymes such as thioesterase (*tesA*) (Davis et al., 2000), acyl-CoA synthase (*fadD*), acetyl-CoA carboxylase (*accABCD*), fatty acid synthase (*fabH, fabB, fabD, fabG, fabF*) (Zha et al., 2009, Edwards et al., 1997, Verwoert et al., 1992), acyl-carrier-protein (ACP) dehydratases (*fabA,fabZ*), enoyl-ACP reductase (*fabI*) wax synthase (*atfA*), pyruvatedecarboxylase (*pdc)*, alcohol acyltransferae, alcohol dehydrogenase (*adhB*), and glycerol-3-phosphate acyltransferase (*PlsB*) (Heath & Rock, 1996). Further, manipulating positions of unsaturated bonds remains an issue through the expression of D9-18:0-ACP desaturase which allow for saturation at different positions (Whittle & Shanklin, 2001). Recently, genetic engineering approach has been used in yeast to improve TAG accumulation through the expression of *gut2 and pox1-6.*

In fungi, most of the studies have been focused on polyunsaturated fatty acids (PUFAs); *Mortierella* was found as the best model organism for producing these types of FA. Overexpression of ω desaturase gene in this strain led to higher production of eicosapentaenoic acid (EPA), accounting for 40% of the total fatty acid profiles (Ando et al., 2009). Further, ω-3 desaturases have also been cloned from the fungi of *Fusarium moniliforme*, *Fusarium graminearum*, and *Magnaporthe grisea* (Damude et al., 2006).

Overall, genetic engineering is likely to have the advantage in the improving the economics of biodiesel production as well as the production of stable transformants that offer advances in industrial process.

2.9 Concluding remarks and future perspectives

The continued accumulation of evidences which demonstrate the important production capacities of FAMEs and FAEEs, has led to consider heterotrophic microorganisms most reliable and attractive source for biodiesel production.

An overview of regulatory mechanism of fatty acids production is given in this review. Although genes involved in the biosynthetic pathways have been cloned and characterized, the understanding of the details of lipid metabolism is still lacking. Many fundamental biological questions are not resolved until date. Most of the questions surround about the difference of metabolic pathways to accumulate fatty acids existing between bacteria (gram positive and gram negative), yeast, fungi and others eukaryotic species. Additionally, the potential interactions between *de novo* and *ex novo* pathways are still misunderstood. For example the fact that for *ex novo* pathway, yeast requires the presence of surfactants and extracellular emulsifiers such as liposan. These mechanisms are absent in non-oleaginous microorganisms and probably in most microbes. Another question of interest is how microorganisms can correlate between two distinctive pathways? So, new experimental approaches have to be adopted for further understanding. Advances in genetic and metabolic pathways will ultimately lead to the efficient biodiesel-producing organisms.

Remarkable progress in engineering fatty acid producing strains has been made by many researchers in the last few years and successful results were obtained for the production of both FAMEs and FAEEs. However, quantities produced remains low. Moreover, most of these approaches are laboratory scale experiments; consequently, they failed when transferred to industrial levels. To overcome these limitations, complete understanding of the metabolic networks is required. Additionally, fermentation engineering, enzyme engineering, and cell engineering are important in order to make the microbial diesel-producing system economically feasible.

More often, processing techniques for production are well documented and many of protocols have been studied as pyrolysis, microemulsions and transesterification but ensuring a suitable process with low cost, less soap formation and less water is still a great challenge. Likewise, lipids obtained from microbial biomass are not all suitable for making biodiesel. Only saponifiable lipids with fatty acid ester linkages and free fatty acids can produce biodiesel.

Microbial diesel offers more unsaturated and extended fatty acids that represent a high iodine value. Actually, PUFA are leading to an exciting future regarding to their role for human health

and dietary requirements, their structural role in the composition of phospholipids and eicosanoids. For this attempt, various hosts have been considered for the PUFA production. It is recognized that in filamentous fungi, such as Mucorales fungi; *M. alpina* has been used for the production of arachidonic acid AA (20:4, n−6), *M. circinelloides* for γ-linolenic acid GLA (18:3, n−6) and both *Crypthecodinium cohnii and Schizochytrium* for docoshexaenoic acid DHA (22:6, n−3) (Meng et al., 2009). Recently, *Shewanella sp.*BR-2 has been reported for the production of eicosapentaenoic acid EPA (20:5n-3) (Lee et al., 2009). Added to PUFA production, oleaginous yeast accumulate large amount of TAGs resembling to plant oils, but only content of stearic acid (C18:0) and oleic acid (C18:1) is of interest. Accordingly, many critical issues and further developments required for the improvement of production of microbial fatty acids are mentioned below. First, better understanding the mechanism of lipid accumulation via *de novo*, *ex novo* and degradation (ω- and β- oxidation) pathways are prominent areas of interest. This requires the knowledge of the expression of key enzymes involved in lipid metabolism and also requires the understanding of the overexpression of genes encoding acyl-CoA oxidases, ATP-citrate lyase, fatty acid synthase, malic enzyme, acyl-CoA carboxylase and other genes encoding glycerol-3-phosphate dehydrogenase and acyl-coenzyme A oxidases, major keys of lipid accumulation. These corresponding enzymes have a defined specificity with different localization and specific regulations are crucial to study and understand them. Besides, efforts have been carried out to enhance lipid production by supplying acetyl CoA and/or NADPH in the cytosol of the cell as necessary precursor for fatty acid biosynthesis.

The selection of cost effective and suitable substrates is another critical area to be considered. This may require consideration of other potential substrates such as hydrophilic and hydrophobic ones, the development of other sources should be envisaged especially lignocellulosic substrates with low cost. Ethanol and sodium acetate can be directly converted into acetyl-CoA by acetyl-CoA synthase in eukaryotic microorganisms, so these substrates can be introduced in the medium to enhance acetyl-CoA supply in prokaryotic microorganisms. Further, recent studies have been carried out to using waste water or sewage sludge as innovative substrates in regard to their environmental effects.

New researches are also focusing on the identification and selection of oil-producing microorganisms possessing high lipid content in their cells or mycelia and growing on cheap substrates. Although a wide variety of oleaginous microorganisms are able to synthesize large amounts of lipids with limited biomass, engineering techniques have been developed to obtain high biomass than the wild types strains. Modulating production through the optimization of culture

conditions is being currently performed. For example, the effect of low temperature in increasing proportion of polyunsaturated fatty acids is studied in both bacteria and yeast. Culture conditions play also an important role in the lipid composition such as C/N ratio and also fed batch mode opens new ways for lipid production.

2.10 Summary

Microorganisms can be utilized as a great source for the production of biodiesel due to their various advantages. This review illustrates the potential of microbes in producing petroleum feedstock and gives a solid evidence for more viable biodiesel production. Likewise, the optimization of biodiesel production involved a set of genes and enzymes described in detail in this review. Similarly, metabolic regulation is well studied for better knowledge. In conclusion, increasing efforts are recommended towards the improvement of lipid synthesis as well as the building up the emergence of biotechnological field with many potential applications.

2.11 Acknowledgments

The authors are thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A 4984, and Canada Research Chair) for their financial support. The views and opinions expressed in this article are those of the authors.

2.12 References

Aggelis, G. 1996. Two alternative pathways for substrate assimilation by *Mucor circinelloides*. *Folia Microbiol*, **41**, 254-256.

Aggelis, G., Sourdis, J. 1997. Prediction of lipid accumulation - degradation in oleaginous micro-organisms growing on vegetable oils. *Antonie Van Leeuwenhoek*, **72**, 159-165.

Alvarez, A.F., Alvarez, H.M., Kalscheuer, R., Wältermann, M., Steinbüchel, A. 2008. Cloning and characterization of a gene involved in triacylglycerol biosynthesis and identification of additional homologous genes in the oleaginous bacterium *Rhodococcus opacus* PD630. *Microbiology*, **154**, 2327-2335.

Alvarez, H.M. 2003. Relationship between b-oxidation pathway and the hydrocarbon-degrading profile in actinomycetes bacteria. *Int Biodeter Biodegr*, **52**, 35-42.

Alvarez, H.M., Steinbu¨chel, A. 2002. Triacylglycerols in prokaryotic microorganisms. *Appl Microbiol Biotechnol*, **60**, 367-376.

Amaretti, A., Raimondi, S., Sala, M., Roncaglia, L., DeLucia, M., Leonardi, A., Rossi, M. 2010. Production of single cell oils by the cold -adapted oleaginous yeast Rhodotorula glacialis AS4.7 :effects of the growth temperature and C :N ratio. *Chem Eng Trans,* **20**.

Ando, A., Sumida, Y., Negoro, H., Suroto, D.A., Ogawa, J., Sakuradani, E. 2009. Establishment of Agrobacterium tumefaciens-mediated transformation of an oleaginous fungus, Mortierella alpina 1S-4, and its application for eicosapentaenoic acid producer breeding. *Appl Environ Microbiol*, **75**, 5529-35.

Andrs, C.D., Espuny, M.J., Robert, M., Mercade, M.E., Manresa, A., Guinea, J. 1991. Cellular lipid accumulation by Pseudomonas aeruginosa 44T1. *Appl Microbiol Biotechnol*, **35**, 813-816.

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

Athenstaedt, K., Daum, G. 2006. The life cycle of neutral lipids: synthesis, storage and degradation. *Cell Mol Life Sci*, **63**, 1355-69.

Athenstaedt, K., Daum, G. 1999. Phosphatidic acid, a key intermediate in lipid metabolism. *Eur J Biochem*, **266**, 1-1.

Bagnat, M., Keranen, S., Shevchenko, A., Shevchenko, A., Simons, K. 2000. Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. *Proc Natl Acad Sci USA, 97*, 3254-9.

Barnwal, B.K., Sharma, M.P. 2005. Prospects of biodiesel production from vegetable oils in India. *Renew Sust Energy Rev*, **9**, 363-78.

Barth, G., JM, B., Dominguez, A., Kerscher, S., Ogrydziak, D., Titorenko, V., Gaillardin, C. 2003. Functional genetics of *Yarrowia lipolytica*. Fuctional Genetics of industrial yeasts. *W. J.H. Berlin-Heidelberg-New YorkSpringer-Verlag*, **1**, 227-271.

Bednarski, W., Adamczak, M., Tomasik, J., Płaszczyk, M. 2004. Application of oil refinery waste in the biosynthesis of glycolipids by yeast. *Bioresour Technol,* **95**, 15-18.

Botha, A., Strauss, T., Kock, J.L.F., Pohl, C.H., Coetzee, D.J. 1997. Carbon source utilization and g-linolenic production by Mucoralean fungi. *System Appl Microbiol*, **20**, 165-170.

Brown, D.E., Hasan, M., Lape-Casillas, M., Thornton, A.J. 1990. Effect of temperature and pH on lipid accumulation by Trichoderma reesei. *Appl Microbiol Biotechnol,* **34**, 335-339.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnol Adv*, **25**, 294-306.

Cronan, J.E., Waldrop, G.L. 2002. Multi-subunit acetyl-CoA carboxylases. *Prog Lipid Res*, **41**, 407-435.

Cupp, J.R., McAlister-Henn, L. 1992. Cloning and characterization of the gene encoding the IDH1 subunit of NAD+-dependent isocitrate dehydrogenase from Saccharomyces cerevisiae. *J Biol Chem*, **267**, 16417-16423.

Cupp, J.R., McAlister-Henn, L. 1991. NAD+-dependent isocitrate dehydrogenase. Cloning, nucleotide sequence, and disruption of the IDH2 gene from *Saccharomyces cerevisiae*. *J Biol Chem*, **266**, 22199-22205.

Czabany, T., Athenstaedt, K., Daum, G. 2007. Synthesis, storage and degradation of neutral lipids in yeast. *Biochim Biophys Acta*, **1771**, 299-309.

Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., Stymne, S. 2000. Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proc Natl Acad Sci US A*, **97**, 6487-6492.

Damude, H.G., Zhang, H., Farrall, L., Ripp, K.G., Tomb, J.-F., Hollerbach, D. 2006. Identifi- cation of bifunctional 12/3 fatty acid desaturases for improving the ratio of 3 to 6 fatty acids in microbes and plants. *Proc Natl Acad Sci USA,* **103**, 9446-51.

Darnoko, D., Cheryan, M. 2000. Kinetics of palm oil transesterification in a batch reactor. *J Am Oil Chem Soc*, **77**, 1263-7.

Daum, G., Lees, N.D., Bard, M., Dickson, Dickson, R. 1998. Biochemistry, cell biology and molecular biology of lipids of Saccharomyces cerevisiae. *Yeast*, **14**, 1471-510.

Davis, M.S., Solbiati, J., Jr, J.E.C. 2000. Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. *J Biol Chem*, **275**, 28593-28598.

Davoli, P., Mierau, V., Weber, R.W. 2004. Carotenoids and fatty acids in red yeasts Sporobolomyces roseus and Rhodotorula glutinis. *Prikl Biokhim Mikrobiol,* **40**, 460-5.

Dey, P., Banerjee, J., Maiti, M.K. 2011. Comparative lipid profiling of two endophytic fungal isolates - Colletotrichum sp. and Alternaria sp. having potential utilities as biodiesel feedstock. *Bioresour Technol*, **102**, 5815-5823.

DuPreez, J.C., Immelman, M., Kock, J.L.F., Kilian, S.G. 1995. Production of γ-linolenic acid by Mucor circinelloides and Mucor rouxii with acetic acid as carbon substrate. *Biotechnol Lett,* **17**, 933-938.

Dyal, S., Narine, S. 2005. Implications for the use of Mortierella fungi in the industrial production of essential fatty acids. *Food Res Int*, **38**, 445-67.

E. Schweizer, Hofmann, J. 2004. Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. *Microbiol Mol Biol Rev,* **68**, 501-517.

Edwards, P., Nelsen, J.S., Metz, J.G., Dehesh, K. 1997. Cloning of the fabF gene in an expression vector and invitro characterization of recombinant fabF and fabB encoded enzymes from Escherichia coli. *FEBS Letters*, **402**, 62-66.

Evans, C.T., Ratledge, C. 1983. A comparison of the oleaginous yeast Candida curvata, grown on different carbon sources in continuous and batch culture. *Lipid,* **18**, 623-9.

Fakas, S., Papanikolaou, S., Batsos, A., M.Galiotou-Panayotou, Mallouchos, A., Aggelis, G., . 2009. Evaluating renewable carbon sources as substrates for single cell oil production by Cunninghamella echinulata and Mortierella isabellina. *Biomass Bioenergy*, **33**, 573-580.

Fei, Q., Chang, H.N., Shang, L., Choi, J., al, e. 2011. The effect of volatile fatty acids as a sole carbon source on lipid accumulation by *Cryptococcus albidus* for biodiesel production. *Bioresour Technol*, **102**, 2695-2701.

Ferreyra, R.G., Burgardt, N.I., Milikowski, D., Melen, G., Kornblihtt, A.R., Angelica, E.C.D., Santome, J.A. 2006. A yeast sterol carrier protein with fatty-acidand fatty-acyl-CoA binding activity. *Arch Biochem Biophys*, **453**, 197-206.

Fouchard, S., J. Pruvost, Degrenne, B., Titica, M., Legrand, J. 2009. Kinetic modeling of light limitation and sulfur deprivation effects in the induction of hydrogen production with Chlamydomonas reinhardtii: Part I. Model development and parameter identification. *Biotechnol Bioeng*, **102**, 232-245.

Gema, H., Kavadia, A., Dimou, D., Tsagou, V., Komaitis, M., Aggelis, G. 2002. Production of c-linolenic acid by Cunninghamella echinulata cultivated on glucose and orange peel. *Appl Microbiol Biotechnol,* **58,** 303-307.

Graham, T., Larson, I.A., Napier, J.A. 2007. Rational metabolic engineering of transgenic plants for biosynthesis of omega-3 polyunsaturates. *Curr Opin Biotechnol,* **18**, 142-147.

Granger, L.M., Perlot, P., Goma, G., Pareilleux, A. 1993. Effect of various nutrient limitations on fatty acid production by Rhodotorula glutinis. *Appl Microbiol Biotechnol*, **38**, 784-789.

Haack, S.H., Garchow, H., D.A.Odelson, Forney, L.J., Klug, M.J. 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. *Appl Environ Microbiol*, **60**, 2483-2493.

Hall, M.J., C.Ratledge. 1977. Lipid accumulation in an oleaginous yeast (Candida 107) growing on glucose under various conditions in a one and two-stage continuous culture. *Appl Environ Microbiol*, **33**, 577-584.

Hassan, M., Blanc, P.J., Granger, L.M. 1993. Lipid production by an unsaturated fatty acid auxotroph of the oleaginous yeast *Apiotrichum curvatum* grown in a single stage continuous culture. *Appl Microbiol Biotechnol*, **40**, 483-8.

Hassan, M., Blanc, P.J., Granger, L.M., Pareilleux, A., Goma, G. 1996. Influence of nitrogen and iron limitations on lipid production by *Cryptococcus curvatus* grown in batch and fed-batch culture. *Process Biochem*, **31**, 355-361.

He, M., Hu, Q., Gou, X., Liu, X., Li, Q., Pan, K., Zhu, Q., Wu, J. 2010. Screening of oleaginous yeast with xylose assimilating capacity for lipid and bio-ethanol production. *Afr J Biotech*, **9**, 8392-8397.

Heath, R.J., Rock, C.O. 1996. Inhibition of beta-ketoacyl-acylcarrier protein synthase III (FabH) by acyl-acyl carrier proteinin *Escherichia coli*. *J Biol Chem*, **271**, 10996-11000.

Hill, J., Polasky, S., Nelson, E., D.Tilman, Huo, H., Ludwig, L., Neumann, J., Zheng, H., Bonta, D. 2009. Climate change and health costs of air emissions from biofuels and gasoline. *Pro Natl Acad Sci USA,* **106**, 2077-2082.

Hobbs, D.H., Lu, C., Hills, M.J. 1999. Cloning of cDNA encoding diacylglycerol acyltransferase from *Arabidopsis thaliana* and its functional expression. *FEBS Lett*, **452**, 145-149.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J*, **54**, 621-639.

Hua, Y.Y., Zhao, X., Zhao, J., Zhang, S.F., Zhao, Z.B. 2007. Lipid production by Rhodosporidium toruloides using Jerusalem artichoke tubers. *Chin Biotechnol,* **27**, 59-63.

Huang, J.Z., Shi, Q.Q., Zhou, X.L., Lin, Y.X., Xie, B.F., Wu, S.G. 1998. Studies on the breeding of Mortierella isabellina mutant high producing lipid and its fermentation conditions. *Microbiology*, **25**, 187-191.

Ivanova, P.E., Bowman, J.P., Lysenko, A.M., Zhukova, N.V., Gorshkova, N.M., Sergeev, A.F., Mikhailov, V.V. 2005. Alteromonas addita sp. nov*. Int J Syst Evol Microbiol*, **55**, 1065-1068.

Jakobsen, A.N., Aasen, I.M., Josefsen, K.D., Strom, A.R. 2008. Accumulation of docosahexaenoic acid-rich lipid in thraustochytrid Aurantiochytrium sp strain T66: effects of N and P starvation and O2 limitation. *Appl Microbiol Biotechnol*, **80**, 297-306.

Jeennor, S., Laoteng, K., Tanticharoen, M., Cheevadhanarak, S. 2006. Comparative fatty acid profiling of Mucor rouxii under different stress conditions. *FEMS Microbiol Lett*, **259**, 60-66.

Jensen-Pergakes, K., Guo, Z., Giattina, M., Sturley, S.L., Bard, M. 2001. Transcriptional regulation of the tow sterol esterification genes in the yeast Saccharomyces cerevisia. *Arch Biochem Biophys*, **296**, 474-81.

Johnson, D.T., Taconi, K.A. 2007. The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environ Prog*, **26**, 338-48.

Kalscheuer, R., Arenskotter, M., Steinbuchel, A.,1999. Establishment of a gene transfer system for *Rhodococcus opacus* PD630 based on electroporation and its application for recombinant biosynthesis of poly(3-hydroxyalkanoic acids). *Appl Microbiol Biotechnol,* **52**, 508-515.

Kalscheuer, R., Stolting, T., Steinbu¨chel, A., 2006. Microdiesel: Escherichia coli engineered for fuel production. *Microbiology*, **152**, 2529-2536.

Kalscheuer, R., Luftmann, H., Steinbüchel, A. 2004. Synthesis of novel lipids in Saccha*romyces cerevisiae* by heterologous expression of an unspecific bacterial acyltransferase. *Appl Environ Microbiol,* **70**, 7119-7125.

Kavadia, A., Komaitis, M., Chevalot, I., Blanchard, F., Marc, I., Aggelis, G. 2001. Lipid and ã-Linolenic Acid Accumulation in Strains of Zygomycetes Growing on Glucose. *JAOCS*, **78**.

Keesler, G.A., Casey, W.M., Parks, L.W. 1992. Stimulation by heme of steryl ester synthase and aerobic sterol exclusion in the yeast Saccharomyces cerevisiae. *Arch Biochem Biophys*, **296**, 474-48.

Kennedy, E.P. 1956. The biological synthesis of phospholipids. *Can J Biochem Physiol* **34**, 334-48.

Kessell, R.H.J. 1968. Fatty acids of Rhodotorula gracilis: Fat production in submerged culture and particular effect of pH value. *J Appl Bacteriol*, **31**, 220-231.

Kim, J.H., Choi, S.K., Park, Y.S., Yun, C.W., Cho, W.D., Chee, K.M., Chang, H.I. 2006. Effect of culture conditions on astaxanthin formation in red yeast Xanthophyllomyces dendrorhous mutant JH1. *J Microbiol Biotechnol*, **16**, 438-442.

Kim, J.W., ParkDDRyu, T.J., Kim, J.Y. 2000. High cell density culture of Yarrowia lipolytica using a one-step feeding process. *Biotechnol Prog*, **16**, 657-60.

Kim, S.W., Seo, W.T., Park, Y.H. 1997. Enhanced synthesis of trispoic acid and β-carotene production in Blakeslea trispora by addition of a non-ionic surfactant, span 20. *J Ferment Bioeng*, **84**, 330-332.

Kimura, K., Yamaoka, M., Kamisaka, Y. 2004. Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. *J Microbiol Methods*, **56**, 331-338.

Ko¨se, O., ter, M.T., Aksoy, H.A. 2002. Immobilized Candida antarctica lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. *Bioresour Technol*, **83**, 125-9.

Kosa, M., Ragauskas, A.J. 2011. Lipids from heterotrophic microbes: advances in metabolism research. *Trends Biotechnol,* **29**.

Kraisintu, P., Yongmanitchai, W., Limtong, S. 2010. Selection and Optimization for Lipid Production of a Newly Isolated Oleaginous Yeast, *Rhodosporidium toruloides DMKU3-TK16*. *Nat Sci*, **44**, 436 - 445.

Kulkarni, M.G., Dalai, A.K., Bakhshi, N.N. 2006. Utilization of green seed canola oil for biodiesel production. *J Chem Technol Biotechnol,* **81**, 1886-93.

Kusdiana, D., Saka, S.I. 2001. Kinetics of transestrification in rapseed oil to biodiesel fuel as tested in supercritical methanol. *Fuel Proc Technol*, **80**, 693-5.

Laoteng, K., Anjard, C., Rachadawong, S., Tanticharoen, M., Maresca, B., Cheevadhanarak, S. 1999. Mucor rouxii Δ9-desaturase gene is transcriptionally regulated during cell growth and by low temperature. *Mol Cell Biol Res Commun*, **1**, 36-43.

Laouar, L., Lowe, K.C., Mulligan, B.J. 1996. Yeast responses to nonionic surfactants. *Enzym Microb Technol*, **18**, 433-438.

Lardizabal, K.D., Mai, J.T., Wagner, N.W., Wyrick, A., Voelker, T., Hawkins, D.J. 2001. DGAT2 is a new diacylglycerol acyltransferase gene family: purification, cloning, and expression in insect cells of two polypeptides from Mortierella ramanniana with diacylglycerol acyltransferase activity. *J Biol Chem,* **276**, 38862-9.

Lee, S.J., Pil-Soo, S., Ho, K.C., K.Ohsuk, H.Byung-Ki, S.Jeong-Woo. 2009. Isolation and Characterization of the Eicosapentaenoic Acid Biosynthesis Gene Cluster from Shewanella sp. BR-2. *Microbiol Biotechnol*, **19**, 881-887.

Leesing, R., Baojungharn, R. 2011. Microbial oil production by isolated oleaginous yeast torulaspora globosa YU5/2. *World Acad Sci Eng Technol,* **76**.

Lehner, R., Kuksis, A. 1996. Biosynthesis of triacylglycerols. *Prog Lipid Res* **35**, 169-201.

Li, S.J., Jr, J.E.C. 1993. Growth rate regulation of Escherichia coli acetyl coenzyme.A carboxylase, which catalyzes the first committed step of lipid biosynthesis. *J Bacteriol*, **175**, 332-340.

Li, Y.H., Liu, B., Zhao, Z.B., Bai, F.W. 2006. Optimized culture medium and fermentation conditions for lipid production by *Rhodosporidium toruloides*. *Chin J Biotechnol,* **22**, 650-6.

Li, Y.H., Zhao, Z.B., Bai, F.W. 2007. High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture. *Enzym Microb Technol*, **41**, 312-317.

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

Libkind, D., Arts, M.T., VanBroock, M. 2008. Fatty acid composition of cold adapted carotenogenic basidiomycetous yeasts. *Argent Microbiol*, **40**, 193-197.

Liu, B., Zhao, Z. 2007. Biodiesel production by direct methanolysis of oleaginous microbial biomass. *J Chem Technol Biotechnol*, **82**, 775-780.

Liu, G.-Q., Lin, Q.-L., Jin, X.-C., Wang, X.-L., Zhao, Y. 2010. Screening and fermentation optimization of microbial lipid-producing molds from forest soils. *Afr J Microbiol Res*, **4**, 1462-1468.

Ma, F., Hanna, M.A. 1999. Biodiesel production: a review. *Bioresour Technol*, **70**, 1-15.

Ma, Y. 2006. Microbial oils and its reaserch advance. *Chin J Bio-process Eng*, **4**, 7-11.

Makri, A., Fakas, S., Aggelis, G. 2010. Metabolic activities of biotechnological interest in Yarrowia lipolytica grown on glycerol in repeated batch cultures. *Bioresour Technol*, **101**, 2351-2358.

Meng, X., Yang, J., Xu, X., Zhang, L. 2009. Biodiesel production from oleaginous microorganisms. *Renew Energ*, **34**, 1-5.

Miao, X., Wu, Q. 2006. Biodiesel production from heterotrophic microalgal oil *Bioresour Technol,* **97**, 841-846.

Michinaka, Y., Aki, T., Shimauchi, T., Nakajima, T., Kawamoto, S., Shigeta, S., Suzuki, O., Ono, K. 2003. Differential response to low temperature of two Ä6 fatty acid desaturases from Mucor circinelloides. *Appl Microbiol Biotechnol,* **62**, 362-368.

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

Mlickova, K., Roux, E., Athenstaedt, K., d'Andrea, S., Daum, G., Chardot, T., Nicaud, J.M. 2004. Lipid accumulation, lipid body formation, and acyl coenzyme A oxidases of the yeast *Yarrowia lipolytica*. *Appl Environ Microbiol,* **70**, 3918-24.

Mohamed, M., Soumanoua, B., Uwe, T., Bornscheuer, A. 2003. Improvement in lipasecatalyzed synthesis of fatty acid methyl esters from sunflower oil. *Enzym Microb Technol*, **33**, 97-103.

Mu, Y., Xiu, Z.L., Zhang, D.J. 2008. A combined bioprocess of biodiesel production by lipase with microbial production of 1,3-propanediol by Klebsiella pneumoniae. *Biochem Eng J*, **40**, 537-41.

Munn, A.L., Riezman, H. 1994. Endocytosis is required for the growth of vacuolar H(+)-ATPase-defective yeast: identification of six new END genes. *J Cell Biol*, **127**, 373-86.

Murphy, D.J. 2001. he biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog Lipid Res*, **40**, 325-438.

Napier, J.A. 2007. The production of unusual fatty acids in transgenic plants. *Annu Rev Plant Biol*, 295-319.

Nomura, C.T., Taguchi K., Gan Z.H., Kuwabara, K., Tanaka, T., Takase, K., Doi, Y. 2005. Expression of 3-ketoacyl-acylcarrier protein reductase (fabG) genes enhances production of polyhydroxyalkanoate copolymer from glucose in recombinant *Escherichia col*i JM109. *Appl Environ Microbiol*, **71**, 4297-4306.

Oelkers, P., Cromley, D., Padamsee, M., Billheimer, J.T., Sturley, S.L. 2002. The DGA1 gene determines a second triglyceride synthetic pathway in yeast. *J Biol Chem.*, **277**, 8877-8881.

Oelkers, P.M., Sturley, S.L. 2004. Mechanisms and mediators of neutral lipid biosynthesis in eukaryotic cells In: Lipid Metabolism and Membrane Biogenesis, edited by Daum G. Berlin. *Springer-Verlag*, 281-311.

Pan, J.G., Kwak, M.Y., Rhee, J.S. 1986. High density cell culture of Rhodotorula glutinis using oxygen-enriched air. *Biotechnol Lett,* **8**, 715-8.

Pan, L.X., Yang, D.F., Shao, L., Li, W., Chen, G.G., Liang, Z.Q. 2009. Isolation of the Oleaginous Yeasts from the Soil and Studies of Their Lipid-Producing Capacities *Food Technol Biotechnol*, **47**, 215-220

Papanikolaou, S., Aggelis, G. 2002. Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. *Bioresour Technol*, **82**, 43-49.

Papanikolaou, S., Aggelis, G. 2003. Modeling lipid accumulation and degradation in *Yarrowia lipolytica* cultivated on industrial fats. *Curr Microbiol,* **46**, 398-402.

Papanikolaou, S., Chevalot, I., Komaitis, M., Aggelis, G., Marc, I. 2001. Kinetic profile of the cellular lipid composition in an oleaginous yeast *Yarrowia lipolytica* capable of producing a cocoa-butter substitute from industrial fats. *Antonie van Leeuwenhoek*, **80**, 215-224.

Papanikolaou, S., Chevalot, I., Komaitis, M., Marc, I. 2002. Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. *Appl Microbiol Biotechnol*, **58**, 308-12.

Papanikolaou, S., Galiotou-Panayotou, M., Chevalot, I., Komaitis, M., Marc, I., Aggelis, G. 2006. Influence of glucose and saturated free-fatty acid mixtures on citric acid and lipid production by *Yarrowia lipolytica*. *Curr Microbiol*, **52**, 134-42.

Papanikolaou, S., Galiotou-Panayotou, M., Fakas, S., Komaitis, M., Aggelis, G. 2007. Lipid production by oleaginous Mucorales cultivated on renewable carbon sources. *Eur J Lipid Sci Technol*, **109**, 1060-1070.

Papanikolaou, S., Komaitis, M., Aggelis, G. 2004. Single cell oil SCO production by Mortieralla isabellina grown on high -sugar content media. *Bioressour Technol,* **95**, 287-291.

Park, M.O. 2005. New pathway for long-chain n-alkane synthesis via 1-alcohol in *Vibrio furnissii M1*. *J Bacteriol*, **187**, 1426-1429.

Peng, X.W., Chen, H. 2008. Single cell oil production in solid-state fermentation by Microsphaeropsis sp. From steam-exploded wheat straw mixed with wheat bran. *Bioresour Technol,* **99**, 3885-3889.

Pinzi, S., I.L.Garcia, Lopez-Gimenez, F.J., Castro, M.D.L.d., Dorado, G., Dorado, M.P. 2009. The ideal vegetable oil-based biodiesel composition: a review of social, economical and technical implications. *Energy Fuels*, **23**, 2325-41.

Rajakumari, S., Grillitsch, K., Daum, G. 2008. Synthesis and turnover of non-polar lipids in yeast. *Prog Lipid Res,* **47**, 157-171.

Ratledge, C. 1988. Biochemistry, stoichiometry, substrates and economics in: Moreton, R. S. (Ed.), Single Cell Oil. *Longman Scientific & Technical, Harlow (UK)*, 33-70.

Ratledge, C. 2004. Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, **86**, 807-815.

Ratledge, C. 2002. Regulation of lipid accumulation in oleaginous micro-organisms. *Biochem Soc Trans*, **30**, 1047-50.

Ratledge, C. 1982. Single Cell Oil. *Enzyme Microb Technol*, **4**, 58-60.

Ratledge, C., Wilkinson, S.G. 1989. Microbial Lipids. *Academic Press London UK*, 555-697.

Ratledge, C., Wynn, J. 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv Appl Microbiol*, **51**, 1-44.

Robles-Medina, A., Gonzalez-Moreno, P.A., Esteban-Cerdan, L., Molina-Grima, E. 2009. Biocatalysis: Towards ever greener biodiesel production. *Biotechnol Adv*, **27**, 398-408.

Rossi, M., Buzzini, P., Cordisco, L., Amaretti, A., Sala, M., Raimondi, S., Ponzoni, C., Pagnoni, U.M., Matteuzzi, D. 2009. Growth,lipid accumulation and fatty acids composition in obligate psychrophilic facultative psychrophilic and mesophilic yeasts. *.FEMS Microbiol.Ecol*, **69**, 363-372.

Ruenwai, R., Cheevadhanarak, S., Laoteng, K. 2009. Overexpression of acetyl-CoA carboxylase gene of Mucor rouxii enhanced fatty acid content in Hansenula polymorpha. *Mol Biotechnol*, **42**, 327-332.

Ruenwai, R., Cheevadhanarak, S., S.Rachdawong, M., T., K.Laoteng. 2010. Oxygen-induced expression of ∆6-, ∆9- and ∆12-desaturase genes modulates fatty acid composition in Mucor rouxii. *Appl Microbiol Biotechnol*, **86**, 327-334.

Rymowicz, W., Rywinska, A., Zarowska, B., Juszczyk, P. 2006. Citric acid production from raw glycerol by acetate mutants of *Yarrowia lipolytica*. *Chem Pap*, **60**, 391-3.

Saenge, C., Cheirsilp, B., Suksaroge, T.T., Bourtoom, T. 2011. Efficient Concomitant Production of Lipids and Carotenoids by Oleaginous Red Yeast Rhodotorula glutinis Cultured in Palm Oil Mill Effluent and Application of Lipids for Biodiesel Production. *Biotechnol Bioprocess Eng,* **16**, 23-33.

Saengea, C., Cheirsilpb, B., Suksarogea, T., Bourtoom, T. 2011. Potential use of oleaginous red yeast Rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochem,* **46** 210-218.

Saha, S., Enugutti, B., Rajakumari, S., Rajasekharan, R. 2006. Cytosolic triacylglycerol biosynthetic pathway oilseeds. Molecular cloning and expression cytosolic diacylglycerol acyltransferase. *Plant Physiol*, **141**, 1533-1543.

Sandager, L., Gustavsson, M.H., Stahl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronneà, H., Stymne, S. 2002. Storage lipid synthesis is non-essential in yeast. *J Biol Chem,* **277**, 6478-648.

Satomi, M., Oikawa, H., Yano, Y. 2003. Shewanella marinintestina sp. nov., Shewanella schlegeliana sp. nov. and Shewanella sairae sp. nov., novel eicosapentaenoic-acid-producing marine bacteria isolated from sea-animal intestines*. Int J Syst Evol Microbiol*, **53**, 491-499

Schirmer, A., Rude, M.A., Li, X., Popova, E., Cardayre, S.B.d. 2010. Microbial biosynthesis of alkanes. *Science*, **329**, 559-62.

Schujman, G.E., Mendoza, D. 2008. Regulation of type II fatty acid synthase in Gram-positive bacteria. *Curr Opin Microbiol,* **11**, 148-152

Schulz, B., C.Boyle, Draeger, S., Rommert, A., Krohn, K. 2002 Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycol Res*, **106**, 996-1004.

Schweizer, E., Bolling, H. 1970. A Saccharomyces cerevisiae mutant defective in saturated fatty acid biosynthesis. *Proc Natl Acad Sci U S A*, **67**, 660-6.

Schweizer, M., Roberts, L.M., Holtke, H.J., Takabayashi, K., Hollerer, E., Hoffmann, B., Muller, G., Kottig, H., Schweizer, E. 1986. The pentafunctional FAS1 gene of yeast: its nucleotide sequence and order of the catalytic domains. *Mol Gen Genet,* **203**, 479-86.

Shi, H., Bao, Z. 2008. Direct preparation of biodiesel from rapeseed oil leached by twophase solvent extraction. *Bioresour Technol*, **99**, 9025-8.

Somashekar, D., Joseph, R. 2000. Inverse relationship between carotenoid and lipid formation in Rhodotorula gracilis according to the C/N ratio of the growth medium. *World J Microbiol Biotechnol*, **16**, 491-393.

Somashekar, D., Venkateshwaran, G., Sambaiah, K., Lokesh, B.R. 2003. Effect of culture conditions on lipid and gamma-linolenic acid production by muccoraceous fungi. *Proc Biochem,* **38**, 1719-1724.

Sorger, D., Athenstaedt, K., Hrastnik, C., Daum, G. 2004. A yeast strain lacking lipid particles bears a defect in ergosterol formation. *J Biol Chem,* **279** 31190-3119.

Sorger, D., Daum, G. 2002. Synthesis of triacylglycerols by the acyl-coenzyme A:diacyl-glycerol acyltransferase Dga1p in lipid particles of the yeast *Saccharomyces cerevisiae*. *J Biol Chem*, **184**, 519-524.

Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., del Cardayre, S.B., Keasling, J.D. 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature*, **463**, 559-563.

Stredanska, S., Sajbidor, J. 1993. Influence of carbon and nitrogen sources on the lipid accumulation and arachidonic acid production by Mortierella alpine. *Acta Biotechnol*, **13**, 185-191.

Strobel, G.A., Knighton, B., Kluck, K., Ren, Y., T.Livinghouse, Griffin, M., Spakowicz, D., Sears, J. 2008. The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus Gliocladium roseum (NRRL 50072). *Microbiology,* **154**, 3319-332.

Subhash, G.V., Mohan, S.V. 2011. Biodiesel production from isolated oleaginous fungi Aspergillus sp. Using corncob waste liquor as a substrate. *Bioresour Technol,* **102**, 9286-90

Tabatabaeia, M., Tohidfara, M., G.S. Jouzania, Safarnejada, M., Pazoukib, M. 2011. Biodiesel production from genetically engineered microalgae: Future of bioenergy in Iran. *Renew Sustain Energy Rev*, **15**, 1918-1927.

Tehlivets, O., Scheuringer, K., Kohlwein, S.D. 2007. Fatty acid synthesis and elongation in yeast., *BBA - Molecular Cell Biol***, 1771**, 255-270.

Turkish, A.R., Sturley, S.L. 2009. The genetics of neutral lipid biosynthesis: an evolutionary perspective. *Am J Physiol Endocrinol Metab,* **297**, 19-27.

VanMeer, G., Voelker, D.R., Feigenson, G.W. 2008. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol*, **9**, 112-24.

Verwoert, I.I., Linden, K.H.V.D., Walsh, M.C., Nijkamp, H.J., Stuitje, A.R. 1995. Modification of Brassica napus seed oil by expression of the Escherichia coli fabH gene, encoding 3-ketoacyl-acyl carrier protein synthase III. *Plant Mol Biol*, **27**, 875-886.

Verwoert, I.I.G.S., Verbree, E.C., Vanderlinden, K.H., Nijkamp, H.J.J., Stuitje, A.R. 1992. Cloning, nucleotide-sequence,and expression of the *Escherichia-coli*-fabD gene, encoding malonyl coenzyme a-acylcarrier protein transacylase. *J Bacteriol*, **174**, 2851-2857.

Vicente, G., Bautista, L.F., Rodriguez, R., Gutierrez, F.J., Sadaba, I., Ruiz-Vazquez, R.M., Torres-Martínez, S., Garre V. 2009. Biodiesel production from biomass of an oleaginous fungus. *Biochem Eng J*, **48**, 22-7.

Wang, D., Li, M., Wei, D., Cai, Y., Zhang, Y., Xing, L. 2007. Identification and functional characterization of the delta 6-fatty acid desaturase gene from Thamnidium elegans. *J Euk Microbiol,* **54**, 110-117.

Wang, L., Sun, Y.M., Wang, P.Z., Zhao, Z.B. 2005. Effects of metal ions on lipid production by fermentation with Trichosporon fermentans. *J. Dalian Inst. Light Indus,* **24,** 259-263.

White, S.W., Zheng, J., Zhang, Y.M., Rock. 2005. The structural biology of type II fatty acid biosynthesis. *Annu Rev. Biochem*, **74**, 791-831.

Whittle, E., Shanklin, J. 2001. Engineering Delta(9)-16:0-acy lcarrier protein(ACP) desaturase specificity based on combinatorial saturation mutagenesis and logical redesign of the castor Delta(9)-18:0-ACPdesaturase. *J Biol Chem*, **276**, 21500-21505.

Woese, C.R., Stackebrandt, E., Weisburg, W.G., Paster, B.J., Madigan, M.T., Fowler, V.J., Hahn, C.M., Blanz, P., R.Gupta, Nealson, K.H., Fox, G.E. 1984. The phylogeny of purple bacteria: The alpha subdivision. *System Appl Microbiol*, **5**, 315-326.

Wu, S., Hu, C., Jin, G., Zhao, X., Zhao, Z.K. 2010. Phosphate-limitation mediated lipid production by *Rhodosporidium toruloides*. *Bioresour Technol*, **101**, 6124-6129.

Wu, S.G., Zhao, X., Hu, C.M., Zhang, S.F., Hua, Y.Y., .Zhao, Z.B. 2008. Screening of fungi for microbial oil production using N-acetyl-D-glucosamine. *China Biotechnol*, **28**, 58-62.

Wu, S.T., Yu, S.T., Lin, L.P. 2005. Effect of culture conditions on docosahexaenoic acid production by Schizochytrium sp. S31. *Process Biochem*, **40**, 3103-8.

Wua, S., Zhao, X., H.Shen, Wang, Q., Zhao, Z.K. 2011. Microbial lipid production by Rhodosporidium toruloides under sulfate-limited conditions. *Bioresour Technol,* **102**, 1803-7.

Wynn, J.P., Ratledge, C. 2000. Evidence that the rate-limiting step for the biosysthesis of arachidonic acid in Mortierella alpine is at the level of the 18:3 to 20:3 elongase. *Microbiol,* **146**, 2325-2331.

Xia, C., Zhang, J., Zhang, W., Hu, B. 2011. A new cultivation method for microbial oil production: cell pelletization and lipid accumulation by *Mucor circinelloides*. *Biotechnol Biofuels*, 4-15.

Xue, F.Y., Miao, J.X., Zhang, X., Luo, H., Tan, T.W. 2008. Studies on lipid production by Rhodotorula glutinis fermentation using monosodium glutamate wastewater as culture medium. *Bioresour Technol,* **99**, 5923-5927.

Yamauchi, H., Mori, H., Kobayashi, T., Shimizu, S. 1983. Mass production of lipids by Lipomyces starkeyi in microcomputer-aided fed-batch culture. *J Ferment. Technol,* **61**, 275-280.

Yang, H., Bard, M., Bruner, D.A., Gleeson, A., Deckelbaum, R.J., Aljinovic, G., Pohl, T.M., Rothstein, R., Sturley, S.L. 1996a. Sterol esterification in yeast: a two-gene process. *Science*, **272**, 1353-6.

Zha, W.J., S.B. Rubin-Pitel, Z.Y.Shao, Zhao, H.M. 2009. Improving cellular malonyl-CoA level in *Escherichia coli* via metabolicengineering. *Metabol Eng*, **11**, 192-198.

Zhao, C.H., Zhang, T., Li, M., Chi, Z.M. 2010a. Single cell oil production from hydrolysates of inulin and extract of tubers ofJerusalem artichoke by *Rhodotorula mucilaginosa TJY15a*. *Proc Biochem*, **45**, 1121-1126.

Zhao, X., Wu, S., Hu, C., Wang, Q., Hua, Y., Zhao, Z.K. 2010b. Lipid production from Jerusalem artichoke by Rhodosporidium toruloides Y4. *J Ind Microbiol Biotechnol,* **37**, 581-585.

Zhao, Z.B.l. 2005. Toward cheaper microbila oil for biodiesel. *China Biotechnol*, **25**, 8-11.

Zheng, Z.M., Xu, Y.Z., Liu, H.J., Guo, N.N., Cai Z.Z., Liu D.H. 2008. Physiologic mechanisms of sequential products synthesis in 1,3-propanediol fed-batch fermentation by Klebsiella pneumoniae. *Biotechnol Bioeng*, **100**, 923-32.

Zhu, L.Y., Zong, M.H., Wu, H. 2008. Efficient lipid production with Trichosporon fermentans and its use for biodiesel preparation. *Bioresour Technol*, **99**, 7881-7885.

Zhu, M., Yu, L.J., Li, W., Zhou, P.P., Li, C.Y. 2006. Optimization of arachidonic acid production by fed-batch culture of Mortierella alpina based on dynamic analysis. *Enzym Microb Technol*, **38**, 735-40.

Zimmerman, A.W., Veerkamp, J.H. 2002. New insights into the structure and function of fatty acid-binding proteins. *Cell Mol Life Sci*, **59**, 1096-116.

Zinser, E., Paultauf, F., Daum, G. 1994. Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. *J Bacteriol*, **175**, 2853-8.

Zweytick, D., Athenstaedt, K., Daum, G. 2000a. Intracellular lipid particles of eukaryotic cells. *Biochim Biophys Acta,* **1469**, 101-120.

Zweytick, D., Leitner, E., Kohlwein, S.D., Yu, C., Rothblatt, J., Daum G. 2000b. Contibution of Are1p and Are2p to steryl ester synthesis in the yeast Saccharomyces cerevisiae. *Eur J Biochem*, **267**, 1075.

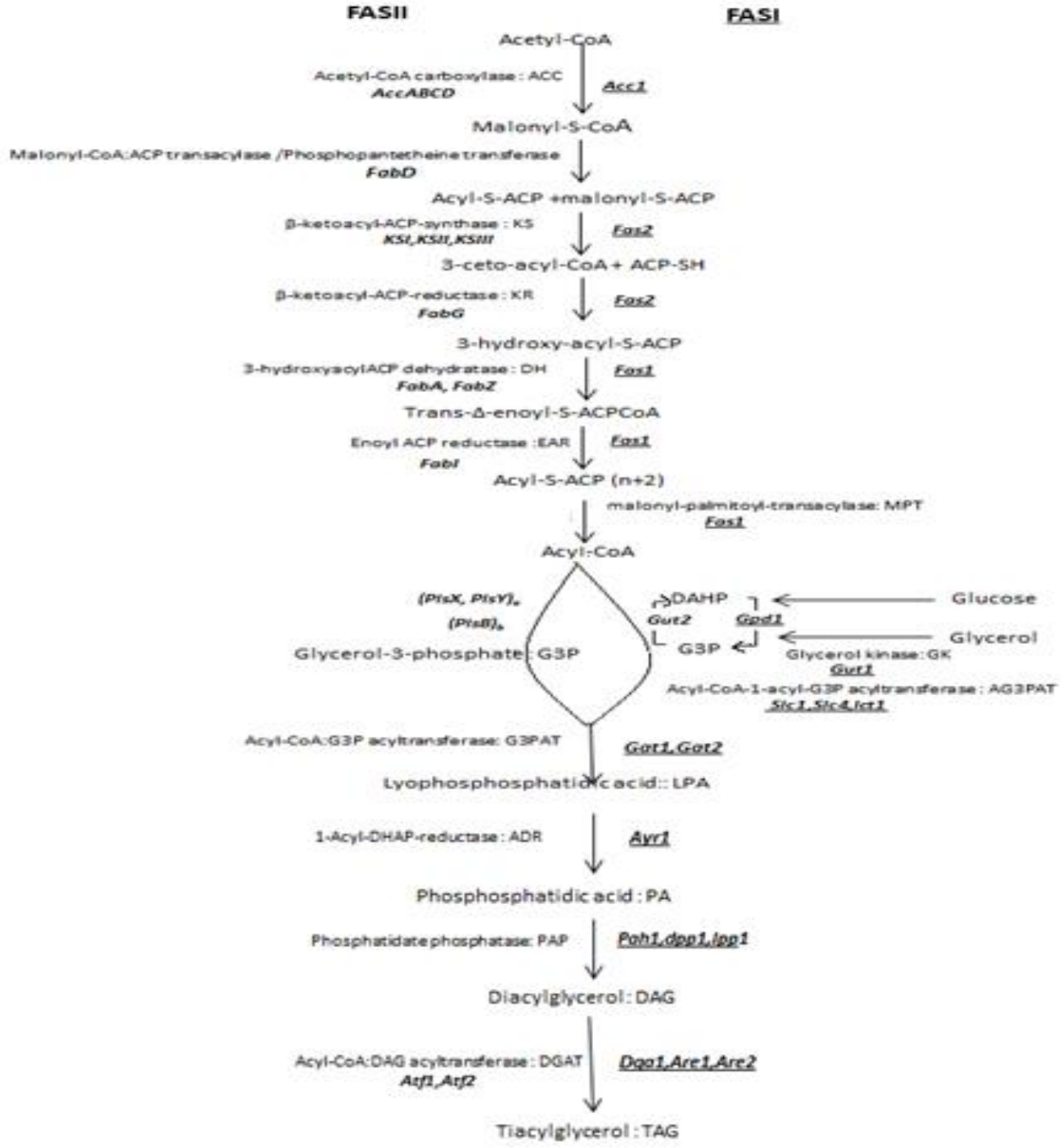


Figure 2.1 **Schematic of FA and TAG synthesis and enzymes involved in heterotrophic microbes. Names of enzymes are mentioned. Underlined genes refer to pathways in yeast : a) *B. subtilis* Gram-positive bacteria model organism enzymes. b) *E. coli* Gram-negative bacteria model**

Tableau 2.1 Oleaginous yeast for lipid accumulation

Strains	Substrates	Mode of culture	Kinetic parameters	References
C. curvatum	*Glucose*	Continuous	Lipid content (31.9%), lipid productivity (0.42 g/h/L)	(Hassan et al., 1993)
C. curvata D	glucose and xylose	Continuous	Lipid content (0.16%), lipid productivity 0.27 (g/h/L)	(Evans & Ratledge, 1983)
C. curvatus	Crude glycerol	Two stage Fed batch	Biomass (32.9 g/L), lipid content (52.9%), lipid yield (0.4 g/L), lipid productivity (1.5 g/L/day)	(Liang et al., 2010)
C.curvatus	Crude glycerol	One stage Fed batch	Biomass (31.2 g/L), lipid content (44.6%), lipid yield (0.6g/L), lipid productivity (1.2 g/day/L)	(Liang et al., 2010)
R. glutinis TISTR 5159	Crude glycerol	Batch	Biomass (8.17 g/L), lipid content (52.91%), lipid yield (4.33 g/L), lipid productivity (0.058 g/h/L)	(Saengea et al., 2011)
R. glutinis TISTR 5159	Palm Oil Mill Effluent	Batch	Biomass (9.15 g/L), lipid content (60.20%), lipid yield (5.55 g/L)	(Saengea et al., 2011)
Y. lipolytica	Glycerol	Batch	Biomass (6.71 g/L), lipid content (20.40%), lipid yield (0.25 g/g)	(Zhao et al., 2010a)
	Glycerol	Batch	Biomass (4.68 g/L), lipid content (22.30%), lipid yield (0.36 g/g)	(Makri et al., 2010)
R. toruloides Y4	Jerusalem arichoke	Batch	Biomass (40 g/L), lipid content (43.30%), lipid yield (17.2 g/L), lipid productivity (0,38 g/h/L)	(Zhao et al., 2010b)
R. toruloides Y4	J. arichoke	Fed batch	Biomass (70 g/L), lipid content (56.5%), lipid yield (39.60 g/L), lipid productivity (33 g/h/L)	(Zhao et al., 2010b)
R. glutinis TISTR 5159	Crude glycerol	Fed batch	Biomass (13.77 g/L), lipid content (60.70%), lipid yield (8.36 g/L), lipid productivity (0.116 g/h/L)	(Saengea et al., 2011)
R .toruloides Y4	Glucose	Fed Batch	Biomass (151.5 g/L), lipid content (48.0%), lipid yield (0.26 g/g), lipid productivity (2.91 g/day/L)	(Li et al., 2007)
R. glutinis TISTR 5159	Palm Oil Mill Effluent	Semi-continuous	Biomass (10.9 g/L), lipid content (67.27%), lipid yield (7.4 g/L)	(Saengea et al., 2011)
T.globosa YU5/2	Glucose	Flask	Biomass (8.81g/L), lipid content (47.2%), lipid yield (4.16 g/L), lipid productivity (0.520 g/L/day)	(Leesing & Baojungharn, 2011)

T.globosa YU5/2	Sweet potato hydrolysate	Flask	Biomass (10.34 g/L), lipid content (23%), lipid yield (72.48 g/L), lipid prodctivity (0.619 g/L/day)	(Leesing & Baojungharn, 2011)
Y. lipolytica	Crude glycerol	Continuous	Biomass (8.1 g/L), lipid content (43%), lipid yield (0.2 g/g), lipid productivity (2.6 g/L/day)	(Papanikolaou & Aggelis, 2002)
Y. lipolytica	Glycerol/stearin	Continuous	Biomass (9 g/L), lipid content (44-54%), lipid yield (2.8 g/L)	(Papanikolaou et al., 2002)

Tableau 2.2 Distribution of fatty acids in oleaginous fungi

Strains	Substrates	Kinetic parameters	*Total fatty acid (%)*							References
			C14 : 0	C16 :0	C16:1	C18:0	C18:1	C18:2	C18:3	
Absidia corymbifera	NA	Lipid content (27%)	1	24	-	7	46	8	10	(Ratledge, 1982)
Aspergillus oryzae	Sabouraud dextrose broth	Biomass (13.6 g/L), Lipid content (23.3%)	0.19	0.73	14.4	48.7	1.60	0.42	0.09	(Subhash & Mohan, 2011)
	Corncob waste liquor	Biomass (2 g/L) Lipid content (21.2%)	0.19	0.73	14.8	43.4	0.42	0.42	-	(Subhash & Mohan, 2011)
Aspergillus terreus	NA	Lipid content (57%)	2	23	-	14	40	21	-	(Ratledge, 1982)
Alternaria sp. (DM09)	Glucose	Biomass (14.6 g/L) Lipid content (58.1%) Lipid yield (7.8 g/L)	2.10	6.12	0.56	5.15	60.15	23.51	3.60	(Dey et al., 2011)
Colletotrichum sp. (DM06)	Glucose	Biomass (16.2 g/L) Lipid content (46.8%) Lipid yield (7.8 g/L)	1.02	5.06	0.26	3.28	58.14	23.21	2.16	(Dey et al., 2011)
C. echinulata ATHUM 4411	Glucose	Biomass (9.2g/L) Lipid content (35%) Lipid yield (3.2 g/L)	tr	19.1	-	11.5	39.9	15.2	-	(Papanikolaou et al., 2007)
C. echinulata ATHUM 4411	Starch	Biomass (13.5g/L) Lipid content (28%) Lipid yield (3.8g/L)	1.5	17.2	-	6.8	43.5	16.9	-	(Papanikolaou et al., 2007)
	Pectin	Biomass (4.1 g/L) Lipid content (10%) Lipid yield (45 g/L)	19.1	24.5	-	9.5	28.2	9.7	-	(Papanikolaou et al., 2007)
M.isabellina	Sweet sorghum	Lipid productivity (0.115 g/L/h)	-	24-39	-	-	37-55	2-11	-	(Kosa & Ragauskas, 2011)

M. isabellina ATHUM 2935	Glucose	Biomass (35.9 g/L) Lipid content (50–55%) Lipid yield (18.1 g/L)	-	20-25	0-5	0-2	48-55	18-20	0-5	(Papanikolaou et al., 2004)
M. isabellina ATHUM 2935	Glucose	Biomass (10.9 g/L) Lipid content (48%) Lipid yield (5.2 g/L)	1.7	25.1	-	3.7	53.6	10.9	-	(Papanikolaou et al., 2007)
M. isabellina ATHUM 2935	Starch	-	2.1	25.5	-	4	51.2	9.9	-	(Papanikolaou et al., 2007)
	Pectin		1.6	25.5	-	8.1	45.1	13.0	-	(Papanikolaou et al., 2007)
	Lactose		-	26.7	-	5.0	52.4	10.2	-	(Papanikolaou et al., 2007)
M. circinelloides	Glucose	Biomass (1.24 g/L), Lipid content (30.87%)								(Xia et al., 2011)
Rhizopus stolonifer LGAM (9)1	Glucose	Lipid content (27.75%)								(Kavadia et al., 2001)
Zygorhychus BPIC 1703	Glucose	Lipid content (23.31%)								(Kavadia et al., 2001)

Tableau 2.3 Lipid production of oleaginous microorganisms with various carbon sources

Strain	Carbon source	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)	References
C. echinulata	Orange peel	NA	1.7	NA	(Gema et al., 2002)
	Starch	13.5	28	3.8	(Papanikolaou et al., 2007)
	Pectin	4.1	10	0.4	(Papanikolaou et al., 2007)
	Glucose	NA	49	4.4	(Gema et al., 2002)
L. starkeyi	Sewage sludge	9.4	68.0	6.4	(Angerbauer et al., 2008)
M. isabellina	Pectin	4.1	10	0.4	(Papanikolaou et al., 2007)
Mucor racemosus	Olive oil+ corn steep liquor	-	-	13.4	(Zhu et al., 2008)
Mucor circinelloides	Thin stillage	20	46	9.2	(Mitra et al., 2012)
	Starch	10.4	36	3.7	(Papanikolaou et al., 2007)
	Glucose	35.9	50–55	18.1	(Papanikolaou et al., 2004)
	Lactose	9.5	37	3.5	(Papanikolaou et al., 2007)
R. toruloides Y4	Glucose		67.5		(Li et al., 2007)
	JA extract		43.3		(Zhao et al., 2010b)
T. fermentans	Molasses	36.4	35.3	12.8	(Zhu et al., 2008)
	Glucose	24.1	56.6	13.6	(Zhu et al., 2008)
	Sucrose	19.5	62.6	12.2	(Zhu et al., 2008)
	Xylose	17.1	57.8	9.9	(Zhu et al., 2008)
	Lactose	16.9	49.6	8.4	(Zhu et al., 2008)
	Fructose	21.5	40.7	8.8	(Zhu et al., 2008)
Y. lipolytica	Stearin		52		(Papanikolaou et al., 2007)
	Glycerol		43		(Papanikolaou & Aggelis, 2002)

3 CHAPITRE 3 : LIPID PRODUCTION BY YARROWIA LIPOLYTICA GROWN ON BIODIESEL-DERIVED CRUDE GLYCEROL : OPTIMIZATION OF GROWTH PARAMETRS AND THEIR EFFECTS ON THE FERMENTATION EFFICIENCY

Lipid production by *Yarrowia lipolytica* grown on biodiesel-derived crude glycerol: optimization of growth parameters and their effects on the fermentation efficiency

Magdouli Sara[a], Satinder Kaur Brar[b], Jean François Blais[c,*]

[a] Ph.D. student, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-4677, Fax: (418) 654-2600, email: magdouli sara@ete.inrs.ca

[b] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-3116, Fax: (418) 654-2600, email: satinder.brar@ete.inrs.ca

[c] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-2575, Fax: (418) 654-2600, email: blaisjf@ete.inrs.ca

*Corresponding author

Email: blaisjf@ete.inrs.ca

RSC Advances (2016) **6**, 90547 - 90558

3.1 Résumé

Yarrowia lipolytica est une souche oléagineuse bien connue pour l'accumulation des huiles d'organismes unicellulaires (HOU). La production a été étudiée et optimisée dans des cultures en carence d'azote. L'effet d'une augmentation de la concentration initiale de glycérol brut et d'azote a été évalué durant la fermentation. Une importante production de biomasse a été observée avec une concentration élevée de glycérol brut de 89 g/L avec 0,54 g/L d'hydroxyde d'ammonium pendant 66 h. Les conditions optimales de culture ont été testées dans un fermenteur de 5 L incluant une variation de la concentration en oxygène dissous de 60% à 30% correspondante à un coefficient de transfert d'oxygène de 80 à 50 h^{-1} . Une concentration en lipides de 13,6 ± 0,8 g/L et une teneur en lipides de 52,7 ± 1,2% (p/p de biomasse sèche) ont été obtenues, des valeurs supérieures à celles rapportées dans la littérature pour des espèces de *Yarrowia* cultivées dans des milieux à base de glycérol brut. Les lipides de la levure contenaient principalement des acides oléique, palmitique, linoléique et stéarique, qui pourraient constituer d'excellents précurseurs pour la synthèse de biodiesel.

Mots-clés : biodiesel, glycérol brut, *Y. lipolytica*, huiles d'organismes unicellulaires, acide citrique

3.2 Abstract

Yarrowia lipolytica, a well-known oleaginous strain for single cells oil (SCO) production was grown in nitrogen-limited flask cultures. The effect of increasing the initial crude glycerol and nitrogen concentration was studied along fermentation process. Significant biomass and SCO production was reported with high initial glycerol concentration of 89 g/L and 0.54 g NH_4OH/L during 66 h. Optimized culture conditions were tested using 5-L fermenter during two-stage cultivation with a dissolved oxygen shift from 60% to 30% of dissolved oxygen corresponding to 50-80 h^{-1}. Lipid concentration of 13.6 ± 0.8 g/L and lipid content 52.7 ± 1.2% (w/w of dry biomass) was obtained which is higher compared with literature values for *Yarrowia* species grown on crude glycerol based media. The yeast lipids contained mainly oleic, palmitic, linoleic and stearic acids which could serve as perfect precursors for the synthesis of biodiesel.

Keywords: biodiesel-derived glycerol, *Y. lipolytica*, single cell oil, citric acid.

3.3 Introduction

Biodiesel has gained interest in recent years due to its contribution to minimize dependence on fossils fuels, especially in transportation sector. Moreover, biodiesel is known to be biodegradable, sustainable, renewable and no toxic fuel. It is reported to reduce sulfur and carbon dioxide emissions compared to fossil engines (Vicente et al., 2004, Antolin et al., 2004).Recently, it was estimated that the biodiesel market will reach 37 billion gallons by 2016 with an annual growth of 42% which is indirectly producing 4 billion gallons of crude glycerol as a by-product. Crude glycerol of 10 kg will be produced from 100 kg of biodiesel (Kerr et al., 2007, Garlapati et al., 2016).Plants oils, e.g, jatropha, corn and canola were reported to produce biodiesel. However, these vegetable oils cannot meet the huge demand of utilization and does not contribute to global energy security. Therefore, oleaginous microorganisms that are reported to produce single cells oils in the presence of high carbon source and a low nitrogenous source represented potential candidates (Rodolfi et al., 2009, Magdouli,et al., 2014). These microorganisms offer advantages to grow faster than higher plants and do not require land. Likewise, a significant number of reports, appearing in most cases in the past few years, indicates the potential of heterotrophic microorganisms to convert crude glycerol into added-value products, such as microbial lipids (also called single cell oils, SCOs) citric acid, microbial mass, enzymes and polyols (Papanikolaou & Aggelis, 2009, Rivaldi et al., 2009, Abghari & Chen, 2014, Kamzolova et al., 2011, 2013). Moreover, oleaginous microorganisms are efficient lipid producers in the presence of a waste (zero energy) (Abreu et al., 2010, Liang et al., 2010) Among natively oleaginous microorganisms, *Yarrowia lipolytica*, is one of the most extensively studied "non-conventional" yeasts due to its biotechnological potential and the availability of genetic tools aiming for the production and the storage of large amounts of lipid. Accordingly, wild *Yarrowia lipolytica* has been reported to accumulate up to 36% of dry weight from glucose and more than 50% in the presence of hydrophobic substrates (Bati et al.,1984, Papanikolaou et al., 2007). In contrast, metabolically engineered strains can achieve more than 90% of dry weight (Blazeck et al., 2014). In addition to SCO production, *Yarrowia* species are reported to secrete various secondary metabolites, such as citric acid (CA) (Kamzolova et al., 2011, 2013, Bellou et al., 2016; Papanikolaou et al., 2008), extracellular enzymes (Barth & Gaillardin, 2014, Gonçalves et al., 2014) and other functional fatty acids of commercial interest such as lipid-derived neutraceuticals and pharmaceuticals using genetically engineered strains (Abghari & Chen, 2014). Several applied studies have focused on increasing SCO production through increasing the overflow of carbon sources. Among common substrates, glucose was widely investigated (Lin et al., 2011) however, this latter competes

directly with food and feed production, which is not the case for other sources (Nakamura & Whited, 2003). Accordingly, glycerol is known to have a greater degree of reduction than other carbohydrates and is less costly and more readily available. Due to carbon rich composition (Polburee et al., 2015, Sarma et al., 2012) .In yeast, the glycolytic pathway produces intermediate compounds from glycerol either via the phosphorylation pathway (Lazar et al., 2014, Joshi et al., 2008) or the oxidative pathway (dehydrogenation of glycerol and the subsequent phosphorylation of the reaction product) (Makkar et al., 1997) and almost exclusive synthesis of reduced products during glycerol fermentation reflects the highly reducible state of glycerol. Additionally, glycerol may be readily incorporated in the core of triglycerides, which are stored in lipid bodies along with steryl esters (Beopoulos et al., 2008). Besides, others studies focused on refining the production process by identifying optimal culture conditions and defining optimal medium composition (Meesters et al.,1996, Tchakouteu et al., 2015, Makri et al., 2010). In this regard, physiological conditions, such as pH, temperature and oxygen concentrations, have also been shown to influence the lipid composition (Rattray et al.,1975, Moreten,1988). Taken together, the aim of the current study was to investigate the potential of biodiesel-derived waste glycerol conversion into metabolic compounds of added-value (SCOs) by yeast strain. After an initial selection, the yeast strains were cultivated on biodiesel-derived waste glycerol utilized as a carbon source under nitrogen-limited conditions (conditions that favour the accumulation of storage lipid by microorganisms). The effect of glycerol and NH_4OH concentration and fermentation time and identification of the most appropriate production conditions, and characterization of the produced lipids was carried out.

3.4 Materials and methods

3.4.1 Strain and culture conditions

Y. lipolytica SM7, isolated from woody forest (Alma, Canada) in a glycerol enriched medium (GEM) composed of 1 g of woody forest soil and 100 g pure glycerol/L, 0.3 g yeast extract/L, 1 g KH_2PO_4/L, 0.5 g $MgSO_4.7H_2O$/L). Enrichment was performed at 28ºC at 180 rpm in 48 h. After that, a serial of decimal dilutions was performed to select strains having the capacity to grow on high rich carbon media. The quantitative selection was based on Nile Red staining. Strains having maximum of lipids droplets were of wide interest in the current study. The newly-isolated strain was identified by means of genetic tools. The genomic identification was based on ribosomal 5.8s sequencing. PCR amplification yielded a 332-bp sequence and rDNA sequence data was subjected to a BLAST search tool of NCBI. Homology results showed that *Y. lipolytica* SM7 has around 99% sequence similarity with *Yarrowia lipolytica*. In this regard, *Y. lipolytica* SM7 was selected and its capacity to produce lipids in crude glycerol based media was optimised in the present study. The strain was grown on YEPD agar (yeast extract peptone dextrose agar) at 28ºC for 2 days, maintained at 4ºC and sub-cultured every three months.

The pre-culture was obtained by inoculating a separate colony of *Y. lipolytica* SM7 in yeast extract peptone dextrose (YPD) medium containing (g/L): Glucose 20, peptone 20 and yeast extract 10 and incubating it at 28°C for 24 h prior to cultivation. Lipid production was performed in duplicates, aerobically, in 2-L Erlenmeyer flasks containing 500 mL of the designed media (crude glycerol, 1 g yeast extract/L, 3 g K_2HPO_4/L, 3 g $NaH_2PO_4.H_2O$/L, 0.5 g $MgSO_4.7H_2O$/L, 0.040 g $ZnSO_4.7H_2O$/L, 0.016 g $FeSO_4.7H_2O$/L, 0.25 µg/L biotin) and inoculated with the pre-culture (initial OD 600 = 0.01), 5% (v/v) and incubated at 28°C in a rotary shaker incubator, under agitation of 180 rpm. Ammonium hydroxide (NH_4OH, 29%, v/v) was used as nitrogenous source and pH was re-adjusted in all solutions by using NaOH and H_2SO_4 4 N. Crude glycerol was provided by Rothsay (Ontario, Canada), this latter was used as carbon source resulted from the transesterification of animal fats, its characterization was presented in Table 3.1. Its high composition of glycerol and low quantities of impurities such soap and salts makes this waste a very potential carbon source for lipid accumulation.

3.4.2 Glycerol and metabolites analysis

For the measurement of glycerol and others organics acids in the broth, LC/MS/MS technique was employed. The technical details of the LC/ MS/MS instrument used for the analysis were: (a)

for sugar estimation: Thermo TSQ Quantum model, equipped with an Electrospray Ionization (ESI) in negative ion mode; Zorbax Carbohydrate (4.6 mm, 150 mm, 5 mm, Agilent) analytical column; 75% acetonitrile; 0.1% NH_4OH; 25% water and 0.1% NH_4OH mobile phase and 10 mL injection volume. Glycerol, citric acid, malic acid, (all from Sigma) was used as the internal standards; and (b) for phenolic compound estimation: Thermo TSQ Quantum model, equipped with an Electrospray Ionization (ESI) in negative ion mode, Thermo Scientific Beta Basic C18 LC column (100 mm, 2.1 mm, 3 mm); mobile phase of methanol and acidified water (0.1% acetic acid) at a ratio of 17.5: 82.5; Flow rate of 0.3 mL/min and 20 mL injection volume.

3.4.3 Biomass determination and lipid extraction

Samples were collected by centrifugation at 5 000 *x g* for 15 min. The resulting pellet was washed once, frozen and lyophilized to a constant mass. The extraction of total cellular lipids was performed according to Folch method. (Folch et al., 1997). Five hundred milligrams of lyophilized cells were suspended in methanol/chloroform (2:1, v/v). After the first extraction, the remaining cell lipids were further extracted twice with methanol/chloroform (1:1, v/v); and then with methanol/chloroform (1:2, v/v). Resulted organic phases were mixed and washed twice with 0.88% (w/v) KCl solution for 10 min and centrifuged for 5 min at 10 000 *x g*. Solvent phase was withdrawn and transferred into a pre-weighed glass vial (W1). Lipids were recovered as dry material after the evaporation of the solvent at 60 ± 1°C, until a constant weight was obtained (W2). The lipid quantity was calculated by the difference between two vials (W2 and W1). The lipid content in the dry biomass was reported to be the difference between two vials extracted/500 mg × 100%. Finally, the obtained lipid was stored in dark at 4°C for further transesterification study.

3.4.4 Lipid analysis and fatty acid composition

Fatty acid profile of the lipid was determined by methylation for conversion of fatty acids to fatty acid methyl esters (FAMES). The lipids (0.01 – 0.1 g) obtained were first dissolved in hexane (50 mL hexane/g lipid), then mixed with methanol. Lipid to methanol molar ratio was 1:6 (0.3 mL methanol per gram lipid). Sodium hydroxide was used as catalyst with concentration of 1% w/w (NaOH/oil). The mixture was then heated at 55 ± 1°C for 2 h. After reaction, 5% NaCl solution was added to 100 mL per gram lipid, and then FAMEs was extracted by two times washing with hexane (100 mL per gram lipid). After washing, the mixture was allowed to stand for phase

separation, and later hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% (w/v) sodium bicarbonate solution (20 mL per gram lipid), and the top layer was then dried at 60 ± 1^{o}C in an oven (Halim et al., 2011). The FAMEs in hexane were analyzed using Gas Chromatography- Mass Spectroscopy (GC–MS) (Perkin Elmer, Clarus 500). The dimensions of the column used were 30 m × 0.25 mm, with a phase thickness of 0.25 µm. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1.3-Dichlorobenzene was also used as an internal standard. All experiments were performed in triplicates, and average results were reported with standard deviation less than 5%.

3.4.5 Optimization study

Box–Behnken model was used for experimental design to optimize key process parameters for enhanced lipid production. Box–Behnken design offers advantages in requiring fewer experimental runs and is rotatable if the variance of the predicted response at any point x depends only on the distance of x from the design center point. The 3K factorial design also allows efficient estimation of second degree quadratic polynomials and obtains the combination of values that optimizes the response within the region of the three dimensional observation space (Annadurai et al., 1999). In developing the regression equation, the relation between the coded values and actual values can be described by the following equation: where xi is the coded value of the independent variable, Xi is the uncoded value of the its independent variable, X is the uncoded value of the independent variable at the center point, and DX_i is the step change value. The levels of the variables and the experimental design are shown in (Table 3.1.1). Lipid concentration was associated with simultaneous changes in glycerol concentration (75, 87.5 and 100 g/L), ammonium hydroxide concentration (0.5, 1.0 and 1.5 g/L) and incubation time (36, 54 and 72 h). A total of seventeen experimental runs decided by the 3K factorial Box–Behnken design were carried out, and the center point was replicated three times to estimate experimental errors. For predicting the optimal conditions, the quadratic polynomial equation was fitted to correlate the relationship between variables and response (i.e. lipid concentration), and estimated with the following equation (3.1):

Équation 3.1 $$Y=\beta_0 \pm \sum \beta_i X_i \pm \beta_{ij} X_i X_i \pm \sum \beta_{ii} X_i^2$$

Where; Y is the predicted response; β0 the intercept, β i is the linear coefficient, β_{ij} the quadratic coefficient, βii is the linear-by-linear interaction between Xi and Xj regression coefficients and Xi, Xj are input variables that influence the response variable Y. The levels of the variables and the experimental design are shown in Table 3.2.

The goodness of fit of the regression model was evaluated using the coefficient of determination (R^2) and the analysis of variance (ANOVA). For tested variable, the quadratic model was represented as contour plots (3D) and response surface curves were generated using Design-Expert Software.

To evaluate the RSM optimized culture parameters, fermentation was conducted in 5-L fermenter (Biostat B plus, Sartorius Stedim Biotech, Allemagne) to assess lipid production in crude glycerol based media. Polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4 and 7 (VWR, Canada). Before sterilization cycle, the oxygen probe was calibrated to zero (using sodium thiosulfate water) and 100% (air saturated water). Propylene glycol (Sigma-Canada) was used as anti-foam agent. The fermenter with the medium was then sterilized in situ at 121ºC for 20 min. After the fermenter cooled down to 28ºC, DO probe was recalibrated to zero and 100% saturation by sparging N_2 gas and air, respectively, at agitation rate of 250 rpm. The pH of the fermenter solution was adjusted to 6.5 with 4 N H_2SO_4. Thereafter, sterilized crude glycerol (83% w/v) and mineral solution was transferred to the fermenter as carbon source under aseptic condition. Agitation was provided to mix the solution, after mixing, pre-culture of *Y. lipolytica* was added to the fermenter.

3.4.6 Morphological study

Cells were analyzed by scanning electron microscopy (SEM, Carl Zeiss EVO® 50) to have a highly magnified view of the surface morphology and the behavior of cells during lipogenesis. To prepare samples for SEM, cells were dried using lyophilizer (VirTis Virtual 50-L pilot lyophilizer). Dried samples were directly mounted on a SEM grid and sputter coated (SPI Module Sputter Coater) with gold before SEM analysis.

3.5 Results and discussion

3.5.1 Evaluation of growth parameters

Despite the higher concentrations reported in the presence of hydrophobic substrates (Bati et al., 1984, Papanikolaou et al., 2007), scarce information was available for hydrophilic carbon sources. The recent data is related to Polburee (2015, 2016) who described the growth of *Rhodosporidium toruloides* on biodiesel-derived crude glycerol with the aim to obtain high lipid content up to 63.8% of dry biomass with a lipid concentration of 8.99 g/L and a lipid yield of 0.16 g/g (Polburee et al., 2015, 2016).

For *Y. lipolytica*, most of the relevant literature emphasized the importance of fatty materials as low cost substrates to produce SCO and other "tailor-made" lipids, such as cocoa-butter substitutes (CBS), illipé substitutes, shea butter, sal fat (Lipp & Anklam,1998, Papanikolaou & Aggelis, 2010, Papanikolaou et al., 2001). Studies have revealed that *Y. lipolytica* is primordially a citric acid producer (Kamzolova et al., 2011, 2013, Ratledge, 1994). Moreover, Cescut has reported that the lipid accumulation in this yeast is a metabolic balance between citric acid production and triglyceride (TAG) synthesis (Cescut, 2009) and the shift from growth phase and CA production phase (i.e. lipogogenisis phase) is not well understood and more research should be performed to study their concomitance. Taken together, the present study investigated whether low-cost raw materials, such as crude glycerol and nitrogenous source, such as NH_4OH could enhance the lipid accumulation and CA production. Experiments showed that varying glycerol concentration from 75 to 100 g/L with the variation of NH_4OH yielded highest biomass and lipid production (Table 3.3).

Herein, both of organic and inorganic nitrogen sources were employed; inorganic one favored mostly product formation (lipid in the present case) rather than the biomass, whereas the organic nitrogen favored biomass and product (lipid) accumulation (Bellou et al., 2016, Papanikolaou et al., 2008, Evans & Ratledge 1984, Azad et al., 2014). Due to this reason, both organic (yeast extract) and inorganic nitrogen source (NH_4OH) were used. Moreover, the lipid production was defined to be the product of lipid content and biomass.

Based on the above information presented in (Table 3.3), the optimization of the whole process via RSM method was required to maximize the biomass and the lipid concentration and to lower the CA production. Thus, when ammonium nitrogen was depleted, some quantities of stored lipids and CA were synthesized (Table 3.3). Following lipogenic phase, glycerol was predominantly converted into cellular lipid, while smaller quantity of CA was secreted in the growth environment

(0.5 - 4.0 g/L), especially in the culture of initial glycerol concentration of 75 and 87.5 g/L. A higher concentration of CA was observed in the presence of higher glycerol concentration 100 g/L and reached around 12.0 ± 2.5 g/L).

Surface curves plots between binary reactions are presented in Figure 3.1. The variation of glycerol concentration, ammonium hydroxide concentration and fermentation time have been reported to have higher impact on lipid production and growth kinetics parameters. The variance analysis and the estimation of parameters by the Design-Expert software, is illustrated in Table 3.4. The *p*-value was used to evaluate the significance of the variable. When the *p*-value of the variable was less than 5%, it represented that the variable had significant effects on the response value. To further assess the effect of the variable, coefficient estimate was applied. Lipid production could increase with increasing concentrations of glycerol, if the coefficient estimate were positive. Conversely, the value of coefficient estimate was negative, indicating that lipid production was negatively correlated with the variable levels. As shown in Table 3.4, ammonium hydroxide concentration had significant effect on the lipid production (*p* value < 0.0001). With increasing glycerol concentration and lowering nitrogenous source concentration from 0.5 to 1.5 g/L, the cellular lipid content in *Y. lipolytica* increased evidently where the *p*-value was less than 0.0001. Therefore, lipid production was observed to be more with the lower nitrogen and higher glycerol concentration. So far, various studies have been carried out to demonstrate that the effect of glycerol concentration on lipid accumulation in many oleaginous strains which is determined by concentration of carbon and nitrogen (C/N molar ratio). Thus, oleaginous potential is critically affected by the C/N ratio of the culture and other factors like aeration, inorganic salt presence, etc (Moreten, 1988, Hassan et al.,1996, Braunwald et al., 2013).

Similar results have been presented by Karanth and Sattur (1991), who found that lipid production in batch fermentation was similar for initial sugar concentrations of 60 and 80 g/L (Karanth & Satur, 1991). Regarding the influence of the initial nitrogen content, at high C/N ratios, the lipid production was shifted to the end of cultivation. Normally an opposite pattern could be anticipated, since lower nitrogen levels would suggest an early shift to lipid synthesis. Most authors recommend a C/N close to 100 as ideal for lipid accumulation (Ageitos et al., 2001; Yousuf, 2012; Angerbauer et al., 2008) In the present study, a C/N ratio of 75 is observed to enhance the biomass production and the lipid around, 25 ± 1.2 g/L and 52% (w/w) of dry biomass, respectively, which is reported also to be closer to the C/N ratio 70 for oleaginous and non- oleaginous (Kolouchová et al., 2016). The C/N ratio was calculated based on the carbon present in the glycerol (39% w/w) and the nitrogen present in the yeast extract approximately (12% w/w).

When glycerol concentration was 87.5 g/L with C/N ratio 112.5, lipid content varied between 40.5 to 45.3% (w/w) of dry biomass. Therefore, increasing glycerol concentration and lowering nitrogen amount would increase remarkably the lipid content inside the cells.

Moreover, for *Cryptococcus sp.,* the highest content of lipids was measured at a C/N ratio of 60-90 and a nitrogen concentration of 0.2% with 60-57% lipids of the dry biomass (Chang et al., 2015). Furthermore, fermentation time had a positive effect, inducing higher lipid accumulation in cells. Additionally, fermentation time was also identified as a significant factor for lipid production. It was obvious that increasing the fermentation time could dramatically promote the growth rate of *Y. lipolytica* (*p*-value lower than 0.0001). The lipid production was improved with fermentation time which accounted for 10.3% (coefficient estimation) of the total contribution. This was in agreement with previous reports that confirmed that higher the fermentation time, more the lipid synthesis is enhanced, however, the time should not exceed the recommended value of 66 h as degradation of lipid occurred after 66 h (Papanikolaou et al., 2004). When nutrients are no longer provided by the medium, lipids stored will be mobilized by TAG lipases and hydrolases to serve as carbon source to maintain the growth of *Y. lipolytica*. In general, microorganisms consume their accumulated lipids mainly through the glyoxylate bypass pathway, and, more specifically, different microbes might preferentially consume different kinds of fatty acids to maintain their growth (Papanikolaou & Aggelis, 2010)

In order to check the fit of the model, R^2 and *F*-value were calculated. Here, R^2 was 0.9907, indicating that 99.07% of the data in Box-Behnken design could be explained by the model; that is, the proposed model was reasonable. Moreover, the model *F*-value of 83.32 demonstrated that the model was significant, as revealed by a *p*-value lower than 0.0001, which further supported that the model fitted in to these data. From the analysis of R adj ^{2} and R pred ^{2}, the R pred^2 of 0.861 was in good agreement with the R adj ^{2} of 0.978.

Based on the previous results, Box–Behnken design was used to further confirm the optimum growth factors of glycerol concentration, nitrogen concentration and fermentation time to maximize lipid production. In order to investigate the adequacy of the model, multiple regression analyses on the data were applied. The results are listed in Table 3.4, which were mainly the individual and the binary effects of all variables and their interactions on lipid production. The multiple correlation coefficient R^2 of 0.990 suggested that the quadratic polynomial model was suitable for revealing the mutual relationship of factors and predicting the response values in the study.

According to the attained results and the equation, the model predicted the maximum lipid production by equation (3.2).

Équation 3.2 $$\text{Lipid content} = -81.046 \pm 0.0527 \times \text{Glycerol} + 26.350 \times NH_4OH + 3.6293 \times \text{Time} - 0.1856 \times \text{Glycerol} \times NH_4OH + 0.0008 \times \text{Glycerol} \times \text{Time} + 0.0347 \times NH_4OH \times \text{Time} + 0.0012 \times \text{Glycerol}^2 - 12.061 \times NH_4OH^2 - 0.0286 \times \text{Time}^2$$

The sign of the coefficient of each term indicates the influence of this term on the response, For instance, from equation (3.2) it can be observed that NH_4OH has a positive effect on lipid production (coefficient: + 26.34). Besides, lipid production is very influenced by the fermentation time (+ 3.62), while glycerol concentration has a very low impact (0.05).

3.5.2 Identifying the best culture conditions for higher lipid production

Under the optimum conditions, glycerol concentration was fixed to 89 g/L and ammonium hydroxide to 0.54 g/L during 66 h, the biomass and lipid content were 25.0 ± 1.5 g/L and 52.7 ± 1.2% (w/w of dry biomass), which was increased by 64% and 20% compared to shake flask under no controlled conditions (9.3 ± 1.1 g/L and 43.5 ± 0.8 (% w/w of dry biomass). The observed lipid production was 52.7 ± 1.2 (% w/w of dry biomass), agreeing well with the predicted values 53.1% (w/w of dry biomass), indicating that the model was valid. Table 3.1.4 presented the reported yields of lipid production in many *Yarrowia* species. Herein, the selected strain presented as a potential candidate for lipid production in the presence of crude glycerol in terms of tolerating higher glycerol concentration up to 100 g/L compared to other oleaginous strains where higher concentration is the threshold. For instance, Meesters et al. (1996) observed that, in *Cryptococcus curvatus*, cell growth was restricted during lipid accumulation when glycerol concentrations were higher than 64 g/L and the optimum of glycerol was fixed to be 16 g/L with a maximum specific growth rate of 0.43 h^{-1} (Meesters et al., 1996).

Accordingly, higher glycerol above 60 g/L is responsible to induce higher osmotic pressure which could inhibit the oxygen uptake or create high osmotic pressure sufficient to inhibit culture growth in other strains (Liang et al., 2010, Beopoulos et al., 2008, Meesters et al., 1996).

However, recent study of Papanikolaou et al. (2008) has demonstrated that *Y. lipolytica* ACA-DC 50109 was tolerating higher concentration of glycerol up to 164 g/L with a maximum biomass concentration of 7.4 g/L, with slight inhibition of the microbial growth was observed and the maximum specific growth rate of around 0.16 h^{-1} (Papanikolaou et al., 2008) . More often, Rymowicz et al. (2006) have found that *Y. lipolytica* mutants can be cultivated in the presence of raw glycerol at extremely high concentrations (i.e. 200 g/L) and can achieve efficient cell growth ranging from 16.5–26.5 g/L (Rymowicz et al., 2006) These observations confirmed that glycerol tolerance using oleaginous microorganisms feature is strain dependant, and the concentration of carbon source should be adjusted accordingly to produce higher yields of CA and SCO. Moreover, Karamerou et al. (2016) have proved that higher concentrations of glycerol had neither a positive nor a negative effect on growth of *Rhodotorula glutinis* and the microorganism could sustain higher glycerol concentrations up to 150 g/L, meanwhile, around 60 g/L of crude glycerol was easily assimilated by the cells and was required to obtain around 29.8% (w/w) of dry biomass, however, lower glycerol concentration of 30 g/L favored effective cell growth 5.28 g/L (Karamerou et al., 2016). Thus, higher glycerol concentrations induced the accumulation of lipids by supressing cellular growth.

Taken together, the inhibition affected generally the glycerol conversion rate (Table 3.3), so that higher the initial crude glycerol, lower the conversion, which was also confirmed by Tchaerou et *al.* (2015), who deduced that high initial crude glycerol concentration (180 g/L) led to lower glycerol conversion in *Rhodosporidium toruloides. H*owever, the decrease in growth resulted in oil production (54% w/w of dry biomass compared to 40% (w/w) at 120 g/L).

Moreover, the analysis of nitrogen concentration showed that ammonium units start to deplete after 16 h (Figure 3.2). Initial nitrogen concentration was around 600 mg/L and after 16 h, remaining concentration was constant (70-100 mg/L) during entire fermentation. This limitation of nitrogen in the media will trigger the pathway towards lipid biosynthesis, In fact, yeast required nitrogen which is furnished by ammonium hydroxide during the growth phase, in contrast to lipogenic phase. Nitrogen at 0.014 g/L has been found to be the critical concentration reported by Cescut (2009) to enhance lipid synthesis (Cescut, 2009). In this study, limiting concentrations of nitrogen in around 70 mg/L into the medium lead to the induction of lipid accumulation.

Thus, the reduction of ammonium concentration activated the ATP citrate lyase enzyme, so that nitrogen limitation could activate diacylglycerol acyltransferase, which converted acyl-CoA to triglyceride (TAG) (Kolouchová et al., 2016) and this point was noted to be a separating phase between growth and lipogenic phase. Lipid concentration started at this stage with a concomitant

increase of biomass concentration. Maximum specific growth rate was around 0.15 h^{-1} during the first stage of growth 12 h. Thus, to distinguish between both phases, the calculation of growth parameters was required and the analysis of nitrogen concentration was analyzed. Nitrogen source started to deplete from 16 h, afterwards, the nitrogen concentration was almost constant along the fermentation.

Besides, transition between growth phase and citric acid production is accompanied by morphological changes. In the first stage of growth phase, Nile red lipid staining revealed that lipid bodies are small and make up very little of the intracellular space at 12 h post-inoculation when the cells are presumably still growing exponentially (Figure 3.2D).

In contrast, in the lipid accumulation stage, large lipid droplets are distinguished by 48h of growth, and cells appear elongated and grow as pseudo-filaments and cells are generally swollen and continue to sprout throughout the time course Figure 3.2 (A,B,C). Besides, the apparition of bud scars after nitrogen depletion, on both poles confirm the accumulation stage of lipids droplets (Figure 3.2). Thus, mycelial transition was indicative of lipogenic phase and was more pronounced during the oxygen limitation. The cell size was notably affected by the different percentage of accumulated lipids among lipogenic and CA production phase. In fact, different conditions were reported to induce the dimorphism transition of yeast to mycelium during lipid accumulation phase. In fact, Zinjarde et al. (1998), showed that micro aerobic conditions were among the reasons of dimorphism in *Yarrowia* species. (Zinjarde et al., 1998). Besides, genetic modifications, nature of culture media and presence of specific compounds, such as N-acetylglucosamine, or bovine serum albumin (BSA) are reported to enhance efficiently the transition phenomena (Kim & Cheon, 2000; Pérez-Campo & Dominguez, 2001).

Chávez et al. (2009) has reported that the dimorphic transition event is related to the activation of protein kinase signaling pathway and other signaling transduction mechanisms specific for some oleaginous strains (Cervantes-Chávez et al., 2009). In fact, Zinjarde et al. (1998) suggested that the dimorphism transition is strain specific and depends ultimately on the nature of carbon source and the microenvironment conditions (i.e. lower dissolved oxygen concentration) (Zinjarde et al., 1998).

The practical outcome of the present study is that a saturation rate of dissolved oxygen 30% is suitable to enhance the morphogenesis changes during growth and lipogenic phase and a control of mechanical agitation during lipogenic and CA production should be monitored to avoid mycelial cells disruption and eventual drop in biomass concentration in the bioreactor.

Most of the accumulated lipids between 0-16 h corresponded to catalytic biomass and lipids corresponds to phospholipids and sterols, components of cell wall of yeasts. The glycerol was used for biomass accumulation and the yield of glycerol conversion to biomass was high compared to lipogenic phase ($Y_{X/S}$= 0.47 ± 0.10 and $Y_{P/S}$=0.08 ± 0.02). Around 4.7 ± 0.5 g/L of lipid concentration was observed with a lipid content of 25.0% (w/w) of dry weight at 36 h (Figure 3.4). The analysis of metabolites in the supernatant showed that many organic acids were produced (pyruvic acid, ketoglutaric acid, acetic acid) but in small traces and the concentration does not exceed 2.0 ± 0.1 g/L. Moreover, citric acid, a non-growth-associated metabolite, was secreted in lower concentration (4.0 ± 0.8 g/L) and was constant during time course. A concomitant production of citric acid is related to the nitrogen exhaustion which also is defined to trigger citric acid as well as SCO (Anastassiadis et al., 2002; Ratledge & Wynn, 2002).

The simultaneous production of SCO and CA permits to classify our isolate as typical "oleaginous" feature, comparable to other *Yarrowia* species reported by Tsisgie et al. (2011) and Fontanille et al. (2012) respectively, where lipid accumulation takes place while glycerol was available in the media and can be used as carbon source. Besides, lower concentration of citrate was reported and this can be explained as a consequence of intracellular nitrogen limitation in yeast overflow metabolism. It does not start until nitrogen in the medium is exhausted, the growth has mainly ceased and intracellular nitrogen decreased. It is possible that nitrogen limitation somehow interrupts the TCA cycle by decreasing the activity of some enzymes, leading to citrate secretion (Anastassiadis et al., 2002).
There are also data on the importance of nitrogen limitation in *Candida oleophila* ATCC 20177 growth for CA production; whereby the optimum [NH_4^+] concentration was found to be 1.2 mg/g (Anastassiadis et al., 2002).

Although *Y. lipolytica* is known to produce CA and the concentration reached around 154 g/L (Rywińska & Rymowicz, 2002) still in this study, the concentration remained stable which was favoured possibly by maintenance of pH during fermentation pH = 6.5. These results are in accordance with Kamzolova et al. (2011), who reported that a pH around 4.5–6.0 was required to enhance CA production (6.10–6.17 g/L) in the presence of crude glycerol (Kamzolova et al., 2011). Accordingly, CA production has a direct relation to pH changes, however, Crolla and Kennedy (2004) suggested that pH showed no direct effect on the mechanism of citric acid synthesis, but influenced the permeability of cell membranes to both substrate and products.

Taken together, CA production in SM7 is not surprising since lipid synthesis and intensive CA production are two competitive processes for acetyl-CoA (i.e. precursor of TAG accumulation)

and both phenomena are triggered by nitrogen depletion. Moreover, the lower CA concentrations can be related to the fact that SM7 may selectively consume the CA produced during lipogenic phase as carbon source to enhance TAG accumulation (Ratledge & Wynn, 2002).

Herein, the majority of the glycerol was converted into SCO in 60 h and the yield of lipid productivity was around 0.20 g/L/h. Thus, the difference in physiological behaviour during lipogenic phase and CA is strain dependent and *Yarrowia* species did not exhibit the same behaviour. In this regard, Dobrowolski et al. (2016) observed that during lipogenic phase in *Y. lipolytica* A101, carbon metabolism is shifted towards lipid accumulation until a threshold is achieved, after which excess carbon is excreted as citric acid in which lipid is stored. However, afterwards, lipid started to degrade and CA production occurred (Dobrowolski et al., 2016). In contrast, Makri et al. (2010) have reported that some of the *Yarrowia* species are termed as atypical "oleaginous" feature, in which, lipid is stored after nitrogen exhaustion, that afterwards is being degraded while simultaneously significant quantities of sugar or glycerol remain unconsumed in the medium and in parallel, citric acid production occurred (Makri et al., 2010).

Herein, *Y. lipolytica* SM7 is belonging to typical "oleaginous" feature, in which nitrogen exhaustion triggered the lipid synthesis and storage while lower quantities of citric acid (4 g/L) and other low-molecular weight metabolites are produced (2 g/L). Hence, *Y. lipolytica* SM7 is very closer to *Yarrowia* species reported by Fontanille et al. (2012) where SCO occurs after nitrogen exhaustion and CA is secreted into the medium (Cervantes-Chávez et al., 2009) without cellular lipid degradation occurring (André et al., 2009).

To further elaborate on the physiological behaviour of SM7 in the presence of crude glycerol, extended fermentation time has been proposed to confirm the choice of operational parameters tested along fermentation time (36-72 h) and to confirm the oleaginous feature of selected strain. Extended time of the process up to 100 h led to a decrease in biomass, lipid quantity and lipid content (24.0 ± 2.1 g/L, 7.3 ± 1.3 g/L and 44.1 ± 0.9% (w/w of dry biomass) respectively and CA production increased gradually after increasing fermentation time and reached around 14.7 ± 2.3 g/L of CA in 100 h. These results agree with the observation of Makri et al. (2010) who noted that CA increased progressively when CA production phase coincided with the lipid turnover phase (Makri et al., 2010).

Besides, Bellou et al. (2016) observed that not only nitrogen depletion was required for CA production, but *Y. lipolytica* needs double limited media (in both nitrogen and magnesium) in the presence of crude glycerol to achieve both lipid and CA in significant quantities, *Y. lipolytica* was cultivated in continuous cultures ($D = 0.028\ h^{-1}$) in media containing glycerol around

86.9 ± 8.5 g/L as carbon source and double limited in both magnesium and nitrogen, lipid accumulation was equal to 24.7 ± 1.3% (w/w of dry weight) (Bellou et al., 2016). In the present study, magnesium was not limited, however it was provided at lower concentration sufficient to induce the lipid accumulation, moreover, during sterilization of medium, minerals can precipitate and become thereafter unavailable for yeast cells (Papanikolaou and Aggelis, 2002). All of these observations strengthened the stimulatory effect of limited nitrogen and magnesium to induce a lipid content of 52% (w/w) of dry weight.

Additionally, Bellou et al. (2016) have noted that higher CA amount of 9.9 ± 0.5 g/L was favored in higher glycerol concentration, however, the amount was reduced to 6.6 ± 0.3 g/L, in media containing glycerol at lower concentrations (i.e. 53.1 ± 2.4 g/L) and was totally absent in the presence of glucose even at higher concentration, 101 g/L and double limited media (Bellou et al., 2016). Similar findings were reported by Rywinska et al. (2010) concluded that CA synthesis was highly favored in the presence of glycerol instead of glucose, which confirmed the potential of crude glycerol to enhance concomitant and concurrent production of lipid and CA.

These observations were in agreement with current study since CA was decreased from 12.5 ± 2.5 to 3.8 ± 0.9 g/L while decreasing glycerol concentration from 100 to 75 g/L (Table 3.3).

This behaviour was found to be a unique feature of *Y. lipolytica* compared to other oleaginous microorganisms reported in the literature. Conventionally, the oleaginous organisms accumulate reserve lipid under nitrogen depletion and degrade it under carbon starvation conditions (Papanikolaou et al., 2007, 2004; Ratledge & Wynn, 2002). During transition from lipogenic to CA production phase, significant quantities of the stored lipid were degraded and converted into CA.

During fermentation, air flow rate was kept constant at 2.5 L/min. Agitation rate was varied during fermentation in order to keep the DO above 30% saturation.

During first growth phase from 0-18 h, higher agitation from 250 to 500 rpm was kept to maintain a high dissolved oxygen of 60% and aeration rate of 3.5 L/min. When DO reached 60% of saturation, the mixing was reduced to 400-350 rpm and then the aeration was reduced to 2.5 L/min in order to maintain the DO at about 30% of saturation. The values of oxygen utilization rate (OUR), oxygen transfer rate (OTR) and oxygen transfer coefficient (K_La) is presented in Figure 3.4. Experiments showed that OUR increased slightly between 24 to 60 h. This increase was accompanied with an increase of K_La value between 60-84 h^{-1}. This value was maintained approximatively in the range due to the variation of agitation rate. A saturation level of 30% of dissolved oxygen was based on previous reported works. For example, Zhao et *al.* (2010)

maintained the dissolved oxygen at 40% of air saturation and achieved around 56.5% (w/w) of lipid production from *Rhodosporidium toruloides* Y4 in the presence of Jerusalem artichoke as carbon substrates (Zhao et al., 2010). Besides, Polburee et al. (2016) have fixed a K_La value of 129 h^{-1} to obtain around 63.8% (w/w of dry biomass) of lipid content with a lipid concentration of 8.99 g/L during the cultivation of *Rhodosporidium fluviale* DMKU-RK253 in crude glycerol. Moreover, an optimum of 88.5 h^{-1} was required to maintain high lipid production of *Schizochytrium* sp. (Qu et al., 2011). In summary, the two-stage cultivation with a dissolved oxygen shift, developed in this study could enhance lipid synthesis. In the first stage, when nitrogen present in the cultured medium and K_La around 48-52 h^{-1}, there was high biomass yield up to 0.47 g/g glycerol with only low lipid yield of 0.08 g/g. Then, the high lipid yield was observed when the dissolved oxygen decreased from 60% to 30% in the second stage (i.e. lipogenic phase). The highest lipid yield of 0.16 g/g glycerol was observed during 66 h. Thus, *Yarrowia* responds to nutrient limitation in the manner typical of oleaginous yeasts, which accumulate intracellular lipids during a stationary phase. This strategy also supported high levels of biomass and lipid concentration when compared with the cultivation of *Yarrowia* species in crude glycerol media Table 2.1.

3.5.3 Lipid analysis and fatty acid composition

Analysis of the fatty acid composition of SCOs produced by *Y. lipolytica* varied as a function of fermentation time aligining with studies of Papanikolaou et al. (2013), who confirmed that fatty acids changed as a function of the glycerol concentration employed and the culture time. In the present study, at crude glycerol concentration of 89 g/L, oleic acid ($^{\Delta 9}$C18:1) was detected at higher concentrations ranging from 39.2% to 43.5% during growth and lipogenic phase, respectively. Similarly, Papanikolaou et al. (2013) found that oleic acid ($^{\Delta 9}$C18:1) was around 47.1 and 59.7% for wild-type *Yarrowia lipolytica* (W29) and genetically engineered strain (JMY1203) respectively, in the presence of 90 g/L of glycerol concentration, during the late exponential phase and the early stationary phase (60–90 h) (Papanikolaoua et al., 2013) corresponding to the lipogenic phase (36-66 h) in the current study. Furthermore, the predominance of ($^{\Delta 9}$C18:1) was in accordance with data reported by André et al. (2009) and Makri et al. (2010), in the presence of crude glycerol. Oleic acid was produced not only in the presence of crude glycerol as carbon source, but also in the presence of hydrophobic substrates, for instance, when *Y. lipolytica* was grown on rapeseed oil, oleic ($^{\Delta 9}$C18:1) and linoleic ($^{\Delta 9,12}$ C18:2) acids, were detected at higher concentration of 61.9 and 29.2% of the total fatty acids, respectively (Kamzolova et al., 2013).

Herein, the analysis of fatty acid profile between different phases is presented in Table 3.1.6 which revealed significant changes along time course. Myristic (C14:0) 8.0%, palmitic (C16:0) 13.2%, stearic (C18:0) 9.68%, oleic ($^{\Delta 9}$ C18:1) 39.2%, linoleic ($^{\Delta 9,12}$ C18:2) 27.0% were the major fatty acids detected at an early growth stage before nitrogen depletion. Moreover, the fatty acid profile of the cells did not change significantly upon entry into the nitrogen limitation phase (between 6 and 16 h). For example, a significant increase of oleic ($^{\Delta 9}$C18:1) content from 39.0% to 43.5%, C16:0 content from 13.2 to 14.4%. Moreover, a smaller decrease of linoleic ($^{\Delta 9,12}$ C18:2) from 27.0 to 17.5% is observed with a small variation of strearic acid content (C18:0). These observations confirmed that the composition is phase- dependent and a fatty acid selectivity towards more unsaturated fatty acids is noted. The mainly produced fatty acids were C16 and C18 long-chain fatty acids, as do other oleaginous yeasts (Makri et al., 2010; Rakicka et al., 2015). Another observation to be concluded from this observation is the high fatty acid desaturase activity during yeast cultivation which is reflected by higher ratio of C18:1/C18:0 which is > 1. The higher ratio, higher activity of D9-desaturase is observed, especially in the lipid production phase which was also confirmed by Kamzolova et al. (2011). Although *Yarrowia* showed good yields of unsaturated fatty acids, it exhibited very low content of the myristic acid (C14:0) and other fatty acids, such as arachidic acid (C20:0), cis-11eicosanoic acid (C20:1) lignoceric acid (C24:0). Nevertheless, these produced fatty acids can constitute perfect precursors for the synthesis of 2nd generation biodiesel (Li et al., 2007; Zhao et al., 2008; Xu et al., 2012).

3.6 Conclusion

Y. lipolytica is a good candidate for glycerol consumption and lipid production. Single cell oil production is comparable to some of the highest in the literature for microorganisms growing on glycerol. Despite large reports of this conventional yeast, this is the first report to deal with the conversion of this residue to SCO with in-depth analysis of metabolites and growth parameters at fermenter scale. Furthermore, when a two-stage cultivation strategy using dissolved oxygen shift cultivation was developed, the highest biomass, lipid quantity and lipid content of 25.8 ± 1.5 g/L, 13.6 ± 0.8 g/L, and 52.7 ± 1.2% (w/w of dry biomass), respectively, were obtained. This two-stage cultivation strategy shows potential for application in industrial processes to achieve high lipid concentration, and the fatty acid composition obtained by this strain show it is favorable for use as the feedstock for biodiesel manufacture. Finally, the actual optimal values of ammonium hydroxide amounts and concentration of crude glycerol and fermentation time should be further studied in response to other operational factors.

3.7 Acknowledgments

Authors would like to acknowledge the Natural Sciences and Engineering Research Council of Canada (grant A4984, Strategic grant 412994 – 11, Canada Research Chair) for financial support.

3.8 References

Abghari, A., Chen, S. 2014. Yarrowia lipolytica as an oleaginous cell factory platform for production of fatty acid-based biofuel and bioproducts. *Front Energy Res*, **2**.

Abreu, A.P., Fernandes, B., Vicente, A.A., Teixeira, J., Dragone, G. 2012. Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source. *Bioresour Technol*, **118**, 61-66.

Ageitos, J.M., Vallejo, J.A., Veiga-Crespo, P., Villa, T.G. 2001. Oily yeasts as oleaginous cell factories. *Appl Microbiol Biotechnol*, **90**, 1219-1227

Anastassiadis, S., Aivasidis, A., Wandrey, C. 2002. Citric acid production by Candida strains under intracellular nitrogen limitation. Appl. Microbiol. Biotechnol, **60**, 81-87.

Antolin G., Tinaut F.V., Briceno Y., Castano, V., Perez, C. Optimisation of Biodiesel Production by Sunflower Oil Transesterification. Bioresour Technol, **83**, 111-114

André, A., Chatzifragkou, A., Diamantopoulou, P., Sarris, D., Philippoussis, A., Galiotou-Panayotou, M., Komaitis, M., Papanikolaou, S. 2009. Biotechnological conversions of bio-diesel-derived crude glycerol by *Yarrowia lipolytica* strains. *Eng Life Sci*, **9**, 468-478.

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

Annadurai, G., Balan, S.M., Murugesan, T. 1999. Box-Behnken design inthe development of optimized complex medium for phenol degrada-tion using Pseudomonas putida (NICM 2174). *Bioprocess Eng*, **21**, 415-421.

Barth G, Gaillardin C. 1996. *Yarrowia lipolytica*. In: Wolf K (ed) Nonconventional yeasts in biotechnology. Springer-Verlag, Berlin, Heidelberg, New York, pp313-388.

Bati N, Hammond EG, Glatz BA. 1984. Biomodification of fats and oils: trials with *Candida lipolytica. J Am Oil Chem Soc*, **61**, 1743-6.

Bellou S., Triantaphyllidou I.-E., Mizerakis P., Aggelis G. 2016. High lipid accumulation in *Yarrowia lipolytica* cultivated under double limitation of nitrogen and magnesium. *J Biotechnol*, **234**, 116-126.

Beopoulos A, Mrozova Z, Thevenieau F, Dall MT, Hapala I, Papanikolaou S. 2008. Control of lipid accumulation in the yeast *Yarrowia lipolytica*. *Appl Environ Microbiol*, **74**, 7779-7789.

Blazeck, J., Hill, A., Liu, L., Knight, R., Miller, J., Pan, A., al., e. 2014. Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production. *Nat Commun*, **4**.

Braunwald, T., Schwemmlein, L., G.-Hönninger, S., French, W.T., Hernandez, R., Holmes, W.E., Claupein, W. 2013. Effect of different C/N ratios on carotenoid and lipid production by Rhodotorula glutinis. *Appl Microbiol Biotechnol,* **97**, 6581-6588.

Cescut, J., 2009. Accumulation d'acylglycérols par des espèces levuriennes à usage carburant aéronautique: physiologie et performances de procédés. Toulouse: Université de Toulouse; 2009. p. 283.

Celińska, E., Grajek, W. 2013. A novel multigene expression construct for modification of glycerol metabolism in *Yarrowia lipolytica*. *Microb Cell Factor*, **12**, 102.

Cervantes-Chávez, J.A., Kronberg, F., Passeron, S., Ruiz-Herrera, J. 2009. Regulatory role of the PKA pathway in dimorphism and mating in *Yarrowia lipolytica*. *Fungal Genet Biol, **46**, 390-399.*

Chang, Y.-H., K.-S.Chang, Lee, C.-F., Hsu, C.-L., C.-W.Huang, Jang, H.-D. 2015. Microbial lipid production by oleaginous yeast Cryptococcus sp. in the batch cultures using corncob hydrolysate as carbon source. *Biomass Bioenergy*, **72**, 95-103.

Crolla, A., Kennedy, K.J. 2004. Fed-batch production of citric acid by Candida lipolytica grown on n-paraffins. *J Bacteriol*, **110**, 73-84.

Demirbas, A. 2009. Biodiesel from waste cooking oil via base-catalytic and supercritical methanol transestérification, *Energ Conver Manage*, **50**, 923-927

Dobrowolski, A., Mituła, P., Rymowicz, W., Mironczuk, A.M. 2016. Efficient conversion of crude glycerol from various industrial wastes into single cell oil by yeast Yarrowia lipolytica. *Bioresour Technol*, **207**, 237-243.

Evans, C.T., Ratledge, C. 1984. Influence of Nitrogen Metabolism on Lipid Accumulation by Rhodosporidium toruloides CBS 14. *J Gen Microbiol*, **130**, 1705-1710.

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

Fontanille, P., Kumar, V., Christophe, G., Nouaille, R., Larroche, C. 2012. Bioconversion of volatile fatty acids into lipids by the oleaginous yeast Yarrowia lipolytica. *Bioresour Technol*, **114**, 443-449.

Garlapati, V.K., Shankar, U., Budhiraja, A. 2016. Bioconversion technologies of crude glycerol to value added industrial products. *Biotechnol Rep*, **9**, 9-14.

Gonçalves, F.A.G., Colen, G. Takahashi, J.A. 2014. *Yarrowia lipolytica* and its multiple applications in the biotechnological industry. *Scientific World J*, **2014**, 14.

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

Hassan, M., Blanc, P.J., Granger, L.M., Pareilleux, A., Goma, G. 1996. Influence of nitrogen and iron limitations on lipid production by *Cryptococcus curvatus* grown in batch and fed-batch culture. *Process Biochem*, **31**, 355-361.

Joshi, S, Bharucha, C, Jha, S., Yadav, S., Nerurkar, A., Desa, A.J. 2008. Biosurfactant production using molasses and whey under thermophilic conditions. *Bioresour Technol*, **99**, 195-199.

Kalam, A.A., Yousuf, A., Ferdoush, A., Hasan, M.M., Karim, M.R., Jahan, A. 2014. Production of Microbial Lipids from Rice Straw Hydrolysates by *Lipomyces starkeyi* for Biodiesel Synthesis. *J Microb Biochem Technol*, **S8.**

Karamerou EE, Theodoropoulos C, Webb C. 2016. A biorefinery approach to microbial oil production from glycerol by *Rhodotorula glutinis*. *Biomass Bioenergy*, **89**,113-122.

Karanth, N.G., Satur, A.P. 1991. Mathematical modelling of production of microbial lipids. *Bioprocess Eng*, **6**, 241-248.

Kerr, B.J., Dozier, W.A., Bregendahl, K. 2007. Nutritional value of crude glycerin for nonruminants. Proceedings of the 23rd Annual Carolina Swine Nutrition Conference, Raleigh, NC, 6-18.

Kim, J., Cheon, S.A., Park, S., Song, Y., Kim, J.Y. 2000. Serum-induced hypha formation in the dimorphic yeast Yarrowia lipolytica. *FEMS Microbiology*, **190**, 9-12

Kolouchová, I., Mátková, O., Sigler, K., Masák, J., Rezanka, T. 2016. Production of Palmitoleic and Linoleic Acid in Oleaginous and Nonoleaginous Yeast Biomass. *Int J Anal Chem*, 2016.

Lazar, Z., Dulermo, T., Neuvéglise, C., Crutz-Le Coq A-M., Nicaud J-M. 2014. Hexokinase-a limiting factor in lipid production from fructose in *Yarrowia lipolytica*. *Metab Eng*, **26**, 89-99.

Li, Y.H., Zhao, Z.B., Bai, F.W. 2007. High density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme Microb Technol*, **41**, 312-317.

Liang, Y., Sarkany, N., Cui, Y., Yesuf, J., Trushenski, J., Blackburn, J.W. 2010. Use of sweet sorghum juice for lipid production by *Schizochytrium limacinum* SR21. *Bioresour Technol*, **101**, 3623-3627.

Lin, J., Shen, H., Tan, H., Zhao, X., Wu, S., Hu, C., Zhao, Z.K. 2011. Lipid production by Lipomyces starkeyi cells in glucose solution without auxiliary nutrients. J Biotechnol, **152**, 184-8.

Lipp, M., Anklam, E. 1998. Review of cocoa butter and alternative fats for use in chocolate. Part A. Compositional data. Food Chem, **62**, 73-97.

Louhasakul, Y., Cheirsilp, B. 2013. Industrial waste utilization for low-cost production of raw material oil through microbial fermentation. Appl Biochem Biotechnol, **169**, 110-122.

M. Rakicka, Z. Lazar, T. Dulermo, P. Fickers, J. M. Nicaud. 2015. Lipid production by the oleaginous yeast *Yarrowia lipolytica* using industrial by-products under different culture conditions. Biotechnol Biofuels, **8**.

Magdouli, S., Yan, S., Tyagi, R.D., Surampalli, R.Y. 2014. Heterotrophic microorganisms: a promising source for biodiesel production. *Crit Rev Environ Sci Technol*, **44**, 416-453.

Makkar RS., Swaranjit S., Cameotra SS. 1997. Utilization of molasses for biosurfactant production by two Bacillus strains at thermophilic conditions. *J Am Oil Chem Soc*, **74**, 887-889.

Makri, A., Fakas, S., Aggelis, G. 2010. Metabolic activities of biotechnological interest in Yarrowia lipolytica grown on glycerol in repeated batch cultures. *Bioresour Technol*, **101**, 2351-2358.

Meesters PAEP, Huijberts GNM, Eggink G. 1996a. High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. *Appl Microbiol* Biotechnol, **45**, 575-579.

Meesters PAEP, van de Wal H, Weusthuis R, Eggink G. 1996b. Cultivation of the oleaginous yeast Carptococcus curvatus in a new reactor with improved mixing and mass transfer characteristics (surer®). *Biotechnol Tech*, **10**, 277-282.

Moreten, R.S. 1988. Physiology of lipid accumulation yeast. In: Moreton RS, editor. Single cell oil. London: Longman. 1-32.

Nakamura, C.E., Whited, G.M. 2003. Metabolic engineering for the microbial production of 1,3-propanediol. *Curr Opin Biotechnol,* **14**, 454-459.

Papanikolaou S, Chevalot I, Galiotou-Panayotou M, Komaitis M, Marc I, Aggelis G. 2007. Industrial derivative of tallow: a promising renewable substrate for microbial lipid, single-cell protein and lipase production by *Yarrowia lipolytica. Electron J Biotechn*, **10**, 425-35.

Papanikolaou, S., Aggelis, G. 2009. Biotechnological valorization of biodiesel derived glycerol waste through production of single cell oil and citric acid by *Yarrowia lipolytica*. *Lipid Technol*, **21**, 83-87.

Papanikolaou, S., Aggelis, G. 2010. Yarrowia lipolytica: A model microorganism used for the production of tailor-made lipids. *Eur J Lipid Sci Technol*, **112**, 639-654.

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., Marc, I. 2003. Accumulation of a cocoa-butter-like lipid by Yarrowia lipolytica cultivated on agro-industrial residues. *Curr Microbiol*, **46**, 124-130.

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

Papanikolaou S., Chevalot I., Komaitis M., Aggelis G., Marc I. 2001. Kinetic profile of the cellular lipid composition in an oleaginous *Yarrowia lipolytica* capable of producing a cocoabutter substitute from industrial fats. Antonie Van Leeuwenhoek, **80**, 215-224

Papanikolaou S., Fakas S., Fick M., Chevalot I., Galiotou-Panayotou M., Komaitis M., Marc I., Aggelis G. 2008. Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: production of 1,3-propanediol, citric acid and single cell oil. *Biomass Bioenergy*, **32**, 60-71.

Pérez-Campo, F.M., Dominguez, A. 2001. Factors affecting the morphogenetic switch in *Yarrowia lipolytica*. *Curr Microbiol*, **43**, 429-433.

Polburee, P., Yongmanitchai, W., Honda, K., Ohashi, T., Yoshida, T., Fujiyama, K., Limtong, S. 2016. Lipid production from biodiesel-derived crude glycerol by *Rhodosporidium fluviale* DMKU-RK253 using temperature shift with high cell density. *Biochem Eng J*, 112, 208-218.

Polburee, P., Yongmanitchai, W., Lertwattanasakul, N., Ohashi, T., Fujiyama, K., Limtong, S. 2015. Characterization of oleaginous yeasts accumulating high levels of lipid when cultivated in glycerol and their potential for lipid production from biodiesel-derived crude glycerol. *Fungal Biol*, **119**, 1194-204.

Qu, L., Ji, X.J., Ren, L.J., Nie, Z.K., Feng, Y., Wu, W.J., Ouyang, P.K., Huang, H. 2011. Enhancement of docosahexaenoic acid production by *Schizochytrium sp*. using a two-stage oxygen supply control strategy based on oxygen transfer coefficient. *Lett Appl Microbiol*, **52**, 22-27.

C. Ratledge, Yeasts, moulds, algae and bacteria as sources of lipids. In: Kamel, and Kakuda (Ed.), Technological advances in improved and alternative sources of lipids, Blackie academic and professional, London, 1994.

Ratledge C., Wynn JP. 2002. The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms. *Adv Appl Microbiol*, **51**, 1-51

Rattray, J.B.M., Schibecl, A., Kidby, D.K. 1975. Lipids of yeasts. *Bacteriol Rev*, **39**, 197-231.

Rivaldi, J.D., Sarrouh, B.F., Silva, S.S.d. 2009. Development of biotechnological processes using glycerol from biodiesel production. In Current Research Topics in Applied Microbiology and Microbial Biotechnology ed. Mendez-Vilas, A. Madrid: World Scientific Publishing Co. *Formatex Research Center*, 429-433.

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

Rymowicz, W., Rywin´ ska A., Z´arowska B., Juszczyk P. 2006. Citric acid production from raw glycerol by acetate mutants of Yarrowia lipolytica. *Chem Pap*, **60**, 391-4.

Rywińska, A., Rymowicz, W. 2010 High-yield production of citric acid by Yarrowia lipolytica on glycerol in repeated-batch bioreactors. *J Ind Microbiol Biotechnol*, **37**, 431-5.

Rywinska A., Rymowicz, W., Zarowska B., Skrzypinski A. 2010. Comparison of citric acid production from glycerol and glucose by different strains of *Yarrowia lipolytica*. *World J Microbiol Biotechnol*, **26**, 1217-1224.

Kamzolova S. V., Fatykhova, A. R., Dedyukhina, E. G., Anastassiadis, S. G., Golovchenko N. P., Morgunov, I. G. 2011. Citric acid production by yeast grown on glycerol-containing waste from biodiesel industry. *Food Technol Biotech*, **49**, 65-74.

Kamzolova S.V., Dedyukhina, E. G., Samoilenko, V. A., Lunina, J. N., Puntus, I. F., Allayarov, R. K., Chiglintseva, M. N., Mironov, A. A., Morgunov, I. G. 2013. Isocitric acid production from rapeseed oil by Yarrowia lipolytica yeast. *Appl Microbiol Biotechnol,* **97**, 9133-9144.

Sarma, S.J., Brar, S.K., Sydney, E.B., Bihan, Y.L., Buelna, G., Soccol, C.R. 2012. Microbial hydrogen production by bioconversion of crude glycerol: a review. *Int J Hydrogen Energy*, **37**, 6473-6490.

Takagi, M., Watanabe, K., Yamaberi, K., Yoshida, Y. 2000. Limited feeding of potassium nitrate for intracellular lipid and triglyceride accumulation of *Nannochloris sp*. UTEX LB1999. *Appl Microbiol Biotechnol*, **54**, 112-117.

Tchakouteu S.S., Kalantzi O., Gardeli C., Koutinas A.A., Aggelis G., Papanikolaou S. Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency. *Appl. Microbiol*, **118**, 911-927.

Vicente G., Martinez M., Aracil J., Integrated Biodiesel Production: A Comparison of Different Homogeneous Catalysts Systems. *Bioresour Technol*, **92**,297

Xu, J., Zhao, X., Wang, W., Du, W., Liu, D. 2012. Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast *Rhodosporidium toruloides* and the individual effect of some impurities on lipid production. *Biochem Eng* J, **65**, 30-36.

Y.A.Tsigie, Wanga, C.-Y., C.-T.Truong, Ju, Y.-H. 2011. Lipid production from Yarrowia lipolytica Po1g grown in sugarcane bagasse hydrolysate. *Bioresour Technol*, **102**, 9216-9222.

Yousuf, A. 2012. Biodiesel from lignocellulosic biomass: Prospects and challenges. *Waste Manage,* **32**, 2061-2067.

Zhao, X., Kong, X., Hua, Y., Feng, B., Zhao, Z. 2008. Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast Lipomyces starkeyi. *Eur J Lipid Sci Technol*, **110**, 405-412.

Zhao, X., Wu, S., Hu, C., Wang, Q., Hua, Y., Zhao, Z.K. 2010. Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides* Y4. *J Indus Microbiol,* **37**, 581-5.

Zinjarde, S.S., Pant, A., Deshpande, M.V. 1998. Dimorphic transition in Yarrowia lipolytica isolated from oil-polluted sea water. *Mycol Res*, **102**, 553-558

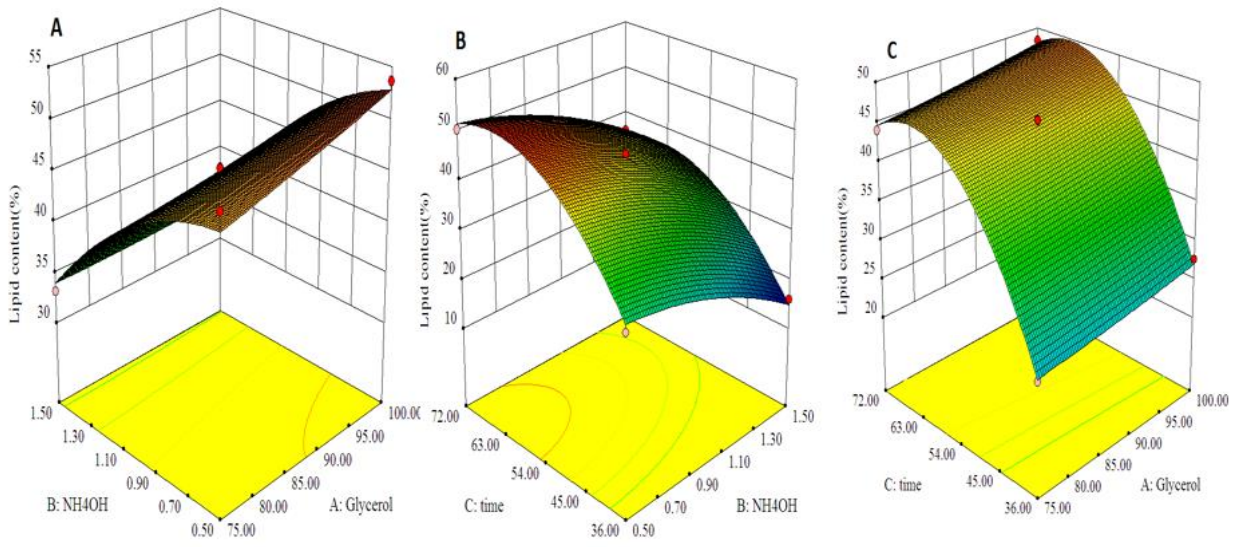


Figure 3.1 **Response surface plots showing binary interaction of different variables. The interaction between (A) NH_4OH concentration and glycerol concentration, (B) glycerol concentration and fermentation time, (C) fermentation time and glycerol concentration**

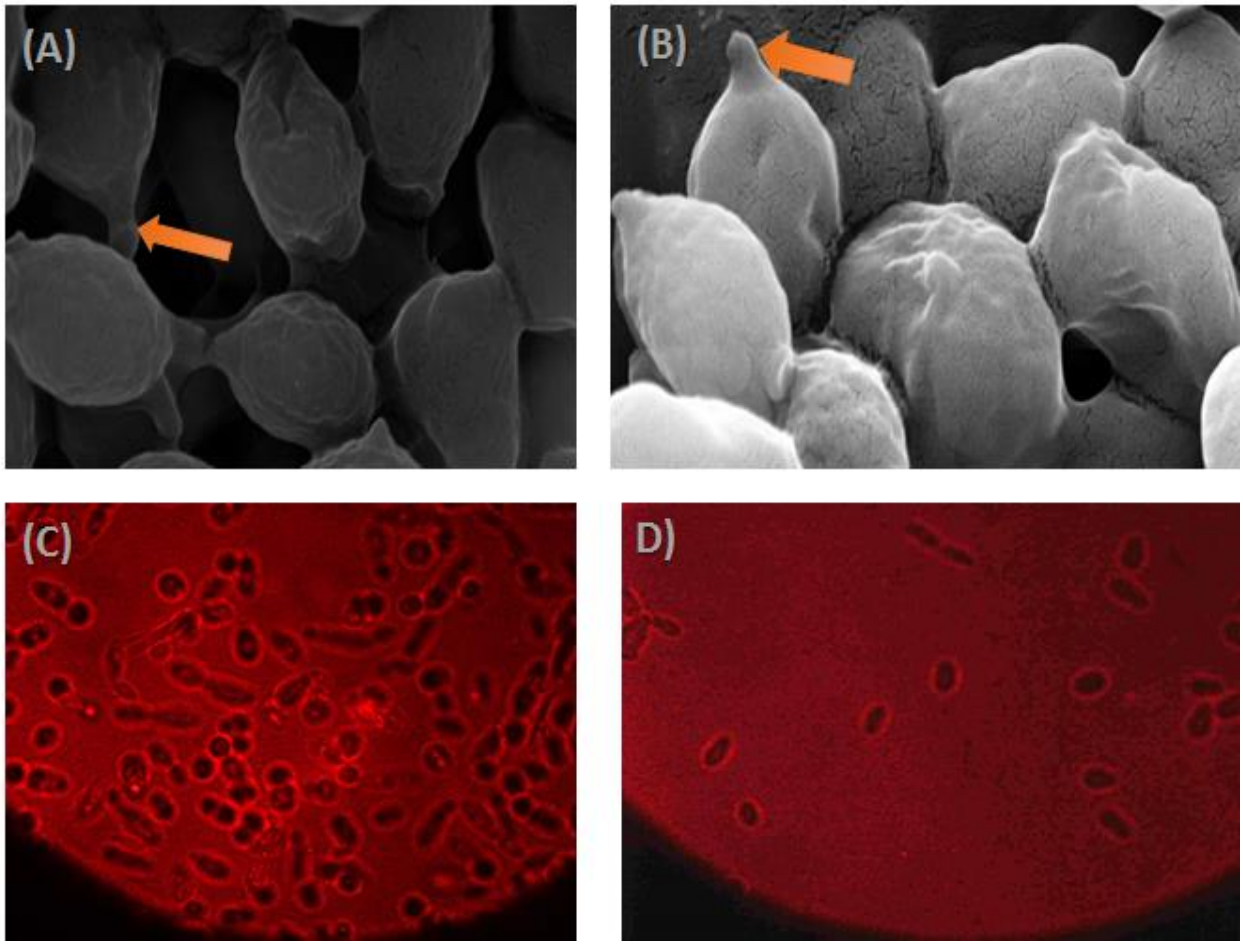


Figure 3.2 **Lipid accumulation of *Y. lipolytica* SM7 over the course of fermentation time. Arrowhead denotes typical bud scarring, (A) and (C) corresponds to the accumulation stage, (B) and (D) corresponds to an early depletion stage**

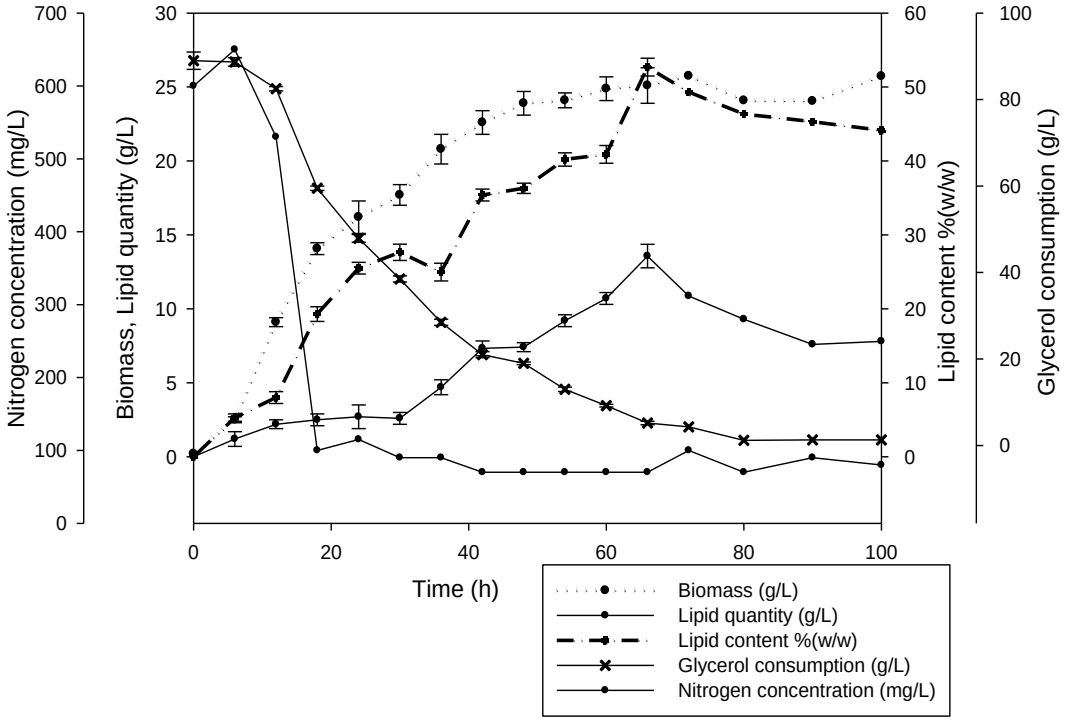


Figure 3.3 **Cell growth and lipid accumulation for *Y. lipolytica* SM7, Culture was performed in the original optimized medium comprising 89 g/L crude glycerol, 0.54 g/L NH_4OH, pH = 6.5 ± 0.3, Temperature = 28 ± 0.5°C**

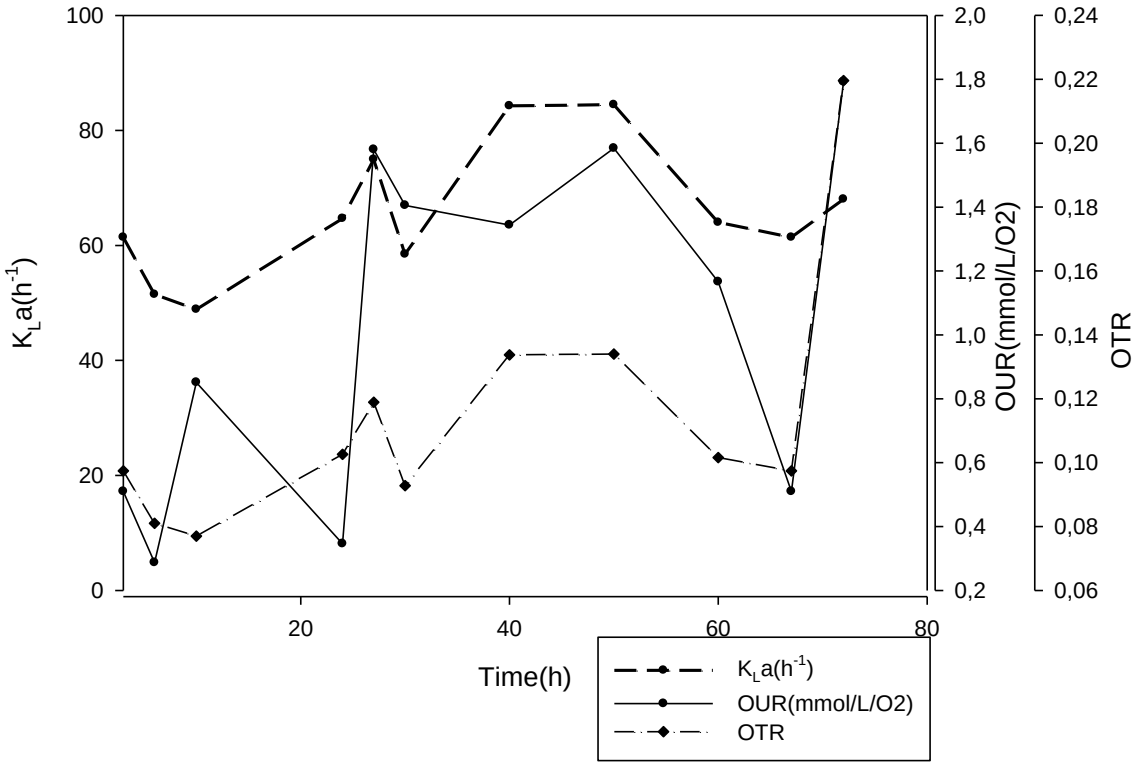


Figure 3.4 **Variation of K_La, OUR and OTR in 5 L fermenter. Culture was performed in the optimized medium on 89 g/L crude glycerol, 0.54 g/L NH_4OH, pH = 6.5 ± 0.3, Temperature = 28 ± 1°C**

Tableau 3.1 Characteristics of crude glycerol waste

Parameters	Method	Value	Unit
Moisture (Karl Fisher)	D 4928	8.83	
pH	Digital pH-meter	3.53	-
Density at 15ºC	Hydrometer	1.264	g/mL
Glycerol concentration	ASTM D7637-10	83.38	%
Methanol	Rotary evaporator	1.5	%

Tableau 3.2 Coded values and levels of experimental factors

Factor	Symbol	Code Levels		
		-1	0	1
Glycerol concentration (g/L)	X1	75	87.5	100
NH_4OH (g/L)	X2	0.5	1.0	1.5
Fermentation time (h)	X3	36	54	72

Tableau 3.3 Statistical analysis of experimental design

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1791.22475	9	199.024972	83.323827	< 0.0001
A-Glycerol	17.2872	1	17.2872	7.23746195	0.0311
B-NH_4OH	504.825313	1	504.825313	211.350247	< 0.0001
C-time	848.926013	1	848.926013	355.411502	< 0.0001
AB	5.3824	1	5.3824	2,25339646	0.1770
AC	0.1156	1	0.1156	0.04839711	0.8322
BC	0.390625	1	0.390625	0.16353913	0.6980
A^2	0.14763184	1	0.14763184	0.06180757	0.8108
B^2	38.2809792	1	38.2809792	16.026721	0.0052
C^2	361.452032	1	361.452032	151.325566	< 0.0001
Residual	16.720005	7	2.38857214		
Lack of Fit	15.472125	3	5.157375	16,5316377	0.0102
Pure Error	1.24788	4	0.31197		

Tableau 3.4 **Growth of Y. lipolytica SM7 in shake flasks and conversion yields in different initial glycerol concentration. Representations of initial substrate (S_o); remaining substrate (S); glycerol consumed (Sconsumed), Biomass produced (X); Lipid content (P/X,% (w/w) of dry biomass; Lipid quantity (L, g/L), Citric Acid produced (CA); and conversion yields ($Y_{X/S}$, $Y_{L/S}$, $Y_{CA/S}$) at different fermentation time. Culture conditions: pH 6.5 ± 0.03; incubation temperature 28°C; agitation rate 180 rpm; for initial glycerol concentration 75, 87.5 and 100 g/L, respectively**

S_o (g/L)	Ferment. time (h)	S (g/L)	$S_{consumed}$ (%)	X (g/L)	L (g/L)	P/X (%)	CA (g/L)	$Y_{X/S}$	$Y_{L/S}$	$Y_{CA/S}$
75	36	25.5 ± 1.5	66.0 ± 2.5	4.6 ± 0.8	1.1 ± 0.1	24.1 ± 0.2	0.5 ± 0.2	0.09 ± 0.01	0.01 ± 0.5	0.01 ± 0.01
	54	16.0 ± 2.7	78.7 ± 2.0	6.7 ± 0.6	3.3 ± 0.2	49.3 ± 1.2	2.5 ± 1.1	0.11 ± 0.11	0.04 ± 0.3	0.04 ± 0.02
	72	8.2 ± 1.2	89.1 ± 2.1	8.7 ± 0.2	3.8 ± 0.3	44.0 ± 0.8	3.8 ± 0.9	0.13 ± 0.01	0.04 ± 0.2	0.06 ± 0.02
87.5	36	28.5 ± 3.2	67.4 ± 1.5	4.9 ± 0.5	1.4 ± 0.3	28.7 ± 0.5	0.5 ± 0.2	0.08 ± 0.02	0.02 ± 0.1	0.01 ± 0.50
	54	13.0 ± 1.8	85.1 ± 2.2	6.9 ± 0.3	3.1 ± 0.3	45.4 ± 1.9	2.4 ± 0.5	0.09 ± 0.01	0.04 ± 0.0	0.03 ± 0.08
	72	4.3 ± 0.9	95.1 ± 1.8	9.4 ± 1.1	4.7 ± 0.4	50.2 ± 1.5	4.0 ± 1.3	0.11 ± 0.09	1 0.05 ± 0.2	0.05 ± 0.02
100	36	77.4 ± 2.2	22.6 ± 3.1	5.1 ± 0.9	1.4 ± 0.1	27.7 ± 0.7	0.5 ± 0.3	0.22 ± 0.07	0.06 ± 0.3	0.02 ± 0.10
	54	70.3 ± 5.4	29.7 ± 2.8	8.1 ± 0.8	4.4 ± 0.1	53.7 ± 1.2	5.6 ± 1.7	0.27 ± 0.10	0.14 ± 0.2	0.18 ± 0.08
	72	45.1 ± 3.3	54.9 ± 2.2	9.8 ± 0.6	4.7 ± 0.1	48.2 ± 1.8	12.0 ± 2.5	0.17 ± 0.10	0.08 ± 0.4	0.21 ± 0.08

4 CHAPITRE 4: MORPHOLOGY AND RHEOLOGICAL BEHAVIOUR OF YARROWIA LIPOLYTICA : IMPACT OF DISSOLVED OXYGEN LEVEL ON CELL GROWTH AND LIPID

Morphology and rheological behaviour of *Yarrowia lipolytica*: Impact of dissolved oxygen level on cell growth and lipid composition

Sarra Magdouli [a], Satinder Kaur Brar [b], Jean François Blais [c,*]

[a] Ph.D. student, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-4677, Fax: (418) 654-2600, email: magdouli.sara@ete.inrs.ca

[b] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-3116, Fax: (418) 654-2600, email: satinder.brar@ete.inrs.ca

[c] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-2575, Fax: (418) 654-2600, email: blaisjf@ete.inrs.ca

* Corresponding author blaisjf@ete.inrs.ca

Article soumis à Energy

4.1 Résumé

Yarrowia lipolytica, une levure ascomycète, a été étudiée pour son potentiel d'accumuler une grande quantité de lipides. La production de lipides sous différents taux d'oxygénation a été testée afin de déterminer le scénario optimal pour obtenir le taux d'accumulation le plus élevé. Une étude métabolomique, morphologique et rhéologique a été réalisée en présence de glycérol brut comme source de carbone. Les résultats ont montré qu'une concentration en oxygène dissous de 30% permet d'obtenir une production de lipides élevés de 44,8 ± 1,2%. L'augmentation ou la diminution du taux d'oxygène peuvent affecter la teneur et le profil des lipides. Cependant, la variation du taux d'oxygène ne modifie pas le comportement rhéologique des cellules ni leur distribution unimodale. Le comportement rhéologique est en fait dépendant de la limitation en éléments nutritifs et de l'aération. La variation du taux d'oxygène (c.-à-d. le coefficient volumétrique de transfert d'oxygène (K_La) et le taux d'absorption d'oxygène) a un impact sur la concentration de biomasse, l'accumulation de lipides et leur composition. Ainsi, la synthèse des lipides dépend de la morphologie et de la rhéologie du milieu. Tous ces paramètres jouent un rôle crucial dans la détermination de la performance du bioprocédé.

Mots-clés : *Yarrowia lipolytica,* oxygène, rhéologie, K_La, production de lipides, composition des acides gras

4.2 Abstract

Yarrowia lipolytica has been investigated as potential ascomycetes yeast for higher lipid productivity. Lipid production under different oxygenation rates was carried out to identify the ideal scenario required for higher accumulation rate. A combination of metabolomic, morphological and rheological profiling was investigated in the presence of crude glycerol as a carbon substrate. Results showed that cells require dissolved oxygen concentration of 30% to achieve a higher lipid production of 44.8 ± 1.2% (w/w). The increase or decrease of oxygen level might affect the lipid content as well as the lipid profiling. However, cells exhibited the same rheological behaviour and presented unimodal distribution despite the oxygen variation. Furthermore, broth rheology was ultimately dependent on nutrient limitation and aeration. The variation of oxygen level (i.e. volumetric oxygen transfer coefficient (K_La) and oxygen uptake rate (OUR)) impacted biomass concentration, lipid accumulation and lipid composition. Moreover, lipid synthesis is dependent on morphology and rheology of the medium. All of these parameters played a crucial role in determining bioprocess performance.

Keywords: Yarrowia lipolytica, oxygen, rheology, KLa, lipid production, fatty acids composition

4.3 Introduction

The increasing emphasis on sustainable and renewable energy and global warming has encouraged scientists to seek for other lipid derived biofuels as potential substitutes of fossils fuels. In this regard, microbial oils have received a high interest owing to their advantages that include the high efficiency and the low land consumption (Beopoulos et al., 2009 a,b). *Yarrowia lipolytica,* "non-conventional" oleaginous ascomycetous yeast, was reported to be an attractive cell factory for the production of single cell oils (SCO) because of its native lipid accumulation, easy engineering and robust growth on diverse variety of industrial by-products, such as raw glycerol and saturated fatty acids to achieve higher lipid productivity (Makri et al., 2010; Dobrowolski et al., 2016). Acccordingly, crude glycerol has been widely investigated for the production of others secondary metabolites such as 1,3-propanediol (Papanikolaou & Aggelis, 2002), CA (Goncalves et al., 2014) and β-carotene (Saenga et al., 2010). Understanding the physical mechanism involved in lipogenesis will guide the easy transition from downstream process to scale up and increase the lipid accumulation task. Thus, the study of the morphogenesis will also provide useful information about the dynamic biological processes during the transition from biomass production to lipid accumulation and could reveal the relevant contribution of operating conditions upon nitrogen limitation on the whole metabolic pathway. Many factors were reported to have a major effect on lipid accumulation, such as nitrogen source, phosphorous, pH, temperature, as well as carbon to nitrogen ratio (Kendrick et al., 1992). Despite the growing scientific literature on the lipid production by *Y*. *lipolytica*, there are significant knowledge gaps regarding the key biological processes involved in relation to oxygen saturation. The study of dissolved oxygen effect on lipid accumulation is not a new concept. Thus, Aiba et al. (1973) have reported that oxygen limitation may affect the growth and biomass production (Aiba et al., 1973). Besides, Kamzolova et al. (2003) have elucidated a correlation between oxygen concentration and citric acid (CA) production. A low pO_2 (5%) enhanced CA production in *Yarrowia* N1 (Kamzolova et al., 2003). Likewise, enzymatic activities of glyoxylate and isocitrate lyase cycles were substantially reduced, for instance, malate synthase was reduced from three to five times and isocitrate lyase four to eight times (Kamzolova et al., 2003).Conversely, an increase in concentration of dissolved oxygen (pO_2) induced CA production in *Aspergillus niger* and *Candida lipolytica* Y1095, respectively (Clark & Lentz, 1991; Rane & Sims, 1994). Not only CA production, but also lipid productivity and profiles were highly affected by oxygen transfer (aeration and agitation). Choi et al. (1982) proved that oxygen concentration between 45 and 234 µM was required for lipid accumulation. Furthermore, a low level of DO (15%) improved the

total lipid accumulation in *Mucor sp. RRl001* and *Candida lipolytica* (Ahmed et al., 2009). The effect of dissolved oxygen (DO) on cell growth and lipid accumulation was also tested by Yen et al. (2001), who observed that low level of DO enhances lipid accumulation, but inhibits cell growth whereas a higher level of DO enhances biomass accumulation rather than lipid accumulation. Moreover, a two stage DO controlled experiment between 25% and 60% was performed to achieve higher biomass and lipid content during the culturing of of *Rhodotula glutinis* (Yen & Zhang, 2011).

The effect of dissolved oxygen on lipid accumulation of *Y. lipolytica* has not been conclusive so far and only few studies dealt with the effects of oxygen on lipid accumulation while using crude glycerol concentration. Another aspect to be studied other than the operational condition is the possible change of physical state of cells during cell culturing in bioreactor. Thus, physical parameters (aeration, mixing) and microorganism physiology and activity are known to closely interact and evolve. Irreducible couplings between heat transfer, mass transfer and fluid mechanics result in a complex and evolving system (Cascaval et al., 2003). Moreover, the rheological behaviour of culture broth stands as a fundamental parameter in bioprocess performances because it simultaneously affects heat and mass transfer as well as flow pattern. Finally, the understanding of rheological behaviour is determinant to drive cell culture up to a defined goal (biomass production, extra or intra cellular metabolite production, substrate biodegradation, etc.) and to optimize bioprocess (Pamboukian & Facciotti MCR, 2005; Petersen et al., 2008). The present investigation is a part of a work aiming to study biological response (growth rate substrate assimilation, lipid accumulation considering accumulation and conversion yields) and physical properties (rheology) of *Y. lipolytica* under controlled operating conditions using crude glycerol as carbon source. Thus, this study will provide a conclusive response to the query as to if lipid metabolism may affect bioprocess performance. Hence, a two-stage DO controlled strategy was also conducted to improve the cell growth during exponential growth, later, the variation of oxygen concentration and its effect on lipid production and rheology of substrate through the variation of DO in a 5-L lab-scale fermenter between the low DO batch (15-20%), (30-35%), the high DO batch (40-60%) was performed. Another aspect investigated was the impact of DO on the scaling up of process during the lipid accumulation.

4.4 Material and methods

4.4.1 Microorganism and medium

Y. lipolytica SM7, having gene bank accession has been newly isolated (Magdouli et al., 2016) and was used in the present study for its capacity to produce lipids in crude glycerol based media. The strain was grown on YEPD agar (yeast extract peptone dextrose agar) at 28°C for 2 days, maintained at 4°C and sub-cultured every three months.

The pre-culture was obtained by inoculating a separate colony of *Y. lipolytica* SM7 in yeast extract peptone dextrose (YPD) medium containing (g/L): Glucose 20, peptone 20 and yeast extract 10 and incubating it at 28°C for 24 h prior to cultivation. Lipid production was performed in duplicates, aerobically, 5 L containing 3 L of the designed media (100 g of crude glycerol, 1 g/L yeast extract, 1.5 g/L $(NH_4)_2SO_4$, 3 g/L K_2HPO_4, 3 g/L NaH_2PO_4 H_2O, 0.5 g/L $MgSO_4.7H_2O$, 0.040 g/L $ZnSO_4.7H_2O$, 0.016 g/L $FeSO_4.7H_2O$, 0.25 µg/L biotin) and inoculated with the pre-culture (initial OD_{600} = 0.01), 5% (v/v) and incubated at 28°C in a rotary shaker incubator, under agitation of 180 rpm. pH was re-adjusted in all solutions by using NaOH and H_2SO_4 4N. Crude glycerol was provided by Rothsay, Ontario, Canada, the latter was used as carbon source resulting from the transesterification of animal fats.

4.4.2 Batch operation in fermenter with controlled dissolved oxygen

About 150 mL of seed medium was transferred into a 5-L stirred desk-top fermenter of 3 L working volume with 100 g/L of crude glycerol. The pH level was automatically maintained at 6.5 by feeding NaOH solution (4N). The fermenter was operated at 28°C with dissolved oxygen (DO) concentration controlled at 15-20%, 30-35%, and 40-60% of saturation respectively. The agitation during the process was limited to the range of 200 to 400 rpm. In the batch process, with DO controlled at 40-60% of saturation, supplemental pure oxygen in the inlet gas was provided to prevent the potential cell damage from the high shear force. In the batch operation with two-stage controlled DO, the DO was set at 60% of saturation for the first 24 h (in the exponential phase) and was adjusted after according to the experiments. The reason of providing higher DO at the beginning was to enhance the cell growth rate and accelerate biomass production.

4.4.3 Glycerol analysis and biomass measurement

For the measurement of yeast growth, dry biomass concentration was determined gravimetrically. For the measurement of glycerol and others organics acids in the broth, LC/MS/MS technique was employed. The technical details of the LC/MS/MS instrument used for the analysis were: (a) for sugar estimation: Thermo TSQ Quantum model, equipped with an Electrospray Ionization (ESI) in negative ion mode; Zorbax Carbohydrate (4.6 mm x 150 mm; 5 mm, Agilent) analytical column; 75% acetonitrile; 0.1% NH_4OH; 25% water and 0.1% NH_4OH mobile phase and 10 mL injection volume. Glycerol, CA and organics acids (all from Sigma) were used as the internal standards.

4.4.4 Lipid extraction and analysis

Extraction of lipids from lyophilized biomass was modified from the procedure of Bligh and Dyer's method. The dry biomass was ground to a fine powder: 0.05 g of powder was blended with 5 mL chloroform/methanol (2:1) and the mixture was agitated for 20 min in an orbital shaker at room temperature. The extraction of total cellular lipids was performed according to Folch method. Five hundred milligrams of lyophilized cells were suspended in methanol/chloroform (2:1, v/v). After the first extraction, the remaining cell lipids were further extracted twice with methanol/chloroform (1:1, v/v); and then with methanol/chloroform (1:2, v/v). Each extraction step consisted of incubation for about 1 day at room temperature under gentle shaking. The four organic phases were mixed and washed twice with 0.88% (w/v) KCl solution for 10 min and centrifuged between each step for 5 min at 10,000 x *g*. finally, lipids were recovered as dry material after the evaporation of the solvent at 40 ± 1^{o}C, until a constant weight was obtained. The fatty acids profile of the lipid was determined by methylation for conversion of fatty acids to fatty acid methyl esters (FAMEs), lipids (0.01–0.1 g) obtained were first dissolved in hexane (50 mL hexane/g lipid), then mixed with methanol. Lipid to methanol molar ratio was 1:6 (0.3 mL methanol per gram lipid). Sodium hydroxide was used as catalyst with concentration of 1% w/w (NaOH/oil). The mixture was then heated at 55 ± 1^{o}C for 2 h. After reaction, 5% NaCl solution was added with a concentration of 100 mL per gram lipid, and then FAMEs was extracted by two times washing with hexane (100 mL per gram lipid). After washing, the mixture was allowed to stand for phase separation, and then hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% (w/v) sodium bicarbonate solution (20 mL per gram lipid), and the top layer was then dried at 60 ± 1^{o}C in an oven (Halim et al., 2011). The FAMEs in hexane were analyzed using a Gas Chromatography linked to Mass Spectroscopy (GC–MS) (Perkin Elmer, Clarus 500). The

dimensions of the column used were 30 m × 0.25 mm, with a phase thickness of 0.25 µm. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1,3-dichlorobenzene was also used as an internal standard. All experiments were performed in triplicates, and average results were reported with standard deviation less than 5%.

4.4.5 Volumetric oxygen transfer coefficient (KLa) oxygen uptake rate (OUR), and (OTR)

The dynamic gassing-out method was used for K_La measurements (Aiba et al., 1973). The DO control was momentarily stopped during the dynamic gassing-out method to facilitate correct K_La measurements. Oxygen uptake rates were obtained by measuring the slope of DO decrease during air-off of the dynamic gassing-out protocol. For the K_La value determination the static gassing out method was employed throughout the experiments. This was performed by firstly purging the system (xanthan solution with palm oil) with nitrogen gas until the dissolved oxygen fell to zero. When DO was stabilized at 0% value, the nitrogen valve was switched off and, simultaneously, the aeration was started and time was marked as zero (t = 0). The gradual increase of DO concentration was monitored and recorded until it reached a steady value at 100% of saturation.

4.4.6 Rheological measurements

Rheological analyses were carried out for fresh samples by using a rotational viscometer (DVII+, Brookfield) equipped with small sample adapter spindle (SC4 34, Brookfield). The calibration and the rheological testing procedure used for the spindle were carried out according to the instrument's manual. The viscosity data were examined by using the software Rheocalc V2.6 (Brookfield Engineering Labs 1999). The shear stress versus shear rate data was analyzed as per the Ostwald deWaele or power law model given in Eq. (4.1):

Équation 4.1 $\tau = K\gamma^n$ **(1)**

where τ is the shear stress (mPa), K is the consistency index (m.Pas^n), γ is the shear rate (s^{-1}) and *n* is the flow behaviour index.

4.4.7 Laser particle size analysis

The particle size distribution of the different fermentation broth samples was determined using a laser particle sizer (Partica Laser Scattering LA-950V2, Laser Particle Size Analyzer, Tokyo, Japan). A cellular suspension was prepared in sterile water and then analyzed. All assays were performed in triplicate.

4.4.8 Scanning electron microscopy

Cells were analyzed by scanning electron microscopy (SEM, Carl Zeiss EVO® 50) to have a highly magnified view of the surface morphology and the behavior of cells during lipogenesis. To prepare samples for SEM, cells were dried using lyophilizer (VirTis Virtual 50L pilot lyophilizer) and no further treatment of cells was done. Dried samples were directly mounted on a SEM grid and sputter coated (SPI Module Sputter Coater) with gold before SEM analysis.

4.5 Results and discussion

4.5.1 Fermentation: growth kinetics in different oxygen saturation conditions

In order to evaluate the lipid production of *Y. lipolytica* using crude glycerol as substrate, effect of DO level was examined. The constant range of DO levels varied between 15-20%, 30-35% and 40-60% of saturation in three independent experiments. The glycerol concentration was fixed to be 100 g/L which reflected the high performance of selected strain to tolerate higher concentrations of crude glycerol compared to other strain, thus, glycerol has been reported to cause growth inhibition at higher concentrations due to osmotic stress on the cells (Beopoulos et al., 2008; Liang et al., 2010; Meesters et al., 1996) which was not presented in the case study and the microorganism was able to tolerate higher glycerol concentration (Magdouli et al., 2016). This observation explains the fact that substrate tolerance is strain specific. Figure 4.1A shows the evolution of cell growth of Y. lipolytica under different oxygenation conditions. As seen from Figure 4.1A, during the biomass production phase, the biomass increased sharply from 0.9 ± 0.1 to 4.8 ± 0.1 g/L, this phase corresponds to steady growth phase. In this case, initial aeration was maintained at 0.15 vvm (volume of air/volume of bioreactor/minute) and dissolved oxygen was kept higher above 60% of saturation.

Herein, the higher supply of oxygen was maintained until 18 h prior to nitrogen depletion. In the different scenarios of oxygen, the higher oxygenation was provided manually by maintaining the air flow rate and the agitation between 400 to 500 rpm. Thus, during the exponential phase, the biomass yield, $Y_{X/S}$ was around 0.86 ± 0.14 g/g, which is higher than the theoretical yield of biomass production reported for carbon hydrate which is estimated to be around 0.5 g/g for xylose and glucose (du Preez et al., 1989). After nitrogen depletion occurred at 18 h, the shift to lipogenesis was controlled by adjusting the oxygen to different saturation concentrations of 15-20%, 30-35%, 40-60%. Thus, the modification of oxygen concentration was carried out during this phase to characterize and identify the best scenario during lipogenic phase.

When cells are cultivated under lower oxygen rate (15%), the biomass was around 12.7 ± 0.5 g/L. Thus, nitrogen deficiency was associated with lower oxygen and had a negative effect on cell growth. The results indicated that lower oxygen influenced the growth rate and the enzymes implicated in the glycerol uptake, in fact only 35.2% of glycerol was consumed during 60 h. The biomass yield $Y_{X/S}$ was estimated to be around 0.34 g/g, while the lipid yield $Y_{P/S}$ = 0.09 g/g and only 28.1% (w/w) was obtained.

Conversely, higher oxygen concentration above 60% of saturation resulted in higher cell biomass of 25.0 ± 0.4 g/L at 100 h, whereas, the lipid concentration decreased from 6.9 ± 0.5 g/L at 60 h to 5.6 ± 0.8 g/L at 100 h at DO concentration of 40-60% of saturation. The higher lipid concentration of 8.0 g/L was obtained at 60 h under DO of 30% of saturation, which corresponded to a lipid content of 44.8 ± 1.2% (w/w). After 60 h, the lipid content was decreased and the conversion yield of lipid was around 0.12 ± 0.05 g/g at compared to 0.19 ± 0.10 g/g at 60 h. A maximum of lipid was recorded with 30-35% of saturation and the higher aeration seemed to lower the lipid content and favorite the biodegradation of SCO accumulated where only 35.5 ± 0.8% (w/w) was obtained, this observation go in line with the findings of Martins et al. (2012), who noted that the aerated cultures can boost oil biodegradation (Martins et al., 2002) and increase acyl-CoA oxidase activities (Papanikolaou et al., 2007). Conversely, lower oxygen was responsible for decrease of lipid content and lipid concentration as shown in Figure 4.1A.

A comparative study of different metabolites produced under different conditions showed that CA was the major organic compound produced during lipid accumulation as well as in the later stage growth which corresponds to the CA phase. In fact, after 60 h, the overall yield of lipid production decreased.

During the first phase of growth, no secondary metabolites were detected, hence, glycerol was essentially used for the synthesis of catalytic biomass. At 30-35% and 40-60% of saturation, there was absence of secondary metabolites coming from the central metabolism of *Y. lipolytica.* During the accumulation phase, the metabolites produced were present but at lower concentrations and their concentration didn't exceed 1.0 ± 0.5 g/L. Among detectable metabolites, acetic, glutamic and ketoglutaric acids were found. Their concentration was constant during the second stage of lipid accumulation and no difference was observed between 40% and 60% of saturation. However, the major difference is noted throughout the production of CA. Its concentration reached 17.4 ± 3.1 g/L during 40-60% DO, 12.2 ± 1.2 g/L at 30-35% and 3.2 ± 2.0 g/L at 15-20% DO. An increase in CA was observed in the following order of DO concentrations: 60% > 30% > 20%. Nevertheless, data dealing with the production of CA by *Y. lipolytica* grown on crude glycerol and in function of dissolved oxygen concentration are not frequently seen in the literature. Thus, CA biosynthesis was favoured under efficient aeration conditions (Kamzolova et al., 2003; Dawson et al., 1988). Recently, Makri et al. (2010) have found that *Yarrowia lipolytica* ACA-DC 50109 was accumulating higher CA concentration in the presence of higher glycerol concentration (105 g/L) to reach around 33.3 ± 8.3 g/L with a conversion yield $Y_{CA/S}$ = 0.52 ± 0.05 g/g and under dissolved oxygen 30% of saturation. Thus, *Y. lipolytica* accumulated significant amounts of SCO during

lipogenic phase and excreted high amounts of CA during CA production phase in aerated cultures which coincided the lipid turnover phase. This behaviour is a unique feature of *Y. lipolytica* compared to other oleaginous micro-organisms. Usually, the lipid turnover phase occurs when the carbon source is depleted or when the carbon source uptake is repressed and doesn't satisfy the metabolic requirement of cells (Holdsworth & Ratledge, 1988). Herein the lipid turnover phase was also dependent on oxygen concentration and the higher aeration seems to accelerate the turnover phase. Besides, unconsumed glycerol concentration remains in the culture and left unconsumed, around 28.7 ± 1.5 g/L of crude glycerol was present in the culture medium during 100 h in the case of DO concentration of 30% of saturation, meanwhile, only 45.0 ± 0.5 g/L was detected in the case of low aerated culture (15% of saturation), which may suggest that not only carbon source is critical parameter, but also the oxygen factor. These findings were in accordance with Papanikolaou and Aggelis (2003) (Papanikolaou & Aggelis, 2003). More often, Dobrowolski et al. (2016) have found that around 55.9 g/L of CA was produced at the end of culture in the presence of higher C/N ratio = 100 and the CA yield was $Y_{CA/S}$= 0.372 g/g in the presence of dissolved oxygen 25 ± 5% of saturation (Dobrowolski et al., 2016).

Likewise, Dawson et al. (1988) revealed that an increase in pO_2 from 25% to 75% saturation resulted in an increase in CA production efficiency from 41% to 60%. Besides, low pO_2 was reported to be vulnerable to enzymatic reactions of TCA and glyoxylate cycles. When pO_2 decreased, it inhibited isocitrate lyase (ICL) and impaired the mechanism of glyoxylate cycle-mediated resynthesis of oxaloacetate (Kamzolova et al., 2003). Thus, lower pO_2 concentrations may affect the functioning of the mitochondrial electron transport chain, which determined the energy supply for various metabolic processes. Therefore, it was proposed that an alternative electron transport pathway was activated at lower oxygen level and the adjustment of the culture media with high iron concentration (3.5 mg/L) was required to mitigate the oxygen requirement level (Kamzolova et al., 2003). As *Y. lipolytica* is a strict aerobe, it is not surprising, therefore, that increasing oxygen concentration showed a positive effect on the lipid content of this aerophilic yeast, even though lipid quantity was reduced at higher dissolved oxygen.

Rather than nitrogen deficiency, O_2 limitation induced lipid accumulation in *Y. lipolytica*. The activation of adenosine monophosphate *(*AMP) deaminase may rather be a general stress or starvation response in oleaginous microorganisms. In fact, lower oxygen is responsible for the shift of intracellular metabolism to lipogenesis and the activation of enzymatic system (e.g. ATP lyase).

4.5.2 OUR, OTR and oxygen transfer coefficient (K_La)

Scale up is a function of rheological parameters that needs to be studied to optimize abiotic parameters, such as agitation and aeration (oxygen transfer) and operating costs (Humphrey, 1998). Therefore, a critical analysis of operating parameters would be a vital step in economizing the mass production of *Yarrowia* based lipids. The operation of low, medium and high DO in a lab-scale fermenter suggested that DO has a potential effect on fermenter variables (e.g. OUR, OTR and oxygen transfer rate (KLa). OUR variations are shown in Figure 4.2. During the first growth phase corresponding to 0-18 h, oxygen concentration reached maximum values of around 0.6-1.28 mmol O_2/L/h. This stage corresponded to the active growth where microbial cells quickly started the consumption of glycerol as carbon source. In the second stage (18-60 h), where the control of oxygen occurred, significant fluctuations of OUR were noticed which may correspond to the transition between two stages of growth and lipid accumulation and stress condition had occurred (low and high oxygen demand). At 15-20% DO, OUR was around 0.9-1.0 mmol O_2/L/h and between 0.27 to 0.33 mmol O_2/L/h for 60% DO and ranged between 1.00 to 1.08 at 30% DO. Oxygen limitation in higher oxygen supplied culture was mainly due to the dramatic decrease of K_La of 50-60 h^{-1}, while lower oxygen supplied culture was due to lower K_La at 25-30% DO of saturation. The OUR was altered corresponding to the change of K_La. The results of the present work also confirmed the OUR is a good indicator for the activities of cultures, even under stress conditions (Zokaei-Kadijani et al., 2013). Further OUR optimization for lipid improvement would be expected which will facilitate the bioreactor design, optimization, scale-up, operation and control for industrial production.

The supply of higher oxygenation (aeration and mixing) during the first growth stage has led to biomass of 10.1 ± 0.5 g/L which corresponded to 2.5 fold increase compared to experiments performed in shake flasks, (i.e. one stage strategy), where only 4.2 ± 0.4 g/L of biomass was achieved. During this stage, cell number increased and nitrogen served for the synthesis of biocatalytic biomass, meanwhile during the second stage, only weight, body and cell size were all increased. Thus, the application of two stages oxygen supply control strategy for lipogenesis is not new. However, few studies have been reported in yeasts. It was first investigated in many oleaginous algal species, such as *Schizochytrium* for the production of docosahexaenoic acid (DHA) (Chi et al., 2009), however, in the current study, this is the first report to deal with the effect of oxygen saturation on lipid production in *Y. lipolytica*. After nitrogen depletion (18 h), *Y. lipolytica* started the accumulation stage. In fact, the nitrogen starvation led to the activation of AMP deaminase. Cellular AMP level in the mitochondria was then reduced which may initiate the citrate

accumulation. The latter, translocated to the cytosol will serve as a precursor of acetyl-CoA during the fatty acid synthesis (Ratledge & Wynn, 2002). In the second experiment of 40-60% DO, OUR reached a very low value of 0.3 mmol/L/h which was due to the excessive anti foam addition. However, it was extremely difficult to maintain DO above >>40% due to design limitations of the fermenter. In addition, higher agitation to maintain caused foam formation and led to probe DO saturation. In terms of oxygen demand in the fermentation process, higher oxygen consumption rate is preferred for the first stage, because cell growth requires large amount of primary metabolites, such as enzymes, nucleic acids and other proteins. In the experiment of 40-60% DO, lower K_La 30-47 h^{-1} was correlated with higher biomass production of around 24.0 ± 1.2 g/L. Although higher biomass was obtained with high K_La, lower K_La was preferable for the lipid' accumulation stage, thus, low oxygen supply is more favourable. These results are in agreement with Chi et al. (2009) who found that high dissolved oxygen concentration set at 50% of saturation during the later phase of fermentation proved to have an adverse effect on lipid production, because cell size remained small and no improvement in dry cell weight was noted. Besides, Bailey et al. (2003) have patented that high dissolved oxygen concentration of 40% of saturation produced 18 ± 2% lipids in the biomass, but low dissolved oxygen at 5% produced 24 ± 4%. These reported results proved that lower oxygen was more beneficial for lipids production in *Schizochytrium sp.* In this study, lower K_La of 20-28 h^{-1} is accompanied with lower lipid concentration 28.9 ± 0.5% (w/w) compared to high K_La of 100 h^{-1} which corresponded to a lipid content of 43.7 ± 1.2% (w/w). High oxygen supply in the lipid accumulation stage may lead to more carbon source used for cell respiration and energy metabolism rather than lipid synthesis. Therefore, the decrease in oxygen supply in the lipid accumulation stage could promote the carbon source utilization efficiency and increase the lipid accumulation. In the figure, K_La decreased at oxygen level 50 h^{-1} > 40% which was possibly due to anti-foam addition.

4.5.3 Rheology and mass transfer

Y. lipolytica is an aerobic mesophilic strain. However, the lipid accumulation process is reported to be a low oxygen dependant process (Choi et al., 1982). As expected, any increase in oxygen rate is presumed to inhibit the lipid accumulation rate. An increase in broth viscosity due the augmentation of oxygen can hamper the oxygen transfer rates and further lower the lipid accumulation process. Consequently, it would be crucial to examine the volumetric oxygen transfer coefficient (K_La), broth viscosity, aeration and agitation as a function of time. This would help to facilitate predicting and ensuring a sufficient amount of oxygen required by *Y. lipolytica* to

reach high lipid productivity. Besides, this study will lead to the increase of biomass and lipid production on one hand, and develop a strategy for scaling-up of the bioreactor design for mass production on the other hand (Bailey et al., 1986).Through this study, the rheological behaviour of supernatant of *Y. lipolytica* SM7 revealed a pseudoplastic behaviour during the accumulation stage with an apparent viscosity ranging between 2 and 3 mPa.s (25°C) for 40% DO and 1-3 mPa.s (25°C) for 15% DO and 1-7 mPa.s (25°C) for 30% DO at 60 rpm. The increase of viscosity is herein closely correlated with the biomass concentration increase and inversely correlated to K_La value (Verma et al., 2006). During the growth step (first 24 h), apparent viscosity (25°C) is stable ranging between 0.0 to 2 mPa.s for three experiments which is correlated to biomass concentration of 10.0 ± 0.4 g/L. Therefore, during cell growth and nitrogen limitation phases (i.e. biomass production phase), a shear-thinning behaviour quickly appeared with biomass concentration increase and flow behavior tended to 0.4 at 24 h. Besides, high K_La and high viscosity showed a direct effect on the lipid accumulation, a decrease of 28.9% (w/w) was also noted. Morphology constituted a limiting factor to heat and mass transfer as well as power consumption if intensification of bioprocess is desired. It is apparent that the accumulation stage seemed to affect broth rheology. Thus, a loss of shear-thinning properties (flow behaviour and consistency index trends to 0.7-0.9 between 36 h and 60 h at 40% DO concentration and between 0.5 to 0.6 in 30%, DO concentration, however, at 20% DO concentration, the flow behaviour index decreased and varied between 0.2 to 0.4, this phase corresponded to lipid accumulation phase. The increase of flow behaviour index during lipogenic stage was related to different physical properties (elasticity, deformation) of particles that are assumed to be modified during lipid accumulation (Trepat et al., 2007). Thus, *Y. lipolytica* is known for formation of some bud scars (filamentous particles) which are responsible to increase the viscosity during this stage (Magdouli et al., 2016). In fact, cells stressed by nutrient limitation appear elongated and grow as pseudo-filaments. This fact is assumed to modify rheological behaviour (Petersen et al., 2008). Herein, the lipid accumulation seemed to be correlated with the viscosity which showed fluctuations owing to the presence of pseudo filaments and cells which were highly sensitive to oxygen variation. This result was in agreement with Fillaudeau et al. (2016) who observed that broth rheology depends on biomass concentration, lipid accumulation and morphology and affects bioprocess performances. Irrespective of dissolved oxygen concentration, a major change in viscosity took place during the second stage of lipid accumulation. In general, for fungi, K_La decreased with viscosity (Verma et al., 2006), however, in the case of 30% of saturation, the profiles of K_La and the viscosity were similar until 36 h (Figure 4. 3). This was probably due to an increase in aeration and agitation rates to maintain 30% of saturation. For 40% DO concentration, K_La varied inversely

to viscosity until 36 h of fermentation. Subsequently, the decrease in K_La was independent of viscosity and followed aeration and agitation variations. Oxygen was imposed to induce a mycelial transition. During the accumulation stage, the culture of *Y. lipolytica* exhibited shear-thinning non-Newtonian fluid behavior, as the viscosity all decreased with the increase of shear rate. The apparent viscosity for each culture increased with increasing biomass concentration. However, with the approachable biomass density, the cultures showed more viscous under higher oxygen supply condition. Therefore, biomass concentration, was reported to be affected by various parameters including the cultivation medium, the size of cells and cell aggregates, biomass concentration, morphological parameters and the products being secreted into culture medium. When oleaginous microorganisms started lipid accumulation, cell size increased in weight. Generally, Chen et al. (1997) have reported that at the same biomass concentration, smaller cells exhibited higher viscosity.

The increase of agitation and oxygen supply culture is responsible to inhibit cell aggregation, which may contribute to the viscosity increase of fermentation broth. However, in most cases, the change of flow behavior in microbial processes was mainly attributed to the increase of extracellular polymer concentration being produced with negligible concentration from the cells (Landon et al., 1993). The viscosity of *Y. lipolytica* is related to the bud scares presented by the strain during accumulation stage (Magdouli et al., 2016).

For the three culture media, the broth apparent viscosity rapidly increased, showing non-Newtonian pseudoplastic behavior. Rheological data could be described in terms of power law model. Figure 4.3 shows also K and n for different experiments performed where K is the consistency index and *n* is the flow behaviour index which indicated the degree of non-Newtonian behavior. As seen, the shear thinning behaviour is also confirmed by $n < 1$ and there is no trend for *n* values with dissolved oxygen, hence the overall average value of $n = 0.3, 0.7, 0.6$ is for 15-20%, 30-35%, 40-60% DO. The Ostwald–de Waele power law model provided the good fit of the experimental shear rate versus shear stress with R^2 value between 0.8970 and 0.9997. The consistency index, K increased over the cultivation time, which was well matched with the increase of broth viscosity. Whereas some fluctuations were presented and corresponded to physiological and metabolic changes during the growth of *Y. lipolytica,* and the fluctuations were more pronounced at lower and higher oxygen saturation level. Besides, the flow index, n during the fermentation process was consistently below 1 and pesudoplastic behavior in the current study can be attributed to the presence of high molecular weight polymers (Garcia-Ochoa et al., 2005).

4.5.4 Morphology and particle size distribution

A narrowing of particle distribution with a higher proportion of ovoid cells was observed in each culture under different oxygenation. Usually, yeast strains were characterized by highly different morphological profiles. To elucidate the effect of particle size distribution on the flow behaviour of liquid broth, the particle size analyses of fermentation broth was performed and results are presented in Figure 4 A,B,C. Cell size distribution was evaluated by suspending the cells in sterile water, results indicated the existence of two distinct populations; population with mean values of 5.4 µm was detected in three independent experiments corresponding to an oval morphology reported generally in *Yarowia* species (Lopes et al., 2007; Aguedo et al., 2003). Nevertheless, a second population of cells was also distinguished where the size varied between 8-15 µm. In the current study, *Y. lipolytica* SM7 exhibited mono-dispersed spherical/ovoid populations as shown by SEM analysis (Figure 4.4). SEM analysis showed that cells were plump and presented buds throughout the time course (Figure 4.4). Besides, during growth and lipogenic phase, cells appear elongated and grow as pseudo-filaments. Although the presence of pseudo filaments, *Y. lipolytica* presented unimodal distribution which has been reported for *Yarrowia* species (Fillaudeau et al., 2009; Cescut, 2009). Generally, filamentous cells represented pseudomycelium. During growth phase, filamentous cells are very low and the predominance of ovoid cells occurred (Magdouli et al., 2016). After nitrogen depletion and during the lipogenic phase, filamentous cells are more predominant, reflected by higher viscosity and dense culture visually observed in the fermenter. After 60 h, the filamentous cells are stabilized between 60 and 70 h. Thus, filamentous process is not dependent on nitrogen limitation since during exponential growth it was not specific for lipogenic phase, however, it accompanied both cell growth and lipogenic phase along fermentation period and at different oxygenation levels of 15%, 30%, 40% DO. To sum up, the filamentous process is omnipresent under different oxygen concentration and it is not dependent on oxygen concentration. *Y. lipolytica* strains exhibited similar behaviour during successive phases in accord with morphological changes or oxygen level. A remarkable difference is noted while SEM analysis is the surface changes between lipogenic and citric acid phase (Figure 4.5). Thus, the cell surface during the biomass production phase is smooth, while it presented lumphy characteristics during the lipogenic phase due to the accumulation of lipid droplets.

Accordingly, many factors were reported controlling the dimorphism of yeast to pseudo filaments, especially culture conditions such as temperature and broth (Makri et al., 2010; Barth & Gaillardin, 1997; Cervantes-Chávez et al., 2009). Thus, the important conclusion that arises from the present

study is that a gentle agitation should be applied during growth as well as lipogenic phase in order to avoid mycelial cells disruption and subsequent drop of biomass concentration in the bioreactor. In summary, *Y. lipolytica* tolerate higher oxygen up to 60% of saturation level in the case study and any higher aeration and/or agitation may have adverse effect.

4.5.5 Analysis of fatty acids produced during different oxygen saturation levels

As discussed earlier, many parameters have been cited to influence the composition of the lipid production of oleaginous microorganisms. In the current study, the analysis of fatty acids accumulated during three independent experiments is presented in Table 4.1, palmitic acid (C16:0) stearic acid (C18:0), linolenic acid (C18:3), and lenoleic acid (C18:2) are the main components produced. Oleic acid (C18:1) and linoleic acid (C18:2) were the major components at 40-48%.and 13-20%, respectively. At lower oxygen level, still detectable levels comprise saturated C20:0, C22:0 and C24:0 chains with the predominance of oleic acid. As observed from the culture of *Y. lipolytica,* the predominance of unsaturated fatty acids was remarkable and lipid composition in *Y. lipolytica showed* specific trends with oxygen concentration that largely reflect the physiological role of oxygen during unsaturation process. More often, at higher oxygen concentration, C18:2 increased, and the unsaturation level was significant at lower oxygen concentration compared to other fatty acids. Thus, the ratios $^{\Delta 9}$ C18:1/C18:0 and $^{\Delta 9,12}$ C18:2/$^{\Delta 9}$C18:1, reflected relative activity of fatty acids desaturases (Fakas et al., 2009). Although *Y. lipolytica* is an obligate aerobe, the variation of fatty acids with change of oxygen concentration under nitrogen-limiting conditions did not significantly affect the overall degree of unsaturation. For other fatty acids, there were no significant fluctuations among the various experiments. This proved that the accumulation of FAMEs especially unsaturated is oxygen-dependent. Metz et al. (2001), have found that major fatty acids synthesized by *Schizochytrium* species were favorable with lower oxygen supply (Metz et al., 2001). This observation was confirmed by Klein and Volkmann, (1975). Similarly, Rogers and Stewart (1974) concluded after observing a decrease in fatty acid content of *Candida parapsilosis* at lower oxygen concentrations without a significant alteration in the degree of unsaturation. However, numerous researchers have found that oxygen limited growth decreases the synthesis of unsaturated acids in a variety of yeasts (Cocucci et al., 1975; Nurminen et al., 1975). Accumulated lipids between the two phases of different% DO are very similar (data not shown). The desaturation of fatty acids is correlated with the no repression of catabolic promotor which is responsible for the stimulation of desaturation activity of cells, thus remarkable desaturase activity is observed for the synthesis of structural lipids of catalytic

biomass. Besides, fatty acids esterified during the growth were C18:1 and C18:2. After 60 h, fatty acids composition is almost stable which suggests other mechanisms of synthesis and degradation that control their synthesis and regulation.

With increase of oxygen concentration, C18:0 and $^{\Delta 9}$ C18:1 decreased slightly, whereas the polyunsaturated fatty acids, linoleic $^{\Delta 9,12}$ C18:2 and linolenic C18:3, increased.. There was also an increase in C16:0 compared with slow decrease of C18:0. The proportionality of C16:0 at the expense of C18:0 is oxygen dependent.

These accumulated FAMEs during the cultivation and oxygen limitation may also influence the diesel quality and the performance of bioprocess to direct the selectivity of FAMEs rather than others. For example, palmitic and stearic C16:0 and C18:0 accumulated along time course are known to have good fuel stability, lower NOx emission, higher ignition quality but lower cold flow properties than conventional diesel making them poor for cold weather while C18:2 (40-48%) accumulation with high percentage during the accumulation phase chains may be too unstable for use as a fuel (Durrett et al., 2008).

4.6 Conclusion

The evolution of yeast morphology and rheological behaviour of *Yarrowia lipolytica* broths under different oxygen saturation levels was scrutinized during production and accumulation of single cell oils. Studies were conducted in 5-L fermenter under perfectly controlled conditions with a culture strategy of two stage growth. Culture strategy enabled biomass concentration up to 18.1 ± 0.5 g/L with a maximum lipid content of 7.99 ± 0.11 g/L under medium aerated with 30-35% DO. During different phases, narrowing of particle distribution with a higher proportion of ovoid cells was noticeable under different oxygen rates. Results clearly demonstrated that lipid synthesis is a complex and vital metabolism and is affected by oxygen transfer rate, OUR, cell morphology and physical properties.

4.7 Acknowledgements

Authors would like to acknowledge the Natural Sciences and Engineering Research Council of Canada (grant A4984, Strategic grant 412994 – 11, Canada Research Chair) for financial support.

4.8 References

Ahmed, S.U., Singh, S.K., Pandey, A., Kanjilal, S., Prasad, R.B.N. 2009. Application of response surface method for studying the role of dissolved oxygen and agitation speed on gamma-linolenic acid production. *Appl Biochem Biotechnol*, **152,**108.

Aiba, S., Humphrey A.E., Millis, N.F. Aeration and agitation. In: Biochemical engineering, 2nd Edition, Academic, New York. 1973.

Aguedo, M., Waché, Y., Mazoyer, V., Le Grand, A.S., Belin, J.M. 2003. Increased electron donor and electron 193 acceptor characters enhance the adhesion between oil droplets and cells of *Yarrowia lipolytica* as 194 evaluated by a new cytometric assay. *J Agric Food Chem*, **51**, 3007-11

Bailey, R.B., DiMasi, D., Hansen, J.M., Mirrasoul, P.J., Ruecker, C.M., Veeder G.T.I. 2003. Enhanced production of lipids containing polyenoic fatty acid by very high density cultures of eukaryotic microbes in fermentors. US Patent 6,607,900

Bailey, J., Bailey, J., Ollis, D. 1986. Biochemical engineering fundamentals. 2nd Edition, McGraw-Hill, New York.

Barth, G., Gaillardin, C. 1997. Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. FEMS *Microbiol Rev*, **19**, 219- 37.

Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.L., MolinaJouve, C., Nicaud, J.M. 2009. *Yarrowia lipolytica* as a model for bio-oil production. *Prog Lip Res*, **48**, 375-87.

Beopoulos, A., Chardot, T., Nicaud, J.M. 2009. *Yarrowia lipolytica*: A model and a tool to understand the mechanisms implicated in lipid accumulation. *Biochimie,* **91**, 692-6.

Beopoulos, A., Mrozova, Z., Thevenieau, F., Dall, M.T., Hapala, I., Papanikolaou, S. 2008. Control of lipid accumulation in the yeast *Yarrowia lipolytica*. *Appl Environ Microbiol,* **74**, 7779-89

Cascaval, D., Oniscu, C., Galaction, A. 2003. Rheology of fermentation broth: influence of the rheological behavior on biotechnological process. *Rev Roum Chim*, **48**, 339-56.

Cervantes-Chávez J.A., Kronberg, F., Passeron, S., Ruiz-Herrera, J. 2009. Regulatory role of the PKA pathway in dimorphism and mating in *Yarrowia lipolytica*. *Fungal Genet Biol,* **46**, 390-9

Cescut, J. 2009. Accumulation d'acylglycérols par des espèces levuriennes à usage carburant aéronautique: physiologie et performances de procédés. Université de Toulouse, Toulouse, France

Cocucci, M.C., Belloni, G., Gianani, L. 1975. Oxygen pressure, fatty acid composition and ergosterol level in *Rhodotorula gracilis*. *Archiv Microbiol*, **105**, 17-20.

Choi, S., Ryu, D.D., Rhee, J. 1982. Production of microbial lipid: effects of growth rate and oxygen on lipid synthesis and fatty acid composition of *Rhodotorula gracilis*. *Biotechnol Bioeng*, **24**, 1165-72.

Chi, Z., Liu, Y, Cea, F. 2009. Study of a two-stage growth of DHA-producing marine algae *Schizochytrium limacinum* SR21 with shifting dissolved oxygen level. *Appl Microbiol Biotechnol*, **81**, 1141.

Chen, C.S, Mrksich, M., Huang, S., Whitesides, G.M., Ingber, D.E. 1997. Geometric control of cell life and death. *Science*, **276**, 1425-8.

Clark, D.S., Lentz, C.P. 1961. Submerged citric acid fermentation of sugar beet molasses: effect of pressure and recirculation of oxygen. *Can J Microbiol*, **7**, 447-53.

Dawson, M.W., Maddox, I.S., Boag, I.F., Brooks, J.D. 1988. Application of fed-batch culture to citric acid production by *Aspergillus niger*: The effects of dilution rate and dissolved oxygen tension. *Biotechnol Bioeng*, **32**, 220-6.

Dobrowolski, A., Mituła, P., Rymowicz, W., Mironczuk, A.M. 2016. Efficient conversion of crude glycerol from various industrial wastes into single cell oil by yeast *Yarrowia lipolytica*. Bioresour Technol, **207**, 237-43

Durrett, T.P., Benning, C., Ohlrogge, J. 2008. Plant triacylglycerols as feedstocks for the production of biofuels. *Plant J*, **54**, 593-607.

Fakas, S., Papanikolaou, S., Batsos A., Galiotou-Panayotou, M., Mallouchos A., Aggelis G. 2009. Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*. *Biomass Bioenergy*, **33**, 573-80.

Fillaudeau, L., Cescut, J., Anne-Archard, D., Nicaud, J.M., Uribelarrea, J-L., Molina-Jouve C. 2009. Morphology and rheological behaviour of *Yarrowia lipolytica* during production of intra-cellular energetic molecules. Impact of lipid accumulation and genetic modifications. In: Proceeding of 8th World Congress of Chemical Engineering Montréal, (Québec),

Canada. Canadian Society for Chemical Engineering (Ontario, Canada), ISBN: 0-9208044-44-6, Session: Bioproducts, Bioreactors and Bioprocesses, L0122, **2016**

du Preez, J.C., van Driessel, B., Prior, B.A. 1989. D-xylose fermentation by *Candida shehatae* and *Pichia stipitis* at low dissolved oxygen levels in fed-batch cultures. *Biotechnol Lett*, **11,** 131-6

Garcia-Ochoa, F, Gomez, E. 2005. Prediction of gas-liquid mass transfer in sparged stirred tank bioreactors. *Biotechnol Bioeng*, **92**, 761-72

Goncalves, F.A.G., Colen, G., Takahashi, J.A. 2014.*Yarrowia lipolytica* and its multiple applications in the biotechnological industry*. Sci World J*, **2014**, 476207

Halim, R., Gladman, B., Danquah, M.K., Webley, P.A. 2011. Oil extraction from microalgae for biodiesel production. *Bioresour Technol*, **102**, 178-85.

Holdsworth, J., Ratledge, C. 1988. Lipid turnover in oleaginous yeasts. *J Gen Microbiol*, **134**, 339-46

Humphrey, A. 1998. Shake flask to fermentor: what have we learned?. *Biotechnol Prog*, **14**, 3-7.

Kamzolova, S.V., Shishkanova, N.V., Morgunov, I.G., Finogenova, T.V. 2003. Oxygen requirements for growth and citric acid production of *Yarrowia lipolytica*. FEMS Yeast Research, **3**, 217-22.

Kendrick, A., Ratledge, C. 1992. Lipid formation in the oleaginous mould *Entomophthora exitalis* grown in continuous culture: effects of growth rate, temperature and dissolved oxygen tension on polyunsaturated fatty acids*. Appl Microbiol Biotechnol*, **37**, 18-22.

Klein, H.P., Volkmann, C.M. 1975. Factors affecting palmitoylcoenzyme A desaturase of *Saccharomyces cerevisiae*. *J Bacteriol,* **124**, 718-23.

Landon, R.S., Law, R.C.S., Webb, C. 1993. Fermentation broth rheology during dextran production by *Leuconostoc mesenteroides* B512 (F). *Appl Microbiol Biotechnol*, **40**, 251-7.

Liang, Y., Sarkany, N., Cui, Y., Yesuf, J., Trushenski, J., Blackburn, J.W. 2010. Use of sweet sorghum juice for lipid production by *Schizochytrium limacinum* SR21. *Bioresour Technol*, **101**, 3623-7

Lopes, M., Gomes, N., Gonçalves, C., Coelho, M.A.Z., Mota, M., Belo, I. 2007.*Yarrowia lipolytica* lipase production enhanced by increased air pressure. *Lett Appl Microbiol*, **46**, 255-60.

Magdouli, S., Brar, S.K., Blais, J.F. 2016. Lipid production by *Yarrowia lipolytica* grown on biodiesel-derived crude glycerol: Optimization of growth parameters and their effects on the fermentation efficiency. *RSC Adv,* **6**, 90547-90558

Martins, F.F., Ferreiraa, T.F., Azevedob, D.A., Alice, M., Coelho, Z. 2012. Evaluation of crude oil degradation by *Yarrowia lipolytica*. *Chem Eng Trans*, **27**, 223-8.

Makri, A., Fakas, S., Aggelis, G. 2010. Metabolic activities of biotechnological interest in *Yarrowia lipolytica* grown on glycerol in repeated batch cultures. *Bioresour Technol*, **101**, 2351- 8.

Meesters, P.A.E.P., van de Wal, H., Weusthuis, R., Eggink, G. 1996. Cultivation of the oleaginous yeast *Cryptococcus curvatus* in a new reactor with improved mixing and mass transfer characteristics. *Biotechnol Technol*, **10**, 277-82.

Metz, J.G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M. 2001. Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science, **293**, 290-3.

Nurminen, T., Konttinin, K., Suomalainen, H. 1975. Neutral lipids in the cells and cell envelope fractions of aerobic bakers yeast and anaerobic brewers yeast. *Chem Phys Lipids*, **14**, 15-32.

Papanikolaou, S., Chevalot, I., Galiotou-Panayotou, M., Komaitis. M., Marc, I., Aggelis, G. 2007. Industrial derivative of tallow: a promising renewable substrate for microbial lipid, single-cell protein and lipase production by *Yarrowia lipolytica*. *Electron J Biotechnol,* **10**, DOI: 10.2225/vol10-issue3-fulltext-8

Papanikolaou, S., Aggelis, G. 2003. Modelling aspects of the biotechnological valorization of raw glycerol: production of citric acid by *Yarrowia lipolytica* and 1,3-propanediol by *Clostridium butyricum*. *J Chem Technol Biotechnol,* **78**, 542-7.

Pamboukian, C.R.D., Facciotti, M.C.R. 2005. Rheological and morphological characterization of *Streptomyces olindensis* growing in batch and fed-batch fermentations. *Braz J Chem Eng*, **22**, 31-40.

Petersen, N., Stocks, S., Gernaey, K. 2008. Multivariable models for prediction of rheological characteristics of filamentous fermentation broth from size distribution. *Biotechnol Bioeng*, **100**, 61-71.

Ratledge, C, Wynn, J.P. 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv Appl Microbio,* **51**, 1-51.

Rane, K.D., Sims, K.A. 1994. Oxygen uptake and citric acid production by *Candida lipolytica* Y 1095. *Biotechnol Bioeng*, **43**, 131-7.

Rogers, P.J., Stewart, P.R. 1974. Mitochondrial protein synthesis in aerobic and micro-aerobic continuous cultures of *Candida parapsilosis*. *Arch Microbiol*, **99**, 25-46.

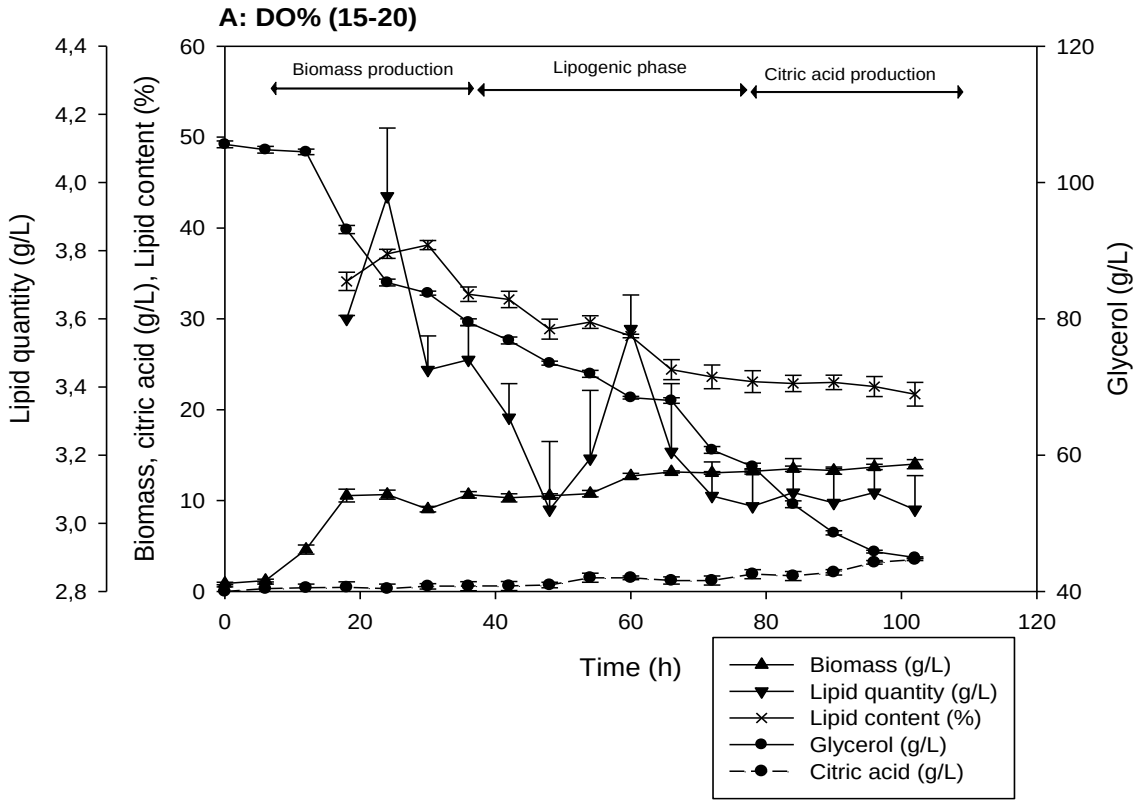
Saenga, C., Cheirsilp, B., Suksaroge, T.T., Bourtoom, T. 2010. Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochem*, **46**, 210-218.

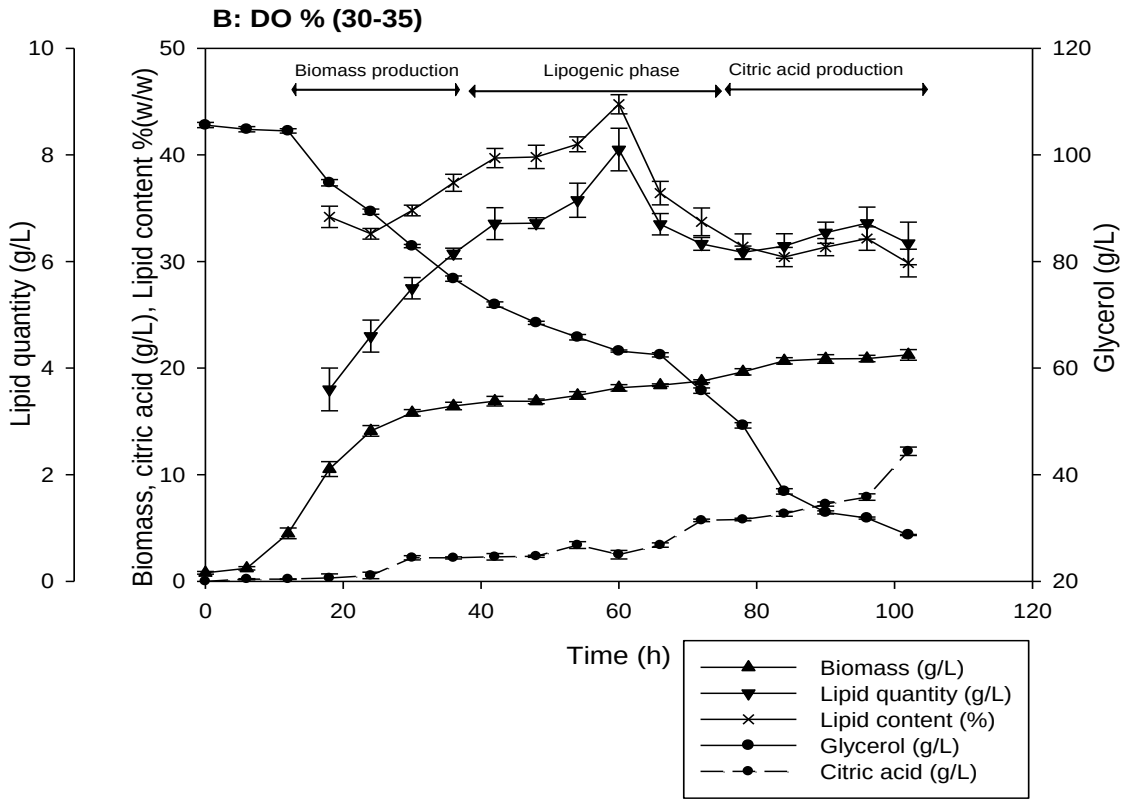
Trepat, X., Deng, L., An, S, Navajas, D., Tschumperlin, D., Gerthoffer, W. 2007. Universal physical responses to stretch in the living cell. Nature, **447**, 592-6.

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y., Valéro, J.R. 2006. Dissolved oxygen as principal parameter for conidia production of biocontrol fungi *Trichoderma viride* in non-Newtonian wastewater. *J Ind Microbiol Biotechnol*, **33**, 941-52

Yen, H.W., Zhang, Z., 2011. Effects of dissolved oxygen level on cell growth and total lipid accumulation in the cultivation of *Rhodotorula glutinis*. *J Biosci Bioeng*, **112**, 71-4.

Zokaei-Kadijani, S., Safdari, J., Mousavian, M.A., Rashidi, A. 2013. Study of oxygen mass transfer coefficient and oxygen uptake rate in a stirred tank reactor for uranium ore bioleaching. *Ann Nuc Energy*, **53**, 280-7


A: DO% (15-20)
Biomass production
Lipogenic phase
Citric acid production
Lipid quantity (g/L)
Biomass, citric acid (g/L), Lipid content (%)
Glycerol (g/L)
Time (h)
Biomass (g/L)
Lipid quantity (g/L)
Lipid content (%)
Glycerol (g/L)
Citric acid (g/L)


B: DO % (30-35)
Biomass production
Lipogenic phase
Citric acid production
Lipid quantity (g/L)
Biomass, citric acid (g/L), Lipid content %(w/w)
Glycerol (g/L)
Time (h)
Biomass (g/L)
Lipid quantity (g/L)
Lipid content (%)
Glycerol (g/L)
Citric acid (g/L)

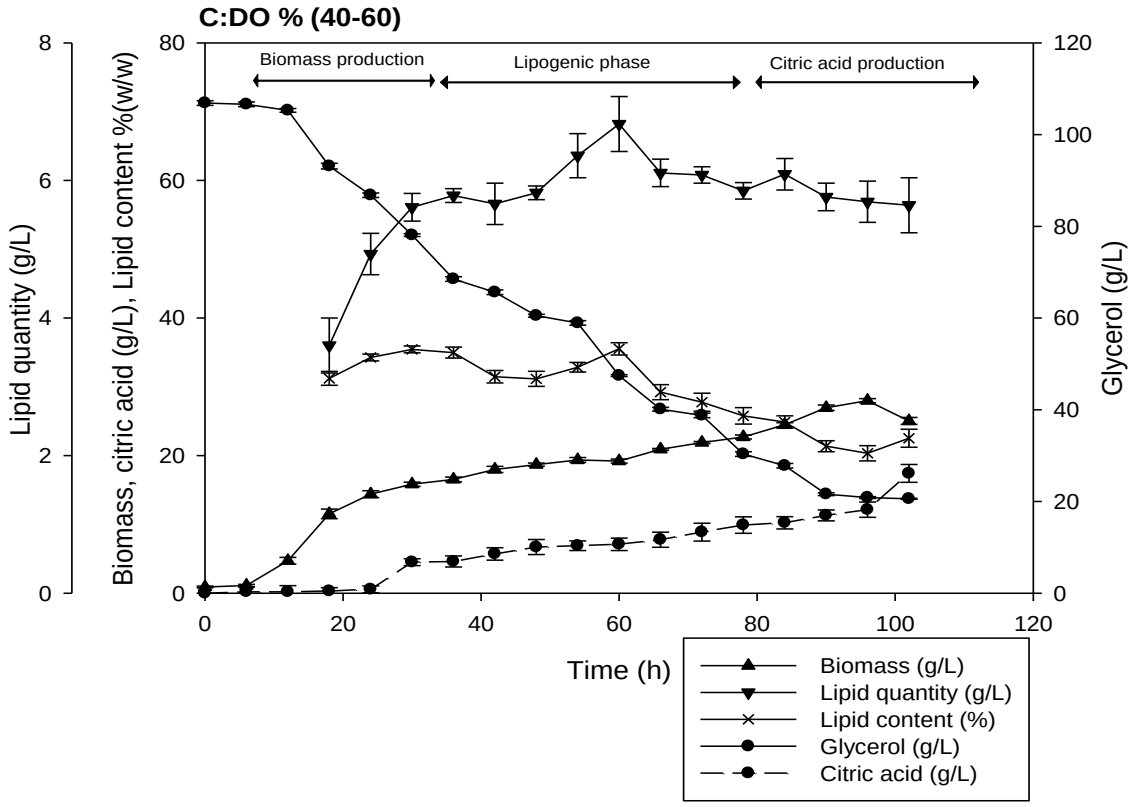


Figure 4.1 **Evolution of biomass concentration, lipid concentration, citric acid, glycerol consumption (g/L) and lipid content% (w/w), under different oxygen concentrations (Culture conditions: incubation temperature = 28 ± 0.5ºC, initial pH = 6.5 ± 0.2, crude glycerol = 100 g/L**

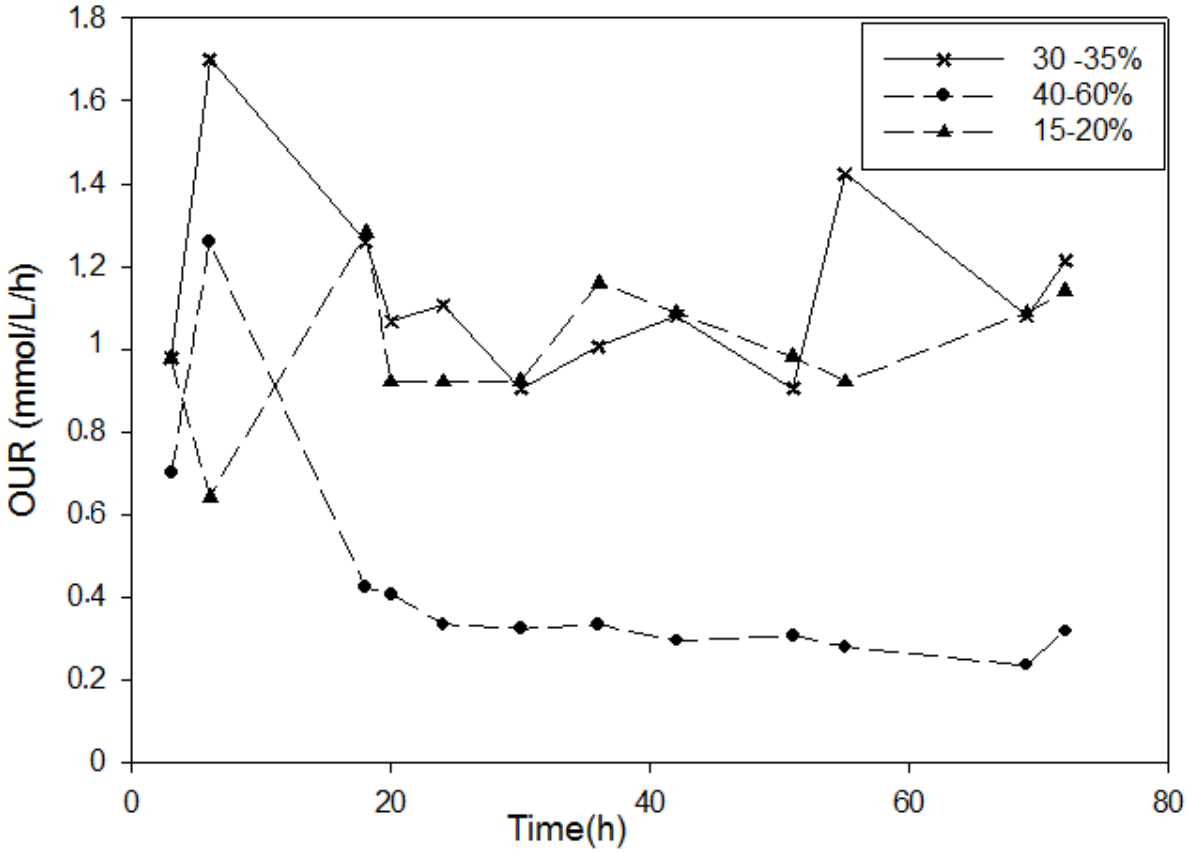
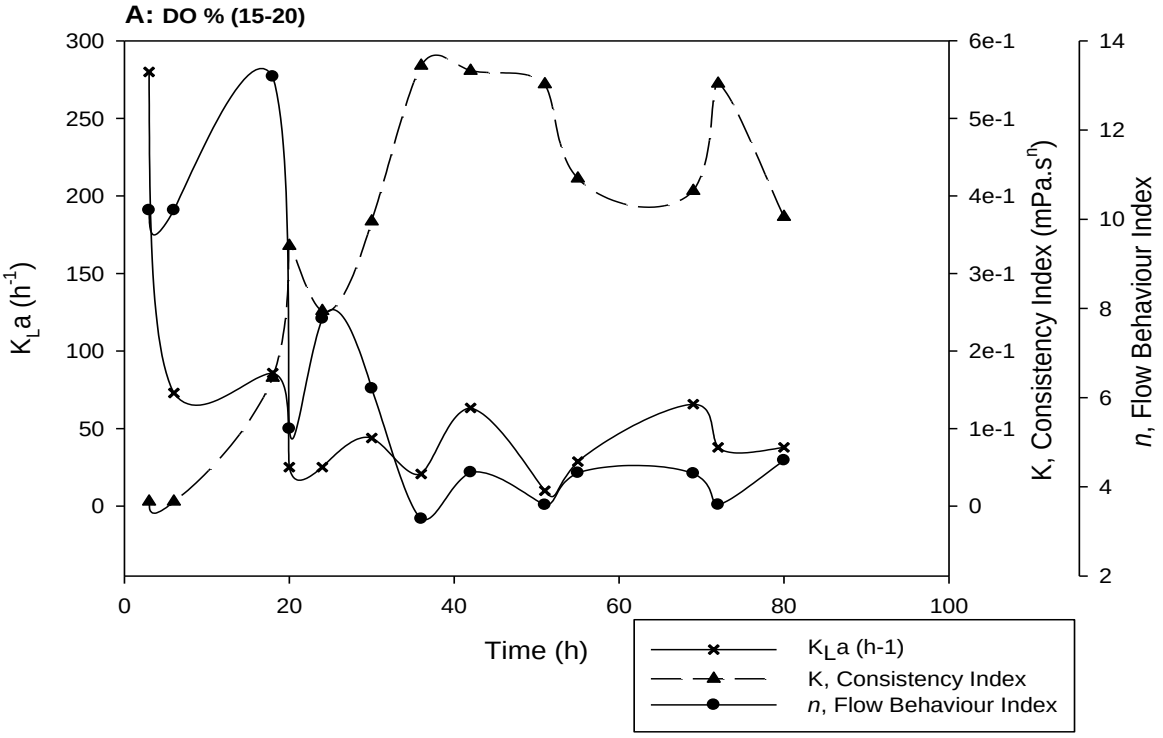
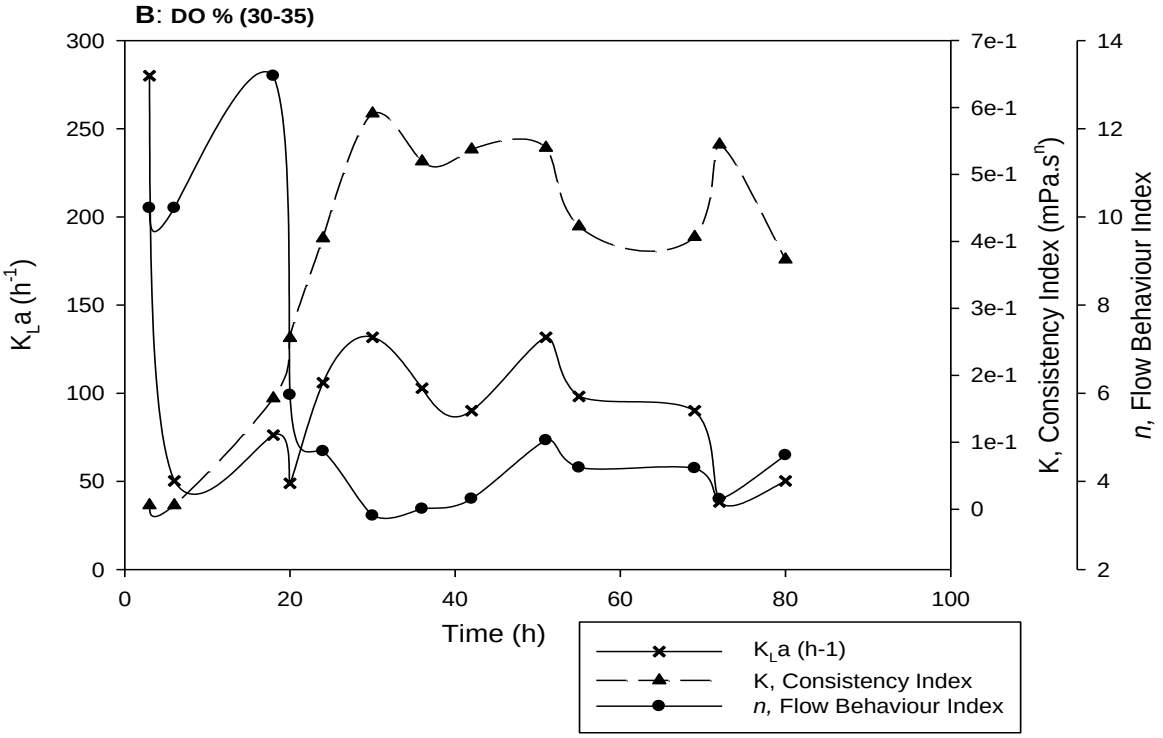


Figure 4.2 **Oxygen uptake rate (OUR) profile of *Y. lipolytica* in crude glycerol at different oxygenation levels (Culture conditions: incubation temperature = 28 ± 0.5°C, initial pH = 6.5 ± 0.2, crude glycerol = 100 g/L)**


A: DO % (15-20)
300
250
200
150
100
50
0
$K_La\ (h^{-1})$
6e-1
5e-1
4e-1
3e-1
2e-1
1e-1
0
K, Consistency Index (mPa.s^n)
14
12
10
8
6
4
2
n, Flow Behaviour Index
0
20
40
60
80
100
Time (h)
K_La (h-1)
K, Consistency Index
n, Flow Behaviour Index


B: DO % (30-35)
300
250
200
150
100
50
0
$K_La\ (h^{-1})$
7e-1
6e-1
5e-1
4e-1
3e-1
2e-1
1e-1
0
K, Consistency Index (mPa.s^n)
14
12
10
8
6
4
2
n, Flow Behaviour Index
0
20
40
60
80
100
Time (h)
K_La (h-1)
K, Consistency Index
n, Flow Behaviour Index

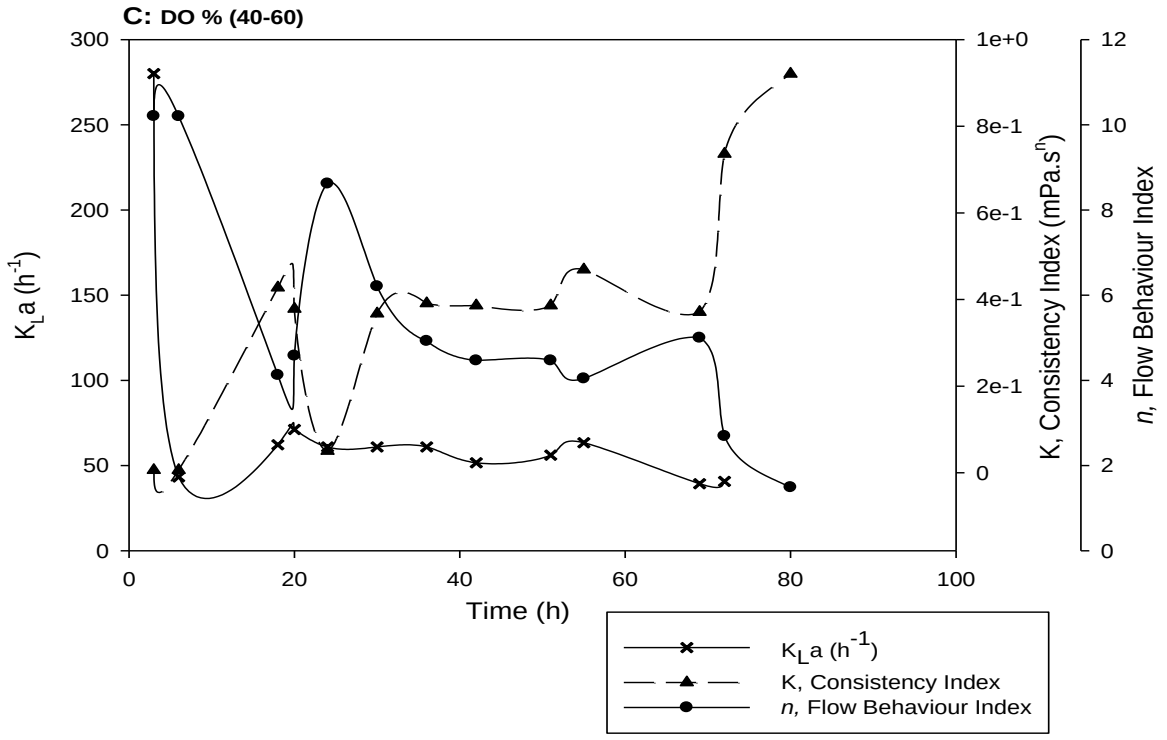
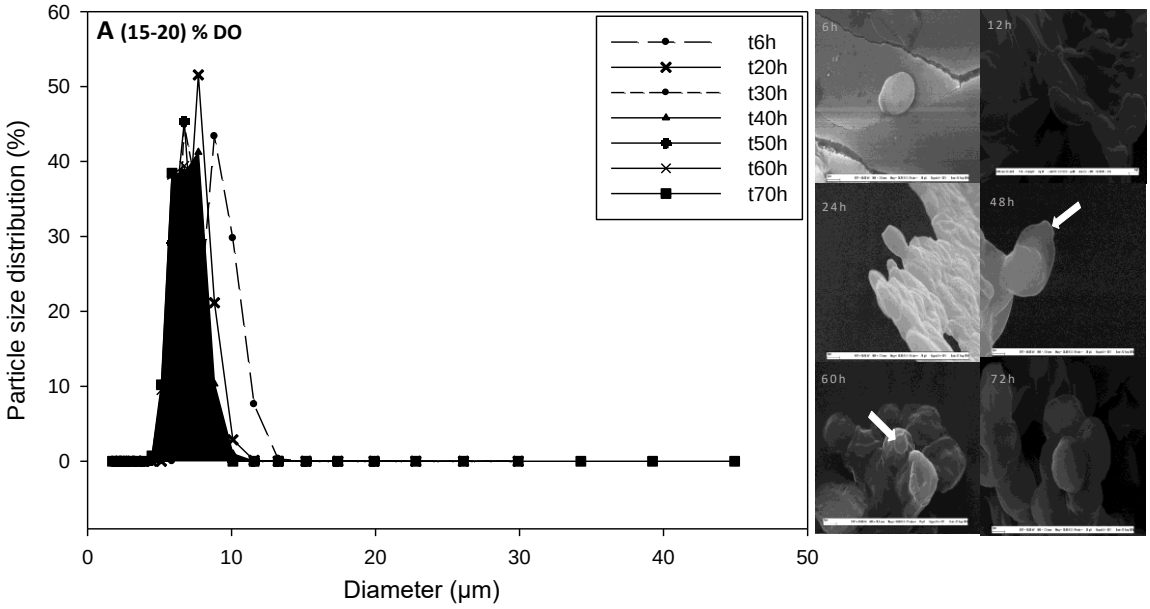
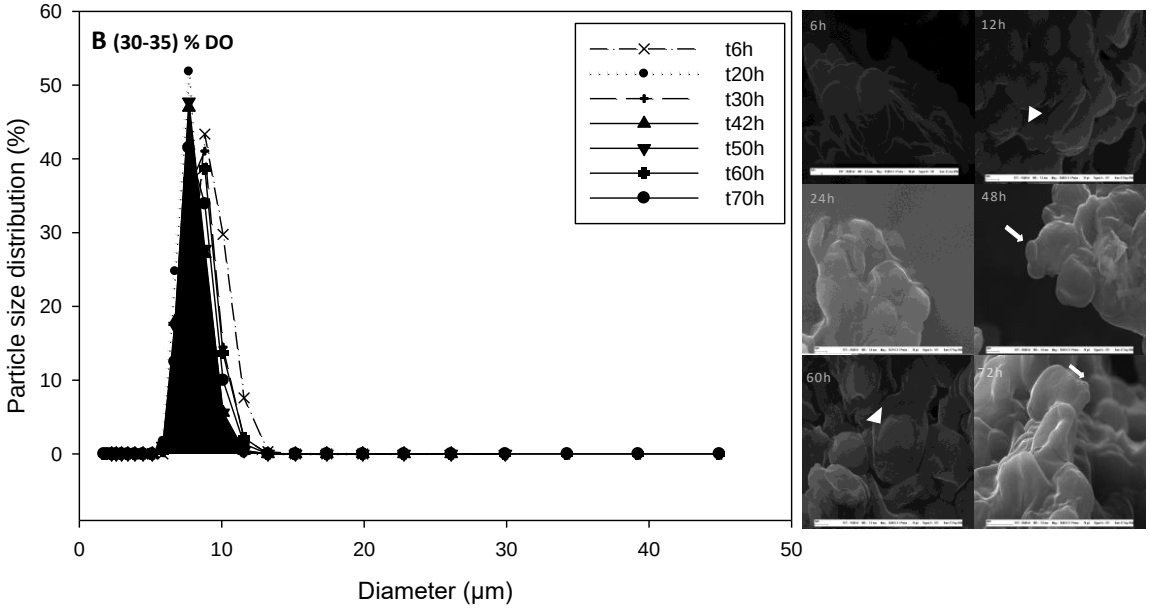


Figure 4.3 **Rheological profile of *Y. lipolytica* at different k_La (Culture conditions: incubation temperature = 28 ± 0.5ºC, initial pH = 6.5 ± 0.2, crude glycerol = 100 g/L)**


A (15-20) % DO
Particle size distribution (%)
Diameter (µm)
t6h
t20h
t30h
t40h
t50h
t60h
t70h
6h
12h
24h
48h
60h
72h


B (30-35) % DO
Particle size distribution (%)
Diameter (µm)
t6h
t20h
t30h
t42h
t50h
t60h
t70h
6h
12h
24h
48h
60h
72h

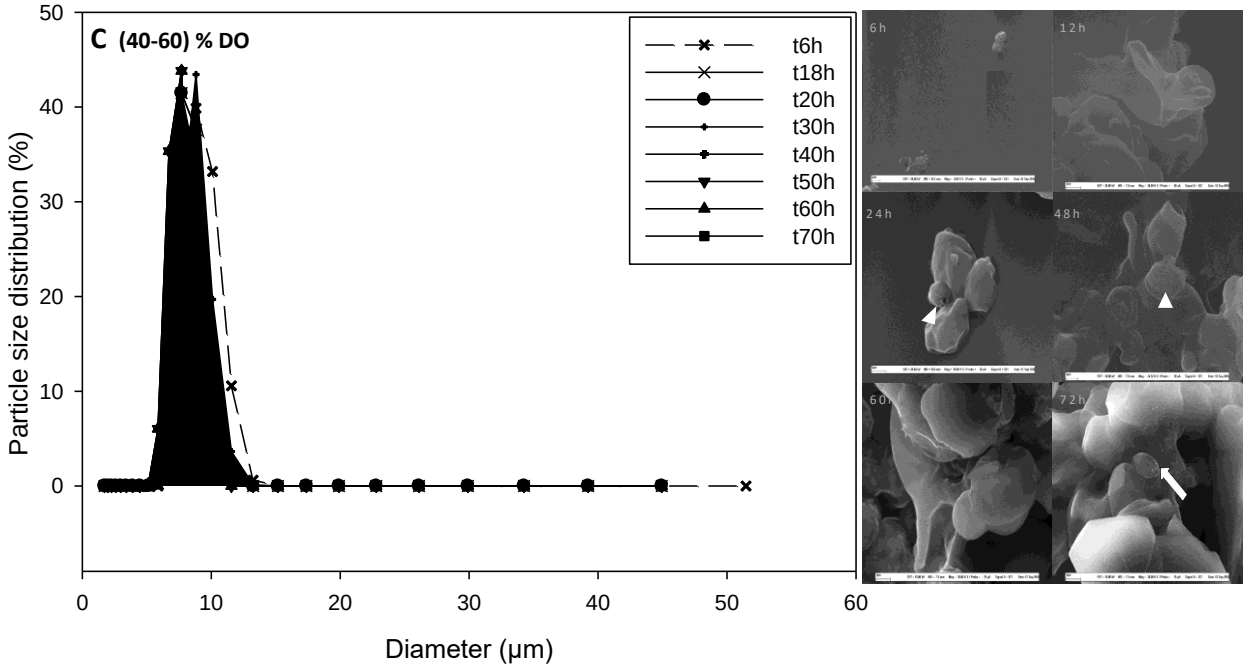


Figure 4.4 **Lipid accumulation of *Y. lipolytica* batch culture under different oxygenation levels. Left, Particle size distribution of fermentation broth. Right, SEM analysis (Arrowhead denotes typical bud scarring, and arrows show areas of lumpy cell wall charact of cells. Scale bar: 1 µm)**

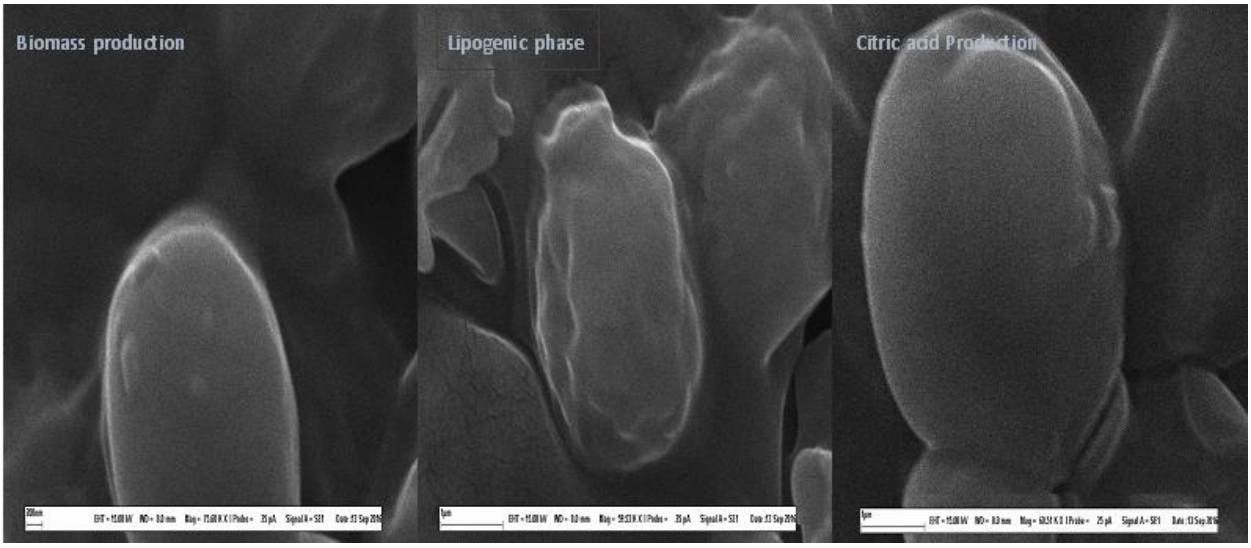


Figure 4.5 **Surface cell morphology of Y. lipolytica cells (SEM analysis) during cell growth (scale bar 1 µm)**

Tableau 4.1 **Fatty acid composition (%) of *Y. lipolytica* cultivated in bench scale fermenter culture conditions: as in Figure 4.1**

Fatty acids (%)/ Conditions	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2
15-20%	12.60	0.97	11.20	48.20	13.44
30-35%	14.60	0.54	10.05	44.20	17.80
40-60%	17.30	0.30	7.58	40.12	20.56

5 CHAPITRE 5: BIO-MODULATOR TRIGGERS AND CITRIC ACID TO IMPROVE LIPID PRODUCTION BY YARROWIA LIPOLYTICA ON CRUDE GLYCEROL BASED MEDIA

Bio-modulator triggers and citric acid to improve lipid production by *Yarrowia lipolytica* on crude glycerol based media

Magdouli S.[a], S.K. Brar[b] and J.F. Blais[c]*

[a] Ph.D. student, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-4677, Fax: (418) 654-2600, email: magdouli sara.@ete.inrs.ca

[b] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-3116, Fax: (418) 654-2600, email: satinder.brar@ete.inrs.ca

[c] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-2575, Fax: (418) 654-2600, email: jean-francois.blais@ete.inrs.ca

* Corresponding author

Email: jean-francois.blais@ete.inrs.ca

5.1 Résumé

L'utilisation de glycérol brut comme source de carbone pour produire des huiles d'organismes unicellulaires (HOU) constitue une stratégie prometteuse pour valoriser les sous-produits de la production de biodiesel et rendre le processus plus rentable. Ce travail vise à améliorer la capacité de *Yarrowia lipolytica* à produire de grandes quantités de lipides et à remplacer l'approche de génie génétique par une approche métabolique basée sur la stimulation des enzymes afin d'augmenter le taux de synthèse des acides gras. Les effets d'un ajout de biotine et de leucine sur la teneur en lipides de *Y. lipolytica* ont été étudiés. La teneur en lipides de *Y. lipolytica* était fortement influencée par l'ajout de biotine, une augmentation de la concentration en biotine de 25 à 200 µg/L a permis d'augmenter la quantité de lipide à 15 g/L. Par ailleurs, pour canaliser le flux métabolique dans la biosynthèse des lipides, un ajout d'acide citrique en tant que précurseur de lipides a conduit à une augmentation de l'activation du catabolisme total et de l'accumulation des lipides pour atteindre environ 63% L'approche biochimique peut être une voie intéressante pour améliorer l'efficacité de la production de lipides plutôt que le génie génétique.

Mots clés : *Yarrowia,* biomodulateurs, biotine, leucine, acide citrique, accumulation de lipides

5.2 Abstract

The use of biodiesel-derived glycerol as a carbon source for single cell oil (SCO) production is a biorefinery engineering strategy that aims to valorize the by-product waste and make the microbial oil process more cost-effective. This work aimed to improve the capacity of *Yarrowia lipolytica* to produce large amounts of lipids and replace genetic engineering by a metabolic approach based on the stimulation of the rate-limiting enzymes and reducing the activation energy thereby increasing the rate of fatty acid synthesis. The effects of biotin and leucine addition on the lipid content of *Y. lipolytica* have been investigated. The lipid content of *Y. lipolytica* was strongly influenced by the addition of biotin, in fact, an increase in biotin concentration from 25 µg/L to 200 µg/L practically increased the lipid quantity up to 15 g/L. Besides, to channel metabolic flux into lipid biosynthesis, the addition of citric acid as lipid precursor led to an increase in total catabolism activation and lipid accumulation to reach around 63% (w/w). Biochemical approach can be a useful target for improving the efficiency of lipid producing yeast strain rather than genetic engineering.

Keywords: *Yarrowia,* biomodulators, biotin, leucine, citric acid, lipid accumulation.

5.3 Introduction

Owing to the high productivity, justified by the shorter duplication time and higher oil content compared to vegetables oils (up to 70% on dry weight) (Blazeck et al., 2014; Magdouli et al., 2014), it is clear that microorganisms accumulating lipids above 20% of the biomass on a dry basis represents an ideal source of lipids for biodiesel production. In fact, oleaginous microorganisms contributed efficiently for fuel oils and responded sufficiently to global needs (Knothe, 2005). Accordingly, increasing lipid accumulation by oleaginous microorganisms has attracted significant attention as single cells oils (SCO) can serve as an important source for petroleum diesel replacement. However, current productivity of microorganisms-based processes is still very low, which has restricted their scale-up application. Although the extent of lipid accumulation in oleaginous microorganisms is influenced by nutrient levels and culture conditions, still the efficient production of lipids of commercial interest ideally requires potential microorganisms with a higher level of lipids, regardless of environmental conditions. Consequently, developing a bioprocess with high production of SCO from renewable carbon source with low cost is very challenging. Lipid overproduction has been pursued through genetic modifications, but the overexpression of one or a few key genes involved in fatty acid biosynthesis produced only modest increase in the lipid content (Courchesne et al., 2009), and the resulted engineered strains have poor stability during scale up.

Magdouli et al. (2014) stated that the higher lipid production is a balance between metabolic and genetic approaches. Unlike genetic modification, metabolic approach relies on phenotypic screening and does not require specific knowledge of molecular targets in metabolic and catabolic pathways involved in synthesis of lipid droplets. In addition to various efforts in strain improvement and cultivation optimization, it was proposed that the higher lipid productivity can also be achieved using various chemicals to trigger or enhance cell growth and accumulation of SCO. Thus, various metabolic triggers or enhancers are able to directly modulate cellular metabolism and can be applied to improve the lipid productivity. Moreover, the application of defined chemicals in large-scale cultures with low concentrations and lower costs, could be a valuable and practical approach in addressing the low productivity issue. Recently, numerous studies have shown that the quantity and quality of lipids in these species can vary as a result of changes in growth conditions (temperature and pH, dissolved oxygen or nutrient media characteristics (carbon source concentration, C/N ratio, macro and micro nutrients) (Ageitos et al., 2011; Karamerou et al., 2016; Kolouchová et al., 2016). Besides, examination of enzymatic activity (Ratledge & Wynn, 2002) and comparative genomics (Vorapreeda et al., 2012) suggest that ATP: citrate lyase (ACL)

may play a crucial role in directing excess carbon to be stored as lipids rather than carbohydrates in oleaginous yeasts. Moreover, acetyl-CoA carboxylase (ACC), is reported to increase during the lipid biosynthesis (Meng et al., 2011; Ruenwai et al., 2009). ACC is a biotin-dependent enzyme that catalyzes the carboxylation of pyruvate to oxaloacetate and acetyl-CoA to malonyl-CoA. Additionally, leucine is a precursor to β-hydroxy-β- methylglutaryl CoA, an intra-mitochondrial intermediate which is a precursor of sterols and helped to the generation of acetoacetate and acetyl CoA. Their mechanism is presented in Figure 5.1.

The activity of this enzyme is reduced when yeast grows on media lacking or deficient in biotin. The decreased activity of acetyl CoA carboxylase has been reported to lead to reduced lipid content due to biotin deficiency (Bunn et al., 1970; Desai et al., 1983). Recently, the highest lipid content of 39% was produced by the culture of *Metschnikowia pulcherrima* without yeast extract but with a supplementation of biotin and nitrogen (Santamauro et al., 2014). Besides, Yu et al. (2015) have illustrated many chemicals to enhance microalgal growth and accumulation of high-value bioproducts and Wahbi et al. (2014) have patented many types of bio-modulators to increase biofuel production in microalgae.

In this regard, this work aimed to increase the lipid content in yeast *Yarrowia lipolytica* as a result of the biomodulators addition. Accordingly, citric acid was defined as a key precursor in lipid accumulation and during the metabolic shift between growth and lipid production, a transient citrate excretion step occurred (Boulton & Ratledge, 1980). More often, CA has been reviewed to activate allosterically ACC enzyme (Gill et al., 1977). Moreover, the metabolism of CA and lipid accumulation are competitive and both phenomena required a nitrogen limitation of 1.4 g/L (Cescut, 2009). However, many points arise as to how oleaginous microorganisms could coordinate both steps and metabolic shift from oxidative to citric acid production over lipid accumulation still need to be elucidated. Hence, the idea was to direct the carbon dissipation from citric acid production towards lipid accumulation. Obviously blocking the activities of tricarboxylic-acid and glyoxylate cycle enzymes and transport processes of CA through the mitochondrial (Holz et al., 2009) presented an advantage, however, this approach remains complex and costly. Thus, a feed batch strategy is aimed principally to block the CA production (feed-back inhibition) and strengthen the effect of biomodulators.

5.4 Materials and methods

5.4.1 Strain

The yeast *Y. lipolytica*, isolated from the woody forest and selected as an oleaginous yeast in previous studies (Magdouli et al., 2016b), was maintained in stock cultures on agar slants at 4°C containing (g/L): 20 glucose, 5 yeast extract, 10 malt extract, and 20 agar, pH = 6.5. This strain was used in the current study and its higher potential to accumulate SCO was investigated.

5.4.2 Inoculum preparation

The yeast stored at 4 °C was reactivated on potato dextrose agar petri dishes. Subsequently, a sloop of reactivated microbial was transferred to Erlenmeyer flasks containing the growth medium (5% inoculum). The culture medium used for inoculum was composed of (g/L): Glucose 20, peptone 20 and yeast extract 10 at pH = 6.5. Experiments were conducted in mineral medium composed of: crude glycerol, 0.54 g/L NH_4OH,1 g/L yeast extract, 3 g/L K_2HPO_4, 3 g/L $NaH_2PO_4.H_2O$, 0.5 g/L $MgSO_4.7H_2O$, 0.040 g/L $ZnSO_4.7H_2O$, 0.016 g/L $FeSO_4.7H_2O$, 0.25 µg/L biotin, pH = 6.5, maintained at 28°C in flasks agitated at 180 rpm for 72 h.

To evaluate the effect of modulators at large scale, fermentation was conducted in 5L fermenter (Biostat B plus, Sartorius) to improve lipid production in crude glycerol based media. pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4 and 7 (VWR, Canada). Before sterilization cycle, the oxygen probe was calibrated to zero (using sodium thiosulfate water) and 100% (air saturated water). Propylene glycol (Sigma-Canada) was used as an anti-foam agent. The fermenter with the medium was then sterilized in situ at 121°C for 20 min. After the fermenter cooled down to 28°C, DO probe was recalibrated to zero and 100% saturation by sparging N_2 gas and air, respectively, at agitation rate of 250 rpm. The pH of the fermenter solution was adjusted to 6.5 with 4N H_2SO_4. Thereafter, sterilized crude glycerol (83% w/v) and mineral solution was transferred to the fermenter as carbon source under aseptic conditions. Agitation at 250 rpm was carried out to mix the solution, after mixing, pre-culture of *Y. lipolytica* was added to the fermenter. During fermentation, air flow rate was kept constant 2.5 L/min. Agitation rate was varied during fermentation in order to keep the DO above 30% saturation.

5.4.3 Biomass determination

Biomass was determined gravimetrically. Samples were centrifuged at 5,000 x *g* and the cells were washed twice to remove residual glycerol. Remaining pellets were frozen and lyophilized (VirTis Lyophilizer, USA) for 36 h. Cell dry weight was calculated by subtracting the weight of the biomass before and after lyophilisation.

5.4.4 Glycerol consumption

For the measurement of glycerol and others organics acids in the broth, LC/MS/MS technique was employed. The technical details of the LC/ MS/MS instrument used for the analysis were: (a) for sugar estimation: Thermo TSQ Quantum model, equipped with an Electrospray Ionization (ESI) in negative ion mode; Zorbax Carbohydrate (4.6 mm x 150 mm x 5 mm, Agilent) analytical column; 75% acetonitrile; 0.1% NH_4OH; 25% water and 0.1% NH4OH mobile phase and 10 mL injection volume. Glycerol and citric acid (all from Sigma) was used as the internal standards.

5.4.5 Determination of lipids and the fatty acid profile (FAME)

Lipids from previously lyophilized biomass were extracted according to the method of Bligh and Dyer (1959), with modifications. Resulting biomass was vortexed with methanol: chloroform for extraction of lipids, followed by centrifugation at 785 x *g* for 10 min for phase separation; the lower phase contained the lipids dissolved in chloroform was evaporated and dried at 60^{o}C to measure the dry lipid mass. The extracted lipid fraction was esterified to obtain the fatty acid methyl esters. For fatty acid, analysis was performed essentially as described earlier (Magdouli et al., 2016a). FAMEs were subsequently analyzed by GC–MS (Perkin Elmer, Clarus 500). The dimensions of the column used were 30 m x 0.25 mm, with a phase thickness of 0.25 µm. A 37-component FAME mix (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA) was used for qualitative analysis. 1,3-dichlorobenzene was used as an internal standard to quantify the fatty acid methyl esters. The results are expressed as mean ± standard deviation. The results were compared by variance analysis (ANOVA) at 5% significance using Excel's Analysis ToolPak.

5.5 Results and discussion

5.5.1 Effect of C/N ratio on cell growth and lipid accumulation in *Yarrowia lipolytica*

Recent studies have showed that *Y. lipolytica,* a potentially attractive organism, can grow at higher C/N ratio with higher glycerol concentrations (Magdouli et al., 2016b), this fact seems to be strain dependent and many strains belonging to *Yarrowia* species can tolerate up to 200 g/L and can achieve efficient cell growth ranging from 16.5–26.5 g/L of glycerol (Rymowicz et al., 2006). The selected strain used in this study can accumulate up 52% in the presence of crude glycerol of 89 g/L and C/N = 75 at 66 h and further increase of C/N will not increase the lipid content as well as biomass Figure 5.1.

The upper limit of lipid content in *Yarrowia lipolytica* SM7 is 47.73% (w/w) in shake flasks at C/N = 75 and higher C/N ratio will lower the biomass content and the lipid content, for example for higher C/N = 200, lipid content and biomass decreased and reached around 7.37 g/L and 24.35% (w/w). Meanwhile, at C/N = 120, 9.54 g/L of dry biomass and a lipid content of 43.19% (w/w) was obtained. As shown earlier, higher C/N ratio above 200 will not be suitable to achieve the higher lipid content and the metabolism of the strain seemed to be inhibited by higher glycerol concentration. These observations were in agreement with Karamerou et al. (2016), who found that a glycerol concentration of 80 g/L and C/N = 182 resulted in lower biomass 4.62 g/L and a lipid content of 17.4% (w/w) in *Rhodotula glutinis* (Karamerou et al., 2016). Consequently, to trigger lipid accumulation, the C/N ratio should be adjusted according to the requirements of cell. Thus, the amount of carbon in the medium must be higher than that required for growth and maintenance of the yeast so that there is surplus for lipids synthesis. However, this surplus cannot exceed since high glycerol concentration up to 150 g/L represses the growth and glycerol at 100 g/L will lead to lower consumption efficiency and more than 50% of glycerol will be left unconsumed (Figure 5.2).

Hence, other metabolic alternatives to increase the lipid productivity were suggested. Thus, the increase in oil production involves the optimization of the two parameters: growth (biomass) and lipid content. For this purpose, improvement of lipid productivity can be achieved by changing physicochemical parameters, such as temperature, pH and nutrient composition of the growth medium since many of these parameters are implicated during lipogenesis. However, these methods are impractical for large scale production. As industrial alternative, the experiments can be divided into two phases to increase the lipid productivity: first phase to stimulate the growth of

strain, and therefore biomass (higher oxygen supply 60%), followed by a second phase of lipid accumulation by applying stress (lower oxygenation 30%) or/and some modulators. For example, the application of biological modulators to promote the growth of yeast followed by nitrogen deficiency phase to stimulate the production of lipids. The choice of the type of stress and its applicability to the efficacy of the process is closely related to the requirements of yeast (i.e. enzymatic system implicated during the synthesis and the accumulation of lipid (Magdouli et al., 2014). The combination of such modulators with bio-osmotic stress by lowering oxygen availability at the end of the active growth phase, would significantly increase the productivity of lipid content.

5.5.2 Effect of modulators to enhance lipid accumulation

The biotin trigger has been tested and optimized on various species, including chlorophytes (Karampudi & Chowdhury, 2011; Ngangkham et al., 2012) and some of baker's yeasts (Desai et al., 1983) and filamentous fungi of *Claviceps* species (Oura & Suomalainen, 1978). In the latter case, the production was principally aimed at biological production of alkaloids. However, Desai et al. (1983) observed a concomitant production of alkaloids and lipids after supplying biotin to the culture. The present study aimed to evaluate how lower concentrations of bio-modulators can induce TAG accumulation and their lower concentrations at an early stage of lipid accumulation associated with a dissolved oxygen shift could enhance efficiently the growth and the lipid accumulation scenario in *Y. lipolytica*.

The supplementation of cultures with low doses of biotin (50, 100 and 200 µg/L) at an early and late stage of lipid accumulation (16 h and 60 h), was evaluated. Briefly, batch cultures of *Y. lipolytica* were grown in crude glycerol medium until just prior to nitrogen depletion 16 h. As expected based on our previous work (Magdouli et al., 2016b), the cultures that did not receive any biotin had maximum of growth rate until 12 h of 0.15 h^{-1}, and biomass tend to increase until reaching stationary growth. The original medium supplemented with 25 µg/L of biotin showed a lipid content of 52.6 ± 1.3% (w/w) with maximum lipid quantity of 13.5 ± 0.5 g/L at 66 h and biomass 25.1 ± 0.4 g/L. The effect of biotin addition on different growth stages is presented in Table 5.1. The biotin addition at an early stage showed improvement in lipid quantities. However, the biomass did not increase and remained almost stagnant 26 g/L. Even though cell dry weight did not increase as much in biotin supplemented media, lipid contents in all biotin supplemented cultures increased. Thus, the addition of 50 µg/L of biotin increased the lipid content up to 14.0 ± 0.3 g/L, and the increase of biotin concentration led to around 15.5 and 15.0 ± 0.5 g/L in the presence of biotin concentration 100 and 200 µg /L respectively. In the later stage of

accumulation at 60 h, the addition of biotin did not improve the lipid quantity and maximum value was obtained in the case of 100 µg/L, where only 13.8 ± 0.4 g/L was reported. Thus, the biotin factor acted preferentially at early stage and the active phase of lipid accumulation was between 16 and 60 h. The action of biotin could be also attributed to effect on intermediary metabolism, correlating with the activity of fatty acid synthases and acetyl-CoA carboxylases, besides, cells require more time for biotin uptake since the entry of biotin into cells is performed by passive diffusion (Piffeteau & Gaudry, 1985). Likewise, microbial transport systems of biotin are dependent upon, or stimulated by, the presence of an energy source carbon source which is in the present case glycerol and only 9.2 ± 0.3 g/L are available in the culture medium at 60 h. The growth seemed to be less affected by biotin addition which was probably due that biotin acts preferentially during lipogenic phase and if the biotin is required for growth and cell wall synthesis, the strain will use the exogenous biotin supplied in the medium , otherwise, it will synthetize biotin according to its needs.

The analysis of glycerol consumption indicated no statistical difference between the control treatments ($Y_{X/S}$ = 0.32 g/g, and those that received biotin addition ($Y'_{X/S}$ = 0.32 g/g). This was due to the delay or arresting of the cellular division, and this phenomenon is assumed to be the result of a fundamental metabolic shift from growth metabolism to lipid accumulation metabolism after nitrogen depletion.

The addition of up to 200 µg/L biotin did not have a negative effect on the lipid accumulation activity of *Yarrowia*. Here, the addition of biotin resulted in a single fold increase of lipid yield over the biotin-free control, which suggested that biotin can enhance fatty acid synthesis in agreement with nitrogen depletion and oxygen limitation (concentration of dissolved oxygen DO = 30% of saturation).

The results provided evidence that the timely addition of biotin, when coupled with nitrogen depletion, can induce significant lipid accumulation. Therefore, the effect of the biotin on lipid production is explained by the stimulation of genes involved in lipid production pathway, such as ACC1 genes (Barber et al., 2005). Generally, biotin participates in carboxylase reactions and various enzymes are activated in the presence of biotin, such as ACC and pyruvate carboxylase which contained biotin. Their mechanism is clarified by the work of the Lynen group (Lynen et al., 1959; Lynen et al., 1961). It was obvious that the only role of biotin in organisms is to function as the prosthetic group of various carboxylases. Meanwhile, the only biotin-enzymes functioning in yeast growing in a synthetic medium containing sugar and ammonium salt are pyruvate

carboxylase and acetyl-CoA carboxylase catalyzing the carboxylation of pyruvate to oxaloacetate and acetyl-CoA to malonyl-CoA.

More often, it was hypothesized that higher lipid productivity might be achieved by adding lower concentrations of leucine near nitrogen depletion. In fact, a recent study revealed that leucine auxotrophy caused a 2.5-fold decrease in cell fatty acid content and that *leuA* gene expression restored its level in *M. circinelloides* strain R7B R7B (Rodríguez-Frómeta et al., 2012). Moreover, acetyl-CoA generated from the endogenous leucine metabolic pathway was postulated to be another rate-limiting step during fatty acid synthesis in *M. circinelloides* (R7B (Rodríguez-Frómeta et al., 2012). Interestingly, Kamisaka et al. (2007) have elucidated a correlation between leucine biosynthesis and oleaginicity. Thus, in that work, the complementation of leucine in *Saccharomyces cerevisiae* increased lipid accumulation in comparison with auxotrophic strains.

In this regard, in an attempt to increase biomass production prior to inducing lipid accumulation, culture was also supplemented with leucine (60 µg/mL) at 16 h, just prior to nitrogen depletion, to induce TAG accumulation. Results are shown in Table 5.2.

As indicated earlier, the leucine enhanced the lipid accumulation and lipid content reached around 14.67 g/L which is better than the control experiment, besides, biotin and leucine, supplemented together enhanced lipid synthesis and the positive effect of leucine was verified. This suggests that leucine metabolic pathway is participating in acetyl coenzyme A (acetyl-CoA) generation which may be critical during fatty acid synthesis (Kohlhaw, 2003; Rodríguez-Frómeta et al., 2012) in the selected strain. The key two-carbon metabolite for lipid biosynthesis, acetyl-CoA, can be produced via degradation of branched chain amino acids, e.g, leucine, in addition to other metabolic pathways presented in Figure 5.1. Interestingly, a recent comparative genome analysis of non-oleaginous and oleaginous species identified a theoretical pathway for leucine degradation that was specific to oleaginous strains and might provide acetyl-CoA for lipid biosynthesis (Vorapreeda et al., 2012). Besides, a higher concentration of leucine up to 120 µg/mL showed a decrease of the lipid quantity only 12.7 ± 0.3 g/L, and the biomass increased comparatively to control experiment up to 30 g/L, this fact may be due to that higher concentration of leucine concentration can induce leucine biosynthesis and block leucine biodegradation and the strain will use the leucine for growth and protein synthesis and not to synthetize lipid.

This may provide a proof that operating leucine metabolic pathway is required for the accumulation of lipids, suggesting that if the strain is endogenously producing leucine, this latter is degraded for the generation of the corresponding acetyl-CoA, which is incorporated into fatty acid biosynthesis, more often, the strain may present smaller amount of leucine coming mainly

from its biosynthesis which is used for lipid synthesis. This fact go in line with the latter observation in which, the selected strain synthetize biotin during the fatty acid biosynthesis as well as leucine which are both indispensable for lipid synthesis and their overproduction via supplementation may have a crucial role in the present study. As shown in Table 5.2, leucine and biotin act in synergy and up to 16.78 g/L was obtained. Thus, the biotin activate the methylcrotonyl-CoA carboxylase involved in leucine catabolic pathway named as branched chain catabolism (BCC) which provide more acetyl-CoA as shown in Figure 5.1.

Consequently, next carbon source, the most prerequisite elements for lipid production are biomodulators, which play pivotal role during lipid synthesis.

5.5.3 Effect of citric acid production to enhance lipid production in Y. lipolytica

The effect of biotin on lipid production is explained by the stimulation of genes involved in lipid production pathway, such as ACC1 genes. Moreover, the addition of citrate can activate allosterically this enzyme (Gill et al., 1977). Herein, the nitrogen depletion occurred at 16 h started and the lipid starts with a percentage. Nitrogen concentration of 90 mg/L that was kept constant along the fermentation. In fact, the nitrogen exhaustion activates AMP deaminase which is responsible for the decrease of AMP concentration and increases cellular ammonium concentration (Ratledge, 2002). The decrease in AMP concentration inhibits isocitrate dehydrogenase, blocking the citric acid cycle at the isocitrate level. Aconitase mediates the accumulation of citrate in mitrochondria, with exit from the mitochondria mediated by the citrate/malate cycle (Botham & Ratledge, 1979; Ratledge, 2002). This reaction provides large amounts of acetyl-CoA for fatty acid synthesis. Thus, acetyl-CoA is provided by the cleavage of citrate coming from the mitochondria by ATP-citrate lyase (ACL) in the cytosol. Later, ACL cleaves the citrate to give oxaloacetate and acetyl-CoA. Both CA production and lipid accumulation are competitive and they are induced by lower nitrogen concentration (Beopoulos et al., 2009; Cescut, 2009). Figure 5.3 showed the kinetic growth of *Y. lipolytica* in the optimized process (e.g. 89 g/L of crude glycerol and C/N = 75), *Y. lipolytica* produced low concentrations of CA 7.8 ± 0.7 g/L and this concentration seemed to be dependent on glycerol concentration. Thus, the variation of glycerol concentration from 75 to 100 g/L, increased the citric acid production around from 2.5 to 14 g/L (Magdouli et al., 2016b). The increase of CA concentration in cells at higher glycerol concentration contributed to low levels of lipid accumulation 12.81 g/L at 72 h within cells which justify that both phenomena are competitive and more knowledge is required to understand this competitiveness. Likewise, Papanikolaou et al. (2002), assumed that ATP-citrate lyase was

inactive in the presence of excess carbon, resulting in low levels of lipid accumulation (Moeller & Strehlitz, 2007; Papanikolaou et al., 2006; Papanikolaou et al., 2002).

Hence, the idea herein was to inhibit the production of CA by generating a feed-back effect which comprises the supply of the culture with citric acid, so that the cells will not produce CA while it available in the media to support the biotin and leucine effect as reviewed earlier. For this purpose, addition of 30 g/L of CA to the culture was investigated, the time was chosen according to the quantities detected in the culture (0.50 g/L) of CA at 36 h. Thus, the feed of the current solution with citric acid at lipogenic phase was assumed to repress the CA metabolism towards lipid accumulation and provides more acetyl Co during lipogenic phase as well as reinforcing the biotin effect. Hence, a higher C/N ratio of 75, combined with a buffered pH, lower oxygenation rate at 30% of dissolved oxygen and higher biotin supplement will have significant effect ($p < 0.05$) on lipid accumulation. The p-values of the models were 0.0002 indicating that the models were significant. Usually, a model term is considered to be significant when its value of ‘‘*p*-value’’ is less than 0.05.

In the control experiment, the final biomass reached around 25.10 g/L. This concentration increased sharply to 28.7 ± 0.5 g/L at 72 h when the culture is supplemented by citric acid and biomodulators. Figure 5.4 represents the growth of *Y. lipolytica* on crude glycerol with different biochemical modulators (citric acid and biotin). As shown in Figure 5.4, *Y. lipolytica* simultaneously consumed crude glycerol and CA. This phenomenon seemed to be not frequent and the simultaneous utilization of two different substrates is not typical of microorganisms, which first assimilate one of the two available substrates, whereas the assimilation of the other substrate starts only after the first substrate is fully consumed from the medium. Herein, *Yarrowia* was assimilating citric acid (3 g/L), followed by glycerol (0.5 g/L). This simultaneous consumption can be attributed to metabolic signaling since some sugars can produce signals which modify the conformation of certain proteins that, in turn, directly or through a regulatory cascade affect the expression of the genes subject to catabolite repression. These genes are not at all controlled by a single set of regulatory proteins (Cho et al., 2009, Esperón et al., 2014). The glycerol consumption rate was decreased principally due to the fact that the cells consumed both substrates at low rates. Thus, the strategy of the CA feeding was efficient to create a pool of CA required during the shift of metabolism to lipid production. More often, the influence of CA on the production phase of SCO has not been described until date. Iske et al. (1983) have determined the effect of CA on the growth rate of the bioprocess and they found that the addition of initial concentrations of CA at 30 g/L into the culture had no remarkable influence on the growth rate of

the organism. Nevertheless, with increasing concentrations of CA, a significant decline of the growth was observed (Iske et al., 1983), which is not the case in the present study where growth was activated and lipid production was more promoted. During the lipogenic phase, K_La was kept between 50-60 h^{-1} and OTR varied between 0.2 to 0.3. During fed batch fermentation, CA was added at 36 h. The supplementation of medium with CA at 30 g/L led to decrease of K_La reaching 21.1 h^{-1}, however manual adjustment of agitation was required to keep the DO around 30% (i.e. agitation 420 rpm and air flow rate 2.5 L/min).

The combined effect of biomodulators and citric acid, which were reviewed to work simultaneously increased the lipid quantity to 18.26 g/L and lipid accumulation up to 63.7 ± 0.9% (w/w). These results were advantageous compared to the control experiment where only 52.7% (w/w) were obtained and to other reported studies on lipid production in *Yarrowia* species such as *Y. lipolytica* TISTR 5151 that accumulate around 50.8% (w/w) (Louhasakul & Cheirsilp, 2013) and *Y. lipolytica* MUCL 28849 which accumulate 34.6% (w/w) (Fontanille et al., 2012). The feasibility of this technology should be assessed using a techno-economic analysis to compare costs associated with biotin addition compared to carbon source addition. Beyond further optimization of modulators supply, additional optimization can perceivably be achieved via improved enzymatic tools.

5.5.4 Fatty acid composition

The fatty acid profile in biomass of *Y. lipolytica* in the presence of citric acid and biomodulators showed that the major fatty acid detected in *Y. lipolytica* biomass was oleic acid (C18:1). This was 33–45% of the fatty acid pool during biomass and lipid production phases. In addition, palmitic (C16:0) and linoleic (C18:2) acids have also been detected in high content in the cells (14–20%). These results agree with previous reports (Dobrowolski et al., 2016; Magdouli et al., 2016b). However, in the current study, *Y. lipolytica* produced more stearic acid 12.98%, compared to the control experiment where 8.30% of stearic acid was obtained (Magdouli et al., 2016b). Despite the minimal changes in fatty acid profiles, the concentration of saturated fatty acids and monounsaturated fatty acids in *Y. lipolytica* biomass was 78.3% of the total lipid pool. This observation presented a significant advantage in fatty acid composition for further potential biodiesel production because polyunsaturated fatty esters have low cetane number, low melting points, and reduced oxidative stability which restrict their use in diesel fuel (Knothe, 2008). Therefore, this research provides new insight in fatty acid production by *Y. lipolytica*. From a technological point-of-view, it is much easier to use this strain to produce biodiesel by only

supplementing biochemical targets products. To sum up, SCO production based on crude glycerol with advanced biochemical approach can yield promising breakthroughs in low cost and effective synthesis of fatty acids.

5.6 Conclusion

The influence of various modulators concentrations on growth and lipid accumulation in *Y. lipolytica* was explored through strategic addition of biotin and leucine to enhance growth and lipid accumulation rates as compared to traditional growth regimes which usually supply elevated concentrations of carbon source up to 89 g/L. These data indicated that the type and strategy (e.g, timing, concentration) of modulators had a significant influence (p values < 0.05) lipid production in *Yarrowia* and at an early stage of lipid accumulation, the strain was accumulating up to 15.46 g/L. An optimized two-phase growth with biochemical approach of biotin and citric acid is an effective strategy to increase its fatty acid biosynthesis and provide pathway precursors for production of targeted products. This could offer a promising strategy for optimizing productivity and reducing resource costs. Moreover, this strain can be regarded as a valuable tool for generating large amounts of lipids owing to the recent identification of additional putative rate-limiting steps required during the lipid synthesis and regulatory elements involved in lipid accumulation.

5.7 Acknowledgements

The authors are thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A 4984, and Canada Research Chair) for their financial support.

5.8 References

Ageitos, J.M., Vallejo, J.A., Veiga-Crespo, P., Villa, T.G. 2011. Oily yeasts as oleaginous cell factories. *Appl Microbiol Biotechnol*, **90**, 1219-1227.

Barber, M.C., Price, N.T., Travers, M.T. 2005. Structure and regulation of acetyl-CoA carboxylase genes of metazoa. *BBA-Mol Cell Biol L*, **1733**, 1-28.

Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.L., Molina-Jouve, C., Nicaud, J.M. 2009. Yarrowia lipolytica as a model for bio-oil production. *Prog Lipid Res*, **48**, 375-387.

Blazeck, J., Hill, A., Liu, L., Knight, R., Miller, J., Pan, A., Otoupal, P.H.S.A. 2014. Harnessing Yarrowia lipolityca lipogenesis to create a platform for lipid and biofuel production. *Nat Commun*, **4**.

Botham, P.A., Ratledge, C. 1979. A biochemical explanation for lipid accumulation in Candida 107 and other oleaginous micro-organisms. *J Gen Microbiol*, **114**, 361-75.

Boulton, C.A., Ratledge, C. 1980. Regulatory studies on citrate synthase in Candida 107 an oleaginous yeast. *J Gen Microbiol*, **121**, 441-7.

Bunn, C. R., McNeill, J.J., Elkan, G.H. 1970. Effect of biotin on fatty acids and phospholipids of biotinsensitive strains of Rhizobium japonicum. *J Bacteriol*, **102**, 24-29.

Cescut, J. 2009. Accumulation d'acylglycérols par des espèces levuriennes à usage carburant aéronautique: physiologie et performances de procédés. Toulouse: Université de Toulouse; 2009. p. 283.

Cho, I.H., Lü, Z.R., Yu, J.R., Park, Y.D., Yang, J.M., Hahn, M.J., Zou, F. 2009. Towards profiling the gene expression of tyrosinase-induced melanogenesis in HEK293 Cells: A functional DNA chip microarray and interactomics studies. *J Biomol Struct Dyn,* **27**, 331-345.

Courchesne, N.M., Parisien, A., Wang, B., Lan, C.Q. 2009. Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. *J Biotechnol,* **141**, 31-41.

Desai, J.D., Desai, A.J., Patel, H.C. 1983. Effect of Biotin on Alkaloid Production During Submerged Cultivation of Claviceps sp. Strain SD-58. *Appl Environ Microbiol*, **45**, 1694-1696.

Dobrowolski, A., Mituła, P., Rymowicz, W., Mironczuk, A.M. 2016. Efficient conversion of crude glycerol from various industrial wastes into single cell oil by yeast Yarrowia lipolytica. *Bioresour. Technol.*, **207**, 237-43.

Esperón, P., Scazzocchio, C., Paulino, M. 2014. In vitro and in silico analysis of the Aspergillus nidulans DNACreA repressor interactions. *J Biomol Struct Dynan*, **32**, 2033-2041.

Fontanille, P., Kumar, V., Christophe, G., Nouaille, R., Larroche, C. 2012. Bioconversion of volatile fatty acids into lipids by the oleaginous yeast Yarrowia lipolytica. *Bioresour Technol*, **114**, 443-9.

Gill, C.O., Hall, M.J., Ratledge, C. 1977. Lipid accumulation in an oleaginous yeast, Candida 107, growing on glucose in single-stage continuous culture. *Appl Environ Microb*, **33**, 231-239.

Holz, M., Förster, A., Mauersberger, S., Barth, G. 2009. Aconitase overexpression changes the product ratio of citric acid production by Yarrowia lipolytica. *Appl Microbiol Biotechnol,* **81**, 1087-1096.

Iske, U., Gwenner, Ch., Bullmann, M., Uhlenhut, G.-J., Schindler G. 1983. Zur Kinetik der Mischsubstratutilisation bei der Citronensäureaccumulation durch Saccharomycopsis lipolytica EH 59. *Acta Biotechnol*, **3** 143-153.

Kamisaka, Y.T.N., Kimura K., Kainou, K., Uemura, H. 2007. DGA1 (diacylglycerol acyltransferase gene) overexpression and leucine biosynthesis significantly increase lipid accumulation in the Δsnf2 disruptant of Saccharomyces cerevisiae. *Biochem J*, **408**, 61-68.

Karamerou, E.E., Theodoropoulos, C., Webb, C. 2016. A biorefinery approach to microbial oil production from glycerol by Rhodotorula glutinis. *Biomass Bioenergy*, **2016**, 1-10.

Karampudi, S., Chowdhury, K. 2011. Effect of Media on Algae Growth for Bio-Fuel Production. *Not Sci Biol*, **3**, 33-41

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

Knothe, G. 2008. ‘‘Designer” biodiesel: optimizing fatty ester composition to improve fuel properties. *Energy Fuels*, **22**, 1358-1364.

Kohlhaw, G.B. 2003. Leucine biosynthesis in fungi: entering metabolism through the back door. *Microbiol Mol Biol Rev*, **67**, 1-15.

Kolouchová, I., Maťátková O., Sigler K., Masák J., Řezanka T. 2016. Production of Palmitoleic and Linoleic Acid in Oleaginous and Nonoleaginous Yeast Biomass. *I J Anal Chem*, **2016**, 9.

Louhasakul, Y., Cheirsilp B. 2013. Industrial waste utilization for low-cost production of raw material oil through microbial fermentation. *Appl Biochem Biotechnol*, **169**, 110-22.

Lynen, F., Knapp, J., Lorch, E., Jutting, G., Ringelmann, E. 1959. Die biochemische Funktion des Biotins. *Angew Chem*, **71**, 481-486.

Lynen, F., Knappc, J., Lorch, E., Jutting, G., Ringelmann, E., Lachance, J.-P. 1961. On the biochemical function of biotin. II. Purification and mode of action of beta-methyl-crotonyl-carboxylase. *Biochem Z*, **335**, 123-67.

Magdouli, S., Brar, S.K., Blais, J.F. 2016a. Comparative study between microwave and ultrasonication aided in situ transesterification of microbial lipids. *RSC Adv,* **6**, 56009.

Magdouli, S., Brar, S.K., Blais, J.F. 2016b. Lipid production by Yarrowia lipolytica grown on biodiesel-derived crude glycerol: Optimization of growth parameters and their effects on the fermentation efficiency. *RSC Adv*, **6**, 90547-90558

Magdouli, S., Yan, S., Tyagi, R.D., Surampalli, R.Y. 2014. Heterotrophic microorganisms: a promising source for biodiesel production. *Crit Rev Environ Sci Technol,* **44**, 416-453.

Meng, X., Yang, J., Cao, Y., Li, L., Jiang, X., Xu, X., Liu, W., Xian, M., Zhang, Y. 2011. Increasing fatty acid production in E. coli by simulating the lipid accumulation of oleaginous microorganisms. *J Ind Microbiol Biotechnol*, **38**, 919-25.

Moeller, L., Strehlitz, B. 2007. Optimization of citric acid production from glucose by Yarrowia lipolytica. *Eng Life Sci,* **7**, 504-511.

Ngangkham, M., Ratha, S.K., Prasanna, R., Saxena, A.K., Dhar, D.W., Sarika, C., Prasad, R.B.N. 2012. Biochemical modulation of growth, lipid quality and productivity in mixotrophic cultures of Chlorella sorokiniana. *Springerplus*, **1**, 33.

Oura, E., Suomalainen, H. 1978. Biotin and the metabolism of baker's yeast. *J Inst Brew,* **84**, 283-287.

Papanikolaou, S., Galiotou-Panayotou, M., Chevalot, I., Komaitis, M., Marc, I., Aggelis, G. 2006. Influence of glucose and saturated free-fatty acid mixtures on citric acid and lipid production by Yarrowia lipolytica. *Curr Microbiol*, **52**, 134-42.

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., Marc, I. 2002. Yarrowia lipolytica as a potential producer of citric acid from raw glycerol. *J Appl Microbiol*, **92**, 737-44.

Piffeteau, A., Gaudry, M. 1985. Biotin uptake: influx, efflux and countertransport in Escherichia coli K12. *Acta Biochim Biophys*, **816**, 77-82.

Ratledge, C. 2002. Regulation of lipid accumulation in oleaginous micro-organisms. *Biochem Soc Trans*, **30**, 1047-50.

Ratledge, C., Wynn, J.P. 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv Appl Microbiol,* **51**, 1-51.

Rodríguez-Frómeta, R.A., Gutiérrez, A., Torres-Martínez, S., Garre, V. 2012. Malic enzyme activity is not the only bottleneck for lipid accumulation in the oleaginous fungus Mucor circinelloides. *Appl Microbiol Biot,* 1-10.

Ruenwai, R., Cheevadhanarak, S., Laoteng, K. 2009. Overexpression of Acetyl-CoA Carboxylase Gene of Mucor rouxii Enhanced Fatty Acid Content in Hansenula polymorpha. *Mol Biotechnol*, **42**, 327-32.

Rymowicz, W., Rywin´ska A., Z´arowska, B., Juszczyk, P. 2006. Citric acid production from raw glycerol by acetate mutants of Yarrowia lipolytica. *Chem Pap*, **60**, 391-4.

Santamauro, F., Whiffin, F.M., Scott, R.J., Chuck, C.J. 2014. Low-cost lipid production by an oleaginous yeast cultured in non-sterile conditions using model waste resources. *Biotechnol Biofuels*, **7**, 34.

Vorapreeda, T., Thammarongtham, C., Cheevadhanarak, S., Laoteng, K. 2012. Alternative routes of acetyl-CoA synthesis identified by comparative genomic analysis: involvement in the lipid production of oleaginous yeast and fungi. *Microbiology*, **158**, 217-228.

Wahby, I., Bennis, I., Hicham, EL A., Benhima, R. 2014. Method for increasing the potential for biofuel production from microalgae by using bio-modulators patent (WO2014003530 A1).

Yu, X., Chen, L., Zhang, W. 2015. Chemicals to enhance microalgal growth and accumulation of high-value bioproducts. *Front Microbiol,* **6**, 56

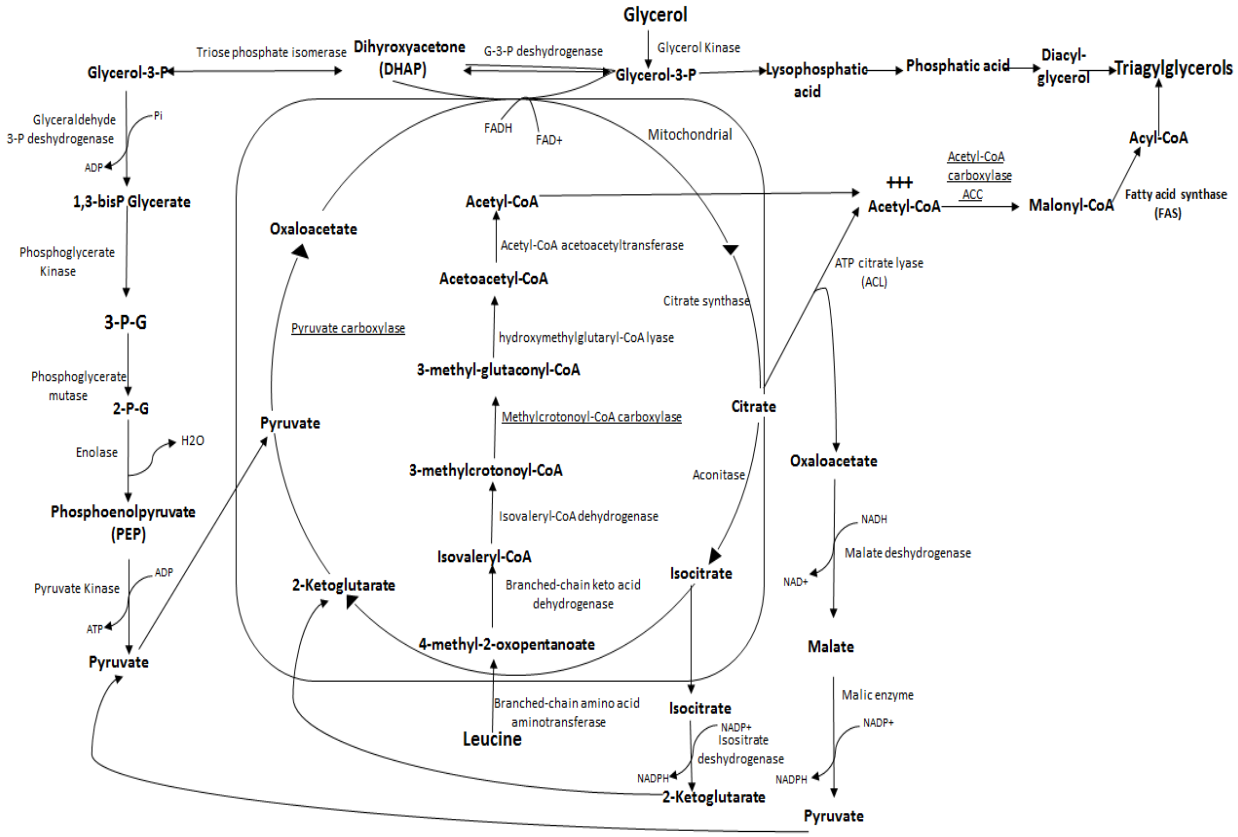


Figure 5.1 **Overview of metabolic pathways for fatty acid synthesis in the presence of glycerol as carbon source, underlined enzymes are activated in the presence of biotin (Pryruvate carboxylase, Acetyl-CoA carboxylase, and methylcrotonyl-CoA carboxylase)**

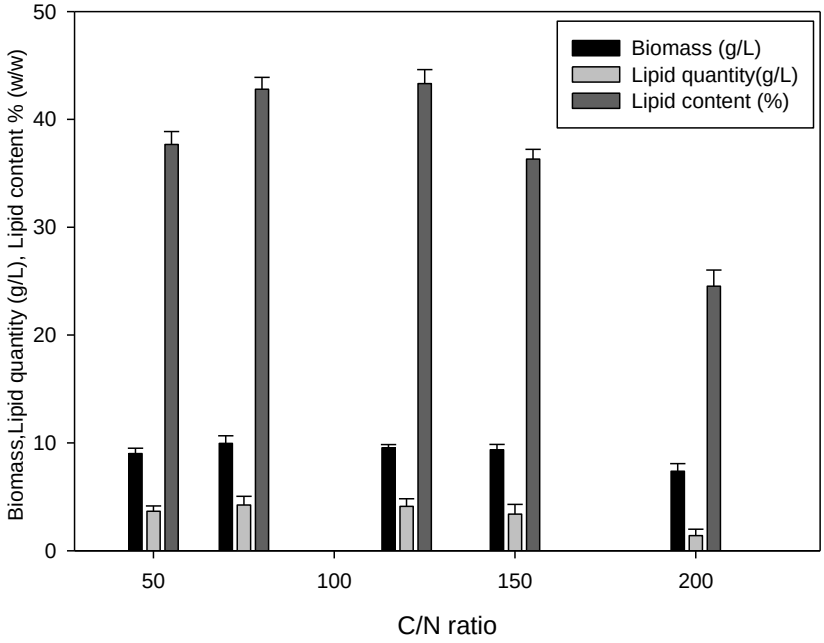


Figure 5.2 **The effect of C/N on the growth and lipid production in *Y. lipolytica*, cultured in shake-flasks (Conditions: T = 28°C, pH = 6.5, 180 rpm, 72 h)**

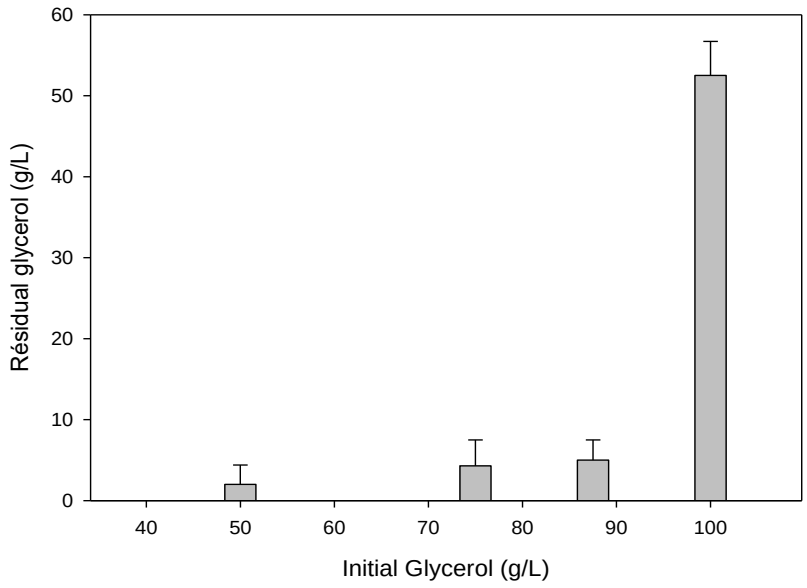


Figure 5.3 **Effect of initial glycerol concentration on the growth of *Y. lipolytica* (Culture conditions: T = 28°C, pH = 6.5, 180 rpm, 72 h)**

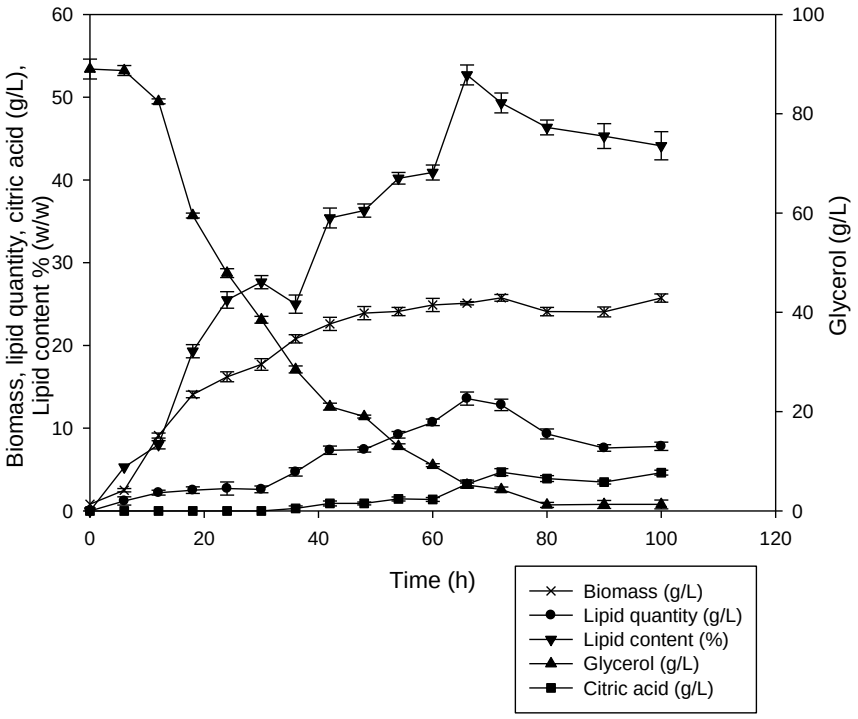


Figure 5.4 **Batch bioreactor fermentation at C/N 75. Crude glycerol was used as a carbon source (89 g/L)**

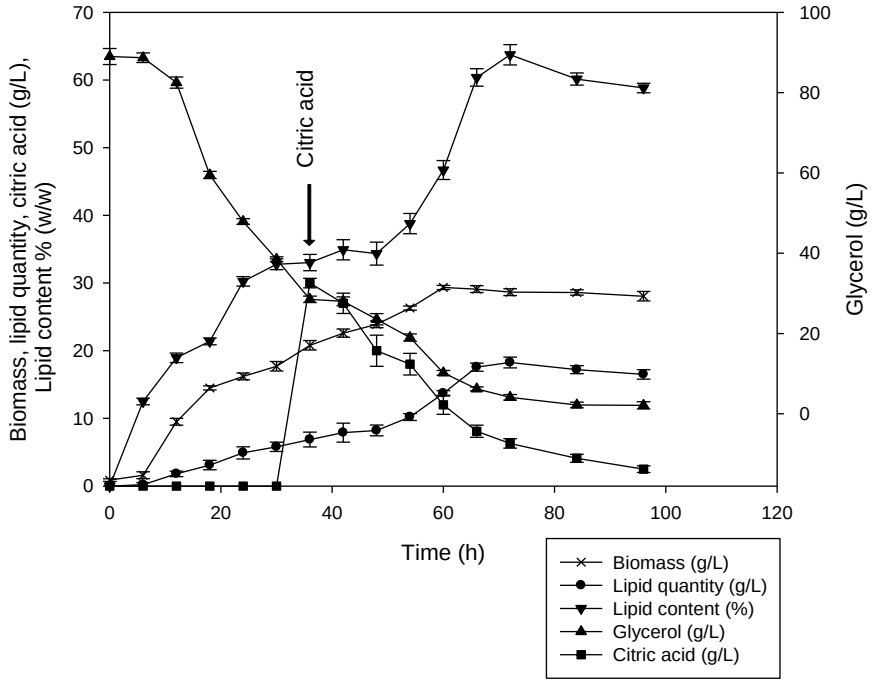


Figure 5.5 **Effect of citric acid and biotin concentration on the lipid by *Y. lipolytica*, Conditions: T = 28°C, pH = 6.5, DO = 30%, 440-480 rpm, 100 h (duplicates)**

Tableau 5.1 ***Y. lipolytica*** **culture characteristics during nitrogen limited growth when supplemented with various concentrations of biotin at different stages in the fermenter 5 L**

	Biotin concentrations (µg/L)					
	50		**100**		**200**	
	Lipid	**CDW**	**Lipid**	**CDW**	**Lipid**	**CDW**
Early stage	14.0 ± 0.3	26.4 ± 0.4	15.5 ± 1.1	25.8 ± 0.5	14.9 ± 0.5	27.1 ± 0.6
Late stage	13.5 ± 0.5	25.9 ± 0.3	13.8 ± 0.9	27.8 ± 0.5	13.8 ± 0.4	25.5 ± 0.7

CDW: cell dry weight (g/L), lipid (g/L), The values are means ± standard deviations of three independent experiments.

Tableau 5.2 **Effect of biomodulators on** ***Y. lipolytica*** **characteristics in fermenter 5 L**

Biomodulator	**CDW (g/L)**	**Lipid concentration (g/L)**	**Lipid content (% w/w)**
With Leucine	26.7 ± 0.6	14.7 ± 0.9	54.9 ± 0.5
With Biotin	25.8 ± 0.6	15.5 ± 1.1	60.1 ± 0.7
With Biotin+leucine	27.4 ± 0.5	16.8 ± 0.5	61.3 ± 0.9

CDW: cell dry weight (g/L), The values are means ± standard deviations of three independent experiments.

6 CHAPITRE 6: VALORISATION OF RAW GLYCEROL AND CRUSTACEAN WASTE INTO VALUE ADDED PRODUCTS BY YARROWIA LIPOLYTICA

Valorization of raw glycerol and crustacean waste into value added products by *Yarrowia lipolytica*

Sarra Magdouli [a], Tayssir Guedri [a], Rouissi Tarek [a], Satinder Kaur Brar[b], Jean François Blais[c,*]

[a] Ph.D. student, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-4677, Fax: (418) 654-2600, E-mail: magdouli.sara@ete.inrs.ca

[b] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-3116, Fax: (418) 654-2600, E-mail: satinder.brar@ete.inrs.ca

[c] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-2575, Fax: (418) 654-2600, E-mail: blaisjf@ete.inrs.ca

*Corresponding author

Email: blaisjf@ete.inrs.ca

6.1 Résumé

L’utilisation de glycérol brut en tant que source renouvelable de carbone pour la production de biodiesel a été amplement étudiée. Dans la présente étude, différents types de tensioactifs et d’huiles ont été utilisés en complément du glycérol brut afin d’améliorer la production de lipides et de lipases par la levure *Yarrowia lipolytica*, une souche nouvellement isolée, Les résultats ont montré qu'un milieu de culture composé de glycérol brut et d'huile d'olive peut augmenter l'activité de la lipase jusqu’à 25 U/mL et la teneur des lipides jusqu'à 35%. La fortification du milieu avec des déchets de crustacés a augmenté l'activité de la lipase jusqu'à 38 U/mL. L'activité hydrolytique des lipases extracellulaires produites dans le milieu était satisfaisante, ceci ouvre la voie à une utilisation dans d'autres procédés biotechnologiques.

Mots-clés : *Yarrowia lipolytica*, glycérol brut, lipides, lipases, déchets de crustacés

6.2 Abstract

Crude glycerol has been widely investigated as a renewable carbon source for biodiesel production. In the present study, this feedstock was supplemented by various inducers surfactants and oils to enhance lipid and lipase production by the newly isolated yeast, *Yarrowia lipolytica*. Results have shown that a culture medium composed of olive oil could enhance lipase activity at 25 U/mL and lipid content up to 35% (w/w). The fortification of the medium with crustacean waste increased the lipase activity up to 38 U/mL. The hydrolytic activity of the extracellular lipases produced in mentioned medium was satisfactory and opened avenues for other biotechnological processes.

Keywords: *Yarrowia lipolytica*, Glycerol, Lipid production, Lipase, crustacean waste

6.3 Introduction

Yarrowia lipolytica has been known to convert many triglycerides, industrial fats and hydrophobic substrates to single cell biomass or to produce environmentally friendly compounds, such as SCO (Kamzolova et al., 2005; Papanikolaou et al., 2007) and has gained interest owing to its high secretory activity of various biomolecules, including citric acid, 1,3-propanediol and enzymes such as protease, RNase, phosphatase, esterase and lipase. Besides, *Yarrowia lipolytica* was reported to utilize various aliphatic and hydrophobic substrates (Rywińska et al., 2013). Crude glycerol has been investigated as by-product residual to produce various metabolites (Rywińska et al., 2013). However, there is scarce information dealing with the possibility of these microorganisms to produce lipids and lipase. In this regard, *Y. lipolytica* NRRL Y-2178 was reported to synthesize alkaline lipase when glycerol is used as a carbon source (Lee et al., 2007). In a recent study, Fabiszewska et al. (2014) have cultivated wild-type strain *Y. lipolytica* KKP 379 in based glycerol media and have determined its efficiency to synthesize lipolytic enzymes. Moreover, the authors have concluded that glycerol utilization in microbiological lipase production is possible, but that this process cannot proceed without the addition of one or more stimulators of lipase synthesis, such as olive oil. Spectacular results were obtained while using a medium containing waste cooking olive oil as inducer (Deive et al., 2003; Goncalves et al., 2013). More often, some oils were reported to increase the surfactant production, which could act as an inducer to enhance lipid accumulation in yeast (Aksu & Eren, 2005). Therefore, the use of these substrates for fermentation aims to decrease the final cost of these enzymes and make the industrial enzymatic processes cost-competitive with chemical ones. For this purpose, researchers have investigated the role of various inducers to enhance lipid and lipase activity in the presence of oils. To the best knowledge of the authors, this is the first report on complete assessment of lipase production by the yeast using crude glycerol and crustacean wastes. In the first part, a suitable inducer was first screened for enhanced production of lipids and lipase activity. Thus, a combination between glycerol and other inducers of lipase synthesis could be a promising alternative.

6.4 Materials and methods

6.4.1 Microorganism

Y. lipolytica SM7 was newly isolated (Magdouli et al., 2016). The micro-organism was maintained at 4°C on (yeast extract peptone dextrose agar) (YEPD) slants. Subcultures were made on fresh agar slants every three months to maintain viability.

6.4.2 Crude glycerol, crustacean waste, reagents and chemicals

All chemicals were of reagent grade. Growth media were purchased from Sigma-Aldrich, Fisher scientific (Mississauga, Ontario, Canada). Domestic vegetables oil and olive oil were obtained from local grocery.

Crude glycerol was obtained from Rothsay Biodiesel (Ontario, Canada), resulting from the transesterification of animal fats was used as carbon source, with high purity of 83.30% with impurities mainly composed of potassium and sodium salts (1-2%), methanol (1-3%), and water (2-5%).

The crustacean waste was obtained from a seafood processing plant located at Gaspesie, Quebec. The seafood waste (shells of crab, shrimp, prawn, krill and lobster) was oven dried, powdered using a grinder and stored at 4 ± 1°C.

6.4.3 Cultivation conditions

Cultures were grown on a minimal medium containing, 1 g/L yeast extract, 1.5 g/L $(NH_4)_2SO_4$, 3 g/L K_2HPO_4, 3 g/L $NaH_2PO_4.H_2O$, 0.5 g/L $MgSO_4.7H_2O$, 0.040 g/L $ZnSO_4.7H_2O$, 0.016 g/L $FeSO_4.7H_2O$, 0.25 µg/L biotin), and 40 g/L of filtered crude glycerol, which is assumed to contain around 1 to 1.5% methanol. Herein, the objective was to provide all components presented in the crude glycerol, especially methanol which can be evaporated after sterilization. The crude glycerol was diluted to obtain 4% glycerol concentration. The final pH of the medium was 6.5. The medium was sterilized by autoclaving at 121°C for 20 min. The inoculum of *Y. lipolytica* SM7 was prepared by transferring cells grown on a slant to 50 mL Erlenmeyer flasks containing (YEPD) broth. The seed culture was incubated in an orbital shaker at 28°C, 180 rpm for 24 h. The YEPD broth was used to initiate growth 5% (v/v). Cultivation was carried out in 2L Erlenmeyer flasks containing 500 mL minimal medium at 28°C with shaking at 180 rpm for 150 h. There was no adjustment of

pH during cultivation. At regular intervals, samples were withdrawn for analyses. All analyses were performed in triplicates and did not vary more than 5%.

To screen a suitable inducer to obtain the maximum lipid and lipase activities, various surfactants and oils were used. After screening step, the effect of inducer on lipase production was investigated.

The experiments were performed in two stages. The first stage experiments were carried out to determine the appropriate inducer to obtain best growth performance and higher metabolites production. In the presence of different inducers, lipase activity, biomass and lipids were monitored throughout the fermentation.

6.4.4 Analytical methods

Five mL aliquot of culture was centrifuged at 5,000 rpm for 5 min. The cell free supernatant obtained after centrifugation was used for estimation of lipase activity. For the determination of lipase activity, titration method was employed which consists of use of olive oil emulsion as substrate (Gargouri et al., 1984). The reaction mixture contains 10 mL of olive oil emulsion (1 mL of olive oil and 9 mL of Arabic gum at 10% v/v), 20 mL of distilled water and 100 L of bovine serum albumin at 12.5% (w/v). The activity was expressed as units per volume of enzymatic solution (mL). One unit (U) of lipase activity was defined as the amount of enzyme that catalyzes the liberation of 1 mol of fatty acid from olive oil per min at pH 8.0 and at 37^{o}C. To study the effects of pH on the enzyme stability, buffered sample was incubated at various pH ranging from 5 to 9 for 1 h at 37°C. To keep the sample pH constant, the following 100 mM buffers systems were used: Na_2HPO_4/citrate, pH 3.0–6 and KH_2PO_4/Na_2HPO_4, pH 6.0–7.0. The thermal stability was studied by incubating the enzyme at various temperatures and measuring the residual activity with time under standard titrimetric assay conditions. For determination of biomass weight (g/L), culture broths were centrifuged at 5,000 rpm for 15 min. After rinsing the pellet once with deionized water, the pellet was frozen at −80°C for 1–2 days, and then dried for 24 h in a lyophilizer for dry cell weight measurement. All experiments were performed in triplicates, and average results were reported with standard deviation less than 5%.

6.5 Results and discussion

6.5.1 Dynamics of lipase and lipids biosynthesis in glycerol based medium

6.5.1.1 Effect of surfactants on growth kinetics and lipase production

Y. lipolytica was cultivated in different media containing filtered crude glycerol (final product of lipase activity that lacks a fatty acid or carbon chain) at initial concentration of 40 g/L and various inducers (e.g. olive oil, vegetable oil and motor oil, Tween 80, Tween 20, Triton X 100) of 5.0% (w/v). These components were investigated for their potential in increasing the metabolites production of SM7 (i.e. biomass, lipid, CA, and lipase). Previously, many agents such as Tween 80, Tween 20, and arabic gum arabic were investigated to enhance biomass and lipid production in oleaginous yeast (Kim et al., 2006; Saenge et al., 2011). Still, the mechanism of their action is not well understood, hence, Kruszeka et al. (1990) reported that these agents appear to cause different alterations in membrane fluidity. It was found that the crude glycerol could serve as a sole carbon source for *Yarrowia* without any additional supplementation of inducers (control experiment). Biomass, lipid and lipid content were around 8.7 ± 0.5 g/L, 2.7 ± 0.5 g/L, 30.8 ± 2.0% (w/w) of dry biomass. The higher biomass concentration was obtained at 120 h and reached around 12.6 ± 0.8 g/L.

Further increase in biomass, lipid content and lipid quantity was observed when surfactant was added in different concentrations (Table 6.1). Among the three investigated surfactants, Tween 80 was found to be most effective in increasing both biomass and lipid content. It gave the highest amount of biomass, lipid content (16.93 g/L, 5.94 g/L, 35.1% (w/w), respectively). Thus, the addition of different detergents as growth promotors and lipid inducers is well illustrated. Their mechanism seems to destabilize the membrane fluidity of the strain and accelerate the uptake of glycerol. These findings are in agreement with Boudour et al. (2003) who found that the higher consumption rate could be attributed to the capacity of the surfactant to emulsify hydrocarbon-based compounds, breaking them down into more manageable molecules, so that the microbes can then more efficiently digest them. Besides, Saengea et al. (2011) have observed that surfactants acted efficiently as activator of TAG accumulation in *R. glutinis* TISTR, which are in concordance with the current study in which higher lipid content up to 35.2 ± 2.5% (w/w) of dry biomass was obtained compared to control experiment at 30.9 ± 1.5% (w/w) of dry biomass.

Besides, the lipid yield decreased between 24 h and 48 h in the early stage of growth, as well as in the late growth stage, thus the exploration of metabolites produced was conducted. Higher quantity of CA was observed and maximum of 3.3 g/L was obtained at 100 h. The production of

CA is also accompanied by lipase secretion at 24 h and 72 h. As seen in Table 6.1, lipase production appeared to be critically influenced by the presence of various surfactants used along experiments, in the flask cultures. In the control experiment, crude glycerol alone was able to induce lipase activity, however, this activity was very low (2.4 ± 1.7 U/mL) and U_{max} = 4.0 ± 1.7 U/mL was observed at 72 h. At 24 h, a lipase activity of 1.66 U/mL was detected, due to the presence of triacylglycerols and fatty acids in the crude glycerol which is estimated by soap content in the crude glycerol of around 1.5 g/L. In addition to soap, the present activity can be attributed to the presence of fatty acids, vitamins and trace elements resulting from the transesterification of animal's fats diffusing to the glycerol phase during the biodiesel formation reactions, and thus enriching the crude glycerol (Cüelik et al., 2008). Accordingly, lipase secretion is known to be induced by the presence of triacylglycerols and fatty acids in different microorganisms (Benjamin & Pandey, 1996; Berto et al., 1997). During lipogenic phase, no activity was detected between 36 h and 60 h, which could be attributed to a repressive phenomenon in the presence of glycerol as sole carbon source, the latter is generated as an end product of lipase activity and triglycerides hydrolysis and when it is available at the beginning of cultivation, there is no immediate need for lipase production by cell. Corzo and Revah (1990) also reported that lipase is not produced in medium containing glycerol as the sole carbon source. Besides, these findings were in accordance with Szczesna-Antczak et al. (2006), who showed that carbohydrates could inhibit biosynthesis of extracellular lipases and addition of an inducer is highly required, since the lipase production is not a constitutive phenomenon in *Y. lipolytica* and the presence of inducer is highly required.

Maximum cell concentration was obtained in the presence of Tween 80 > Tween 20 > Triton X100 (X_{max} = 16.97 g/L > 16.55 g/L > 14.73 g/L respectively, the higher biomass was associated with higher lipase activity (U_{max} = 15.1 ± 2.5, U_{max} = 14.3 ± 1.9, U_{max} = 12.5 ± 2.5 U/mL) respectively detected at 72 h. The pH dropped from an initial value of 6.5 to 4.5 from day 3 onwards, probably due to the action of the produced lipases and the subsequent increase of organic acids concentration in the medium, especially citric acid (Magdouli et al., 2016). Generally, surfactants have been well reviewed to enhance extracellular lipase activity by causing changes in cell permeability of the cell (Kruszewska et al., 1990; Wei et al., 2003). However, their role in the lipase production is not conclusive since some authors reported that surfactants do not increase the lipase production in some species (Lin et al., 1995). Therefore, the effect seems to be strain dependant. Herein, the presence of surfactants seemed to bring about a slight increase in biomass production: maximum cell concentration was found to be between 15.0-19.0 ± 2.5 g/L between 72 h and 100 h, compared with the control. Thus, Tween, a polysorbate surfactant,

enhanced permeability of cell membrane, accelerates the glycerol uptake, and might be used as a carbon source (Taoka et al., 2011).

No activity was observed in the late growth phase (96 h) due to the possible degradation of lipase since *Yarrowia* species are known to produce alkaline extracellular protease (AEP) (Davidow et al., 1987), which is responsible for the degradation of extracellular lipase produced by selected strain. Between 120 h and 150 h, the activity fell down to undetectable levels.

In the present study, whatever the surfactant used, lipase activity peaked twice at 24 h and 72 h medium, however, it was lower in the early stage (i.e. 24 h) and accounts around (9.0 ± 3.5 U/mL for Tween 80, 5.3 ± 2.5 U/mL for Triton X 100 and 6.0 ± 3.0 U/mL for Tween 20). The double peak of lipase activity was correlated with sequential consumption of glycerol and surfactant, indicating that *Yarrowia* consumed methanol, soap (free fatty acids in the early stage were mobilized to consume glycerol) and later, the surfactant employed.

Furthermore, the quantification of lipid content in the presence of these stimulators was carried out, and lower content of lipid during the phase of biomass production 36 h corresponded to lipid quantity between (0.2 ± 0.3 and 1.13 ± 0.25 g/L). Between 48-70 h (i.e. lipogenesis phase), a higher lipid content was observed and maximum of 35.1% (w/w) at 60 h, however, during the CA phase between 72 h-120 h (lipid content was decreased to reach 17.6 ± 3.5% (w/w) due to the presence of lipolytic activity, to the exhaustion of carbon source and the induction of lipase owing to the presence of accumulated TAG. Besides, another hypothesis can be evolved corresponding to lipase activity during extraction which remains bound to the cells and subsequently induced lysis of intracellular lipid and lower the content below 17% (w/w). Hence, the rapid extraction is required to avoid such phenomena since in the presence of chloroform/methanol, lipase activity disappeared (Najjar et al., 2011). Moreover, lipid accumulated can be preserved owing to different strategies (Zhang et al., 2015).

6.5.1.2 Effect of oil inducers on growth kinetics and lipase production

Addition of oils to flask cultures induced an increase in biomass value as found with surfactants, compared with the medium in which no addition of oil was carried out. As shown in Table 6.2, *Y. lipolytica* showed efficient cell growth when fermentation was carried out in the presence of olive oil and glycerol based medium (X = 17.12 g/L). During the lipogenic phase, biomass was around 17.0 ± 0.5 g/L compared to 8.7 ± 0.5 g/L. A higher biomass was recorded with olive oil (X_{max} = 21.9 ± 0.8 g/L at 120 h).

However, in the presence of motor oil, a little improvement of biomass production was observed (X_{max} = 10.3 g/L) and visually, the oil remained on the surface, this oil was not consumed, thus, the composition of engine oil inhibited the uptake of glycerol and the microorganism was unable to degrade and use the oil as carbon source while glycerol was there. Accordingly, engine oil is reported to be composed of synthetic antioxidant and corrosion inhibitor, required to minimize its susceptibility to oxidation (Meira et al., 2014). These components seemed to inhibit the growth in the present case. Conversely, the higher lipid content is obtained in the presence of olive oil at 35.8% (w/w) compared to 33.1% (w/w) in the presence of vegetable oil.

Accordingly, olive oil was constituted by complex mixture of triacylglycerols (99%), and free fatty acids, mono- and diacylglycerols, and an array of lipids, such as hydrocarbons, sterols, aliphatic alcohols, tocopherols, and pigments (Boskou, 1996), that activateds the metabolite production of biomass and lipid reaching up to 38% (w/w). Secondly, vegetable oil exhibited the same effect of olive oil with small differences. Therefore, olive oil and vegetable oil, composed of fatty acids (70% of oleic acid), promoted the novo synthesis without affecting the glycerol uptake. These results suggested the high performance of *Y. lipolytica* during growth in various oil-glycerol based media. In fact, *Yarrowia* is known to produce an emulsifying bio surfactant (Lyposan) in the presence of hydrophobic substrates, which emulsify the medium and enhance the uptake of oleic acid and traicylglycerols present in the olive oil. The higher capacity to accumulate lipids when grown on these substrates is probably related to protrusions formed on cell surfaces, facilitating the uptake of hydrophobic substrates from the medium (Mlickova et al., 2004).

The lipid content increased rapidly compared to control experiment. Therefore, *Y. lipolytica* is capable of to use triglycerides as carbon source and the first step of this metabolism involved hydrolysis of olive oil by lipases to produce fatty acids and glycerol, and a higher activity was detected at 24 h, 15.0 ± 3.2 U/mL and 12.8 ± 2.5 U/mL for olive oil and vegetable oil, respectively.

Besides, a double peak was observed at 72 h and lipase activities increased sharply and attained (25.1 U/mL and 22.5 U/mL) for olive and vegetable oils, respectively which corresponded to the initiation of the degradation of TAG accumulated. The higher lipase activity could be attributed to the composition of olive oil as discussed earlier. In this regard, the microorganism starts degrading triglycerides and glycerol present in the medium and produced free fatty acids and glycerol due the action of lipase produced at 24 h, around 15 U/mL for olive oil and 12.8 U/mL for vegetable oil. Afterwards, resulting glycerol is reverted to biomass without lipase production and represses the uptake of fatty acids. During lipogenic phase, the activity remains low and does not exceed 3 U/mL with is mainly due to the production of organics acids that lower the pH of the medium

and inactivate the lipase secretion as well as the catabolic repression exerted owing to the presence of glycerol. It is noteworthy that the activity was slow in the later growth stage, which questioned stability during the culturing or carbon source availability where the substrate was completely consumed at 72 h. This observation was in agreement with Kamzolova et al. (2005) and Papanikolaou et al. (2007), who found that lipase activity significantly decreased when fermentation progressed and the quantity of substrate lipid into the medium decreased (Kamzolova et al., 2005; Papanikolaou et al., 2007). The selected microorganism, thus is a potential candidate for cell growth, lipid and lipase production in the presence of oils.

Thus, olive and vegetable oils from which oleic acid can be released are herein inducers of lipase activity as do other reported oils, such as elaedic and linoleic acids (Hadeball, 1991). The oleic acid induction of gene expression is attributed to activation sequences, such as the oleate response element which exists in the upstream regions of genes encoding enzymes involved in β- oxidation, fatty acid metabolism, and proteins involved in peroxisomal biogenesis (Hadeball, 1991).

Besides, the activity detected in the presence of motor oil was lower as compared to those recorded in the presence of olive oil and vegetable oil (Figure 6.1B).

Thus, the lipase activity is correlated to biomass growth and both work in synergy. In the presence of motor oil, growth and lipase activity was repressed. Moreover, the lipid quantity seemed to be affected by the components present in motor oil. And even the remarkable lipid quantity was detected at 60 h (i.e. 3.33 g/L), possibly coming from the residual motor oil which is not used and bound to cell membrane before extraction. On the other side, the absence of lipase activity in the presence of motor oil may be due to nature and the composition of the oil (Meira et al., 2014), and Mafakher et al. (2010) showed that the lipase activity could be inhibited in the presence of hydrocarbons.

These results lead to consider *Y. lipolytica* as an efficient producer of SCO and make the current investigation base for the use of other hydrophobic residues such tallow derivatives (Papanikolaou et al., 2007) and olive mill wastes (D'Annibale et al., 2006; Finogenova et al., 2008; Imandi et al., 2008; Moftah et al., 2013; Papanikolaou et al., 2008) and also other metabolites of industrial significance such as lipases. As described earlier, the evolution of lipase production was different depending on the lipid material used (i.e. surfactants or oils). The highest lipase activity levels were obtained when olive oil was added to the medium U_{max} = 25.1 ± 4.5 U/mL. Data are in accordance with data reported by Corzo and Revah (1990) and Pereira-Meirelles et al. (1997) which indicates that Yarrowia strains produce high levels of lipase on medium containing olive oil

(Corzo & Revah, 1999; Pereira-Meirelles et al., 1997). These results could be also justified by the fact that *Y. lipolytica* strains display a lipase activity, which acts preferentially on oleyl residues at positions 1 and 3 of the glyceride and the extracellular lipase requires oleic acid as stabilizer/activator (Barth & Gaillardin, 1996). Moreover, in the later stage of growth, biomass continues to gradually increase and cells are using fatty acids as well as the main components of degradation of olive oil to support their growth (Del Río et al., 1990; Pereira-Meirelles et al., 1997).

Herein, due to the presence of motor oil and glycerol, the activity of lipase seemed to be lower compared to the activity recorded in the presence of olive oil and vegetable oil. *Yarrowia* was able to metabolize glycerol which had a repressive phenomenon on motor oil uptake and subsequently lowered the activity to 5 U/mL. Thus, the concentration of glycerol and motor oil should be optimised to avoid such inhibitory effect since the role of glycerol to induce lipolytic activity is not conclusive and authors confirmed that a certain amount of glycerol in the culture medium may not have a repressive nature in relation to the production of lipolytic enzymes. For example, Volpato et al. (2008) observed that maximum lipolytic activity was achieved in the presence of 30 g/L glycerol and 3 g/L olive oil. Moreover, Corzo and Revah (1999), found that the addition of 0.5–6.0 g/L glycerol to the medium did not significantly inhibit the synthesis of extracellular lipase by *Y. lipolytica* 68*1.* Similarly, Fabiszewska et al. (2015) have found an activity in the presence of 30 g/L compared to lower activity at higher glycerol concentration (up to 150 g/L). The upper limit is 37.5 g/L (Volpato et al., 2008). These conclusions are in accordance with the present results, where the activity is recorded at 40 g/L in the presence of olive oil and vegetable oil, and a lower activity is obtained at 40 g/L glycerol and 5% (w/v) motor oil. Thus, a hypothesis has to be evolved to understand the possible inhibitory effect of both substrates on the lipase activity. Besides, the microorganism will start using the simple carbon source available, and later on moving to the degradation of hydrophobic substrate which can justify such results.

The extension of fermentation time up to 150 h decreased the lipid content and the lipid productivity to around 19% (w/w). The main cause of lipid degradation was attributed to the lipase activity recorded at 72 h. However, to the best of author knowledge, with an exception of a recent study (Szczena-Antczak et al., 2006), the simultaneous lipid and lipase production is not frequent.

6.5.2 Use of crustacean waste to enhance more lipase production

Y. lipolytica was also tested for the secretion of lipase and maximum activities were found in the presence of crude glycerol and olive oil, vegetable oil, Tween 80, Tween 20 and Triton X-100 in

the following order. Recent attempts have been made to increase the lipase production via supplementation of medium with highly nitrogenous source. Literature reports use of organic nitrogen source (i.e. urea, peptone, yeast extract) to enhance lipase activity (Fickers et al., 2005a,b; Sharmaa et al., 2001). Thus, crustacean waste (CW) was added into the medium as nitrogen source to improve the lipase production. Usually, the nitrogen content in CW is assumed to be around 7% by weight. (Figure 6.2 A,B) represents the evolution of lipase production in the presence of CW, surfactants and oils.

As seen from (Figure 6.2 A,B), lipase activity drastically increased during the early stage of fermentation and reached a maximum level at 48 h in the presence of different oils and surfactants. A higher lipase activity of 38 ± 3 U/mL is observed in the case of CW and Tween 80 and CW and olive oil. Surprisingly, a higher activity was recorded in the presence of motor oil and CW, where only 5.5 U/mL was obtained. Thus, motor oil composed of hydrocarbons stimulated the lipase production and nitrogen rich source (CW) was required to obtain 30 U/mL at 48 h. The activity continued to be present in the medium and decreased around 72 h. Afterwards, undetectable activities were observed which is in agreement with the previous studies, where no activity was observed after 72 h although the biomass still increased. In the presence of CW, around 38 ± 3 U/mL was obtained and this value was higher than the values reported by *Candida rugosa* ATCC which produced around (2-15 U/mL) (Montesinos et al., 2003) and values reported by Pereira-Meirelles et al. (2000) who obtained extracellular lipase around 5- 8.5 U/mL in media enriched with both yeast extract and peptone. Besides, these activities were significant compared to the activities reported in the presence of 13 g/L glycerol and 10 g/L peptone 1% of olive oil (12.2 U/mL) (Galvagno et al., 2011). Thus, the addition of CW to the culture medium is very promising alternative to enhance the lipase production.

In fact, this is the first report to deal with the production of lipase in the presence of CW and motor oil. Most of the reports so far investigated the possibility of using wastes such as palm oil mill effluent (POME) (Louhasakul et al., 2016; Moftah et al., 2013) and other organic nitrogenous sources (Fickers et al., 2005 a,b; Sharmaa et al., 2001). This bioprocess avoided possible glycerol inhibition on lipase in the case of motor oil where 30 U /mL were obtained. Therefore, the current study provided alternative ways of valorization of CW, by using it as substrate by *Yarrowia* species, in order to produce lipases enzymes, thus, lipases exhibited hydrolytic activities against hydrocarbons, this fact, opens the door for economical process of bioremediation oil spillage contaminated site by enzymatic tools and the optimization of the lipase production should be carried out.

6.5.3 Characterization of lipase produced by *Y. lipolytica*

The effect of pH on lipase produced by *Y. lipolytica* was determined at pH values ranging from 6 to 8 in phosphate buffer (Figure 6.3), this pH was chosen according to the literature where the lipase activity is recorded in this pH range. The activity of lipase increased by increasing the pH up to 7.5 and the optimal showed an activity around 7 which is expected since the optimum pH for growth of *Y. lipolytica* in this study is 6.5. The increase of pH leads to the decrease of the activity of the enzyme by 60%. Besides, the enzyme was stable at pH 6 and 7 and decreased while increasing pH values more than 7. These results are in accordance with Corzo and Revah (1999), who found that the lipase activity of *Y. lipolytica* 681 was higher at pH ranging from 5 to 9 and decreased in basic pH (Corzo & Revah, 1999). Moreover, Brigida et al. (2014) found that lipases from *Y. lipolytica* are active at pH ranging from 6 to 10 and the optimum varied depending on the strain used (Brigida et al., 2014). The effect of temperature on lipase activity has been also studied and investigated at temperature ranging from 25 to 50^{o}C using 50 mM phosphate buffer (pH = 7.0). Results showed that higher activity was observed at 30^{o}C. The maintenance of enzymes in phosphate buffer at pH 7 for 12 h resulted in high thermal stability at temperatures from 25 to 35^{o}C. Such increase in temperature to 50^{o}C resulted in loss of enzyme activity, which justified the effect of high temperature on enzyme denaturation, responsible for its loss of activity. Yadav et al. (2011) reported that the lipase from *Y. lipolytica* NCIM 3639 showed a higher activity at 25^{o}C and its activity decreased at higher temperatures starting from 30^{o}C. However, Yu et al. (2007) showed an optimum of lipase activity obtained at 40^{o}C and decreased at 45^{o}C until no activity was observed at 60 ^{o}C.

6.6 Conclusion

The potential of *Y. lipolytica* SM7 for the biotechnological valorisation of crude glycerol (industrial derivative) to single-cell oil 35% (w/w) and lipase 25 U/mL was investigated. It was found that he use of crustacean residue as nitrogen source can contribute to costs reduction in lipase production as the latter permitted to obtain around 38 U/mL. The lipase so produced showed high thermal stability, activity in a broad range of pH values, with the highest stability at a slightly acidic pH of 6. Further studies on the concomitance production of cited metabolites are purposeful and higher lipase activity can be used for degradation of waste lipids, as well as the synthesis of esters of fatty acids via enzymatic transesterification.

6.7 Acknowledgments

The authors are grateful the Natural Sciences and Engineering Research Council of Canada (Grant A 4984, Strategic Grant-STPGP 412994-11, Canada Research Chair) for their financial support. The views and opinions expressed in this paper are those of the authors.

6.8 References

Aksu, Z., Eren, A.T. 2005. Carotenoids production by Rhodotorula mucilaginosa: use of agricultural wastes as a carbon source. *Process Biochem,* **40**, 2985-91.

Barth, G., Gaillardin, C. 1996. Yarrowia lipolytica, In: Wolf K (ed) Nonconventional yeasts in biotechnology. Springer-Verlag, Berlin, Heidelberg, New York, .

Benjamin, S., Pandey, A. 1996. Optimization of liquid media for lipase production by Candida rugosa. *Bioresour Technol,* **55**, 167-170.

Berto, P., Belingheri, L., Dehorter, B. 1997. Production and purification of a novel extracellular lipase from Alternaria brassicicola. *Biotechnol Lett*, **19**, 533-536.

Bodour, A., Drees, K., Maier, R. 2003. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. *Appl Environ Microbiol,* **69**, 3280-3287.

Boskou, D. 1996. Olive oil composition. In D. Boskou (Ed.), Olive oil chemistry and technology (pp. 52-83). Champaing, IL: AOCS Press. Correa, P. (1988). Epidemiology of gastric cancer. *Rev Cancer*, **2**, 49-52.

Brigida, A.I.S., Amaral, P.F.F., Coelho, M.A.Z., Goncalves, L.R.B. 2014. Lipase from Yarrowia lipolityca: production, characterization and application as an industrial catalyst. *J Mol Catal B Enzym,* **101**, 148-158.

Corzo, G., Revah, S. 1999. Production and characteristics of the lipase from Yarrowia lipolityca 681. *Bioresour Technol,* **70**, 173-180

Cüelik, E., Ozbay, N., Oktar, N., Cüalık, P. 2008. Use of biodiesel byproduct crude glycerol as the carbon source for fermentation processes by recombinant Pichia pastoris. *Ind Eng Chem Res,* **47**, 2985-90.

D'Annibale, A., Sermanni, G.G., Federici, F., Petruccioli, M. 2006. Olive-oil wastewaters: a promising substrate for microbial lipase production. *Bioresour Technol,* **97**,1828-33.

Davidow, L.S., O'Donnell, M.M., Kaczmarek, F.S., Pereira, D.A., DeZeeuw, J.R, Franke, A.E. 1987. Cloning and sequencing of the alkaline extracellular protease gene of Yarrowia lipolytica. *J Bacteriol,* **169**, 4621-4629.

Deive, F. J., Costas, M., Longo, M.A. 2003. Production of a thermostable extracellular lipase by Kluyveromyces marxianus. *Biotechnol Lett,* **25**, 1403-1406.

Del, Río J.L., Serra, P., Valero, F., Poch, M., Sola, C. 1990. Reaction scheme of lipase production by Candida rugosa growing on olive oil. *Biotechnol Lett,* **12**, 835-838.

Fabiszewska, A.U., Stolarzewicz, I., Zamojska, W., Białecka-Florjańczyk, E. 2014. Carbon sources influencing profile of lipases produced by yeast Yarrowia lipolytica. *Appl Biochem Microbiol,* **50**, 404-41.

Fickers, P., Fudalej, F., Le Dall, M.T., Casaregola, S., Gaillardin, C., Thonart, P., Nicaud, J.M. 2005a. Identification and characterisation of LIP7and LIP8 genes encoding two extracellular triacylglycerol lipasesin the yeast Yarrowia lipolytica. *Fungal Genet Biol,* **42**, 264-274.

Fickers, P., Fudalej, F., Nicaud, J.M., Destain, J., Thonart, P. 2005b. Selection of new over-producing derivatives for the improvementof extracellular lipase production by the non-conventional yeast Yarrowia lipolytica. *J Biotechnol,* **115**, 379-386.

Finogenova, T.V., Puntus, I.F., Kamzolova, S.V., Lunina, Y.N., Monastyrskaya, S.E., Morgunov, I.G., Boronin, A.M. 2008. Mutant Yarrowia lipolytica strains producing citric acid from glucose. *Appl Biochem Microbiol,* **4**, 197-202.

Galvagno, M.A., Iannone, L.J., Bianchi, J., Kronberg, F., Rost, E., Carstens, M.R., Cerrutti, P. 2011. Optimization of biomass production of a mutant of Yarrowia lipolytica with a an increased lipase activity using raw glycerol. *Rev Argent Microbiol,* **43**, 218-225.

Gargouri, Y., Julien, R., Sugihara, A., Verger, R., Sarda, L. 1984. Inhibition of pancreatic and microbial lipases by proteins. *Biochim Biophys Acta,* **795**, 326-331.

Goncalves, F.A.G., Colen, G., Takahashi J.A. 2013. Optimization of cultivation conditions for extracellular lipase production by Yarrowia lipolytica using response surface method. *Afr J Biotechnol,* **12**, 2270-2278.

Hadeball, W. 1991. Production of lipase by Yarrowia lipolytica. *Acta Biotechnol,* **11**, 159-167.

Imandi, S.B, Bandaru, V.V.R., Somalankab, S.R., Bandaru, S.R., Garapati, H.R. 2008. Application of statistical experimental designs for the optimization of medium constituents for the production of citric acid from pineapple waste. *Bioresour Technol*, **99**, 4445-4450.

Kamzolova, S.V. , Morgunov, I.G., Aurich, A., Perevoznikova, O.A., Hishkanova, N.V.S, Stottmeister, U., Finogenova, T.V. 2005. Lipase secretion and citric acid production in Yarrowia lipolytica yeast grown on animal and vegetable fat. *Food Technol Biotechnol,* **43**, 113-122.

Kim, J.H., Choi, S.K., Park, Y.S., Yun, C.W., Cho, W.D., Chee, K.M., et al. 2006. Effect of culture conditions on astaxanthin formation in red yeast Xanthophyllomyces dendrorhous mutant JH1. *J Microbiol Biotechnol,* **16**, 438-42.

Kruszewska, J., Palamarczyk, G., Kubicek, C.P. 1990. Stimulation of exoprotein secretion by choline and Tween 80 in Trichoderma reesei QM 9414 correlates with increased activities of dolichol phosphate mannose synthase. *J Gen Microbiol* **136**, 1293-8.

Lee, G.H., Bae, J.H., Suh, M.J., Kim, I.H., Hou, C.T., Kim, H.R. 2007. New finding and optimal production of a novel extracellular alkaline lipase from Yarrowia lipolytica NRRL Y-2178. *J Microbiol Biotechnol,* **17**,1054-7.

Lin, S.F., Chiou, C.M., Tsai, Y.C. 1995. Effect of Triton X-100 on alkaline lipase production by Pseudomonas pseudoalcaligenes F-111. *Biotechnol Lett*, **17**, 959-962.

Louhasakul, Y., Cheirsilp, B., Prasertsan, P. 2016. Valorization of Palm Oil Mill Effluent into Lipid and Cell-Bound Lipase by Marine Yeast Yarrowia lipolytica and Their Application in Biodiesel Production. *Wast Biomass Valor,* **7**, 417-426.

Mafakher, L., Mirbagheri, M., Darvishi, F., Nahvi, I., Zarkesh-Esfahani, H., Emtiazi, G. 2010. Isolation of lipase and citric acid producing yeasts from agro-industrial wastewater. *N Biotechnol*, **27**, 337-340.

Magdouli, S., Brar, S.K., Blais, J. F. 2016. Lipid production by Yarrowia lipolytica grown on biodiesel-derived crude glycerol: Optimization of growth parameters and their effects on the fermentation efficiency. *RSC Adv***, 6**, 90547-90558

Meira, M., Santana, P.M.B., Araújo, A.S., Silva, C. L., Filho, J.R.L.L., Ferreira, H.T. 2014. Oxidative degradation and corrosiveness of biodiesel. *Corros Rev,* **32**, 143-161.

Mlickova, K., Roux, E., Athenstaedt, K., d'Andrea S., Daum, G., Chardot, T., Nicaud, J.M. 2004. Lipid accumulation, lipid body formation, and acyl coenzyme A oxidases of the yeast Yarrowia lipolytica. *Appl Environ Microbiol,* **70**, 3918-3924.

Moftah, O., ŽGrbavčić, S., Moftah, W.A.S., Knežević-Jugović, Z. 2013. Lipase production by Yarrowia lipolytica using olive oil processing wastes as substrates. *J Serb Chem Society*, **78**, 781-794.

Montesinos, J.L., Dalmau, E., Casas, C. 2003 Lipase production in continuous culture of Candida rugosa. *J Chem Technol Biotechnol,* **78**, 753-761.

Najjar, A., Robert, S., Gu'erin, C., Violet-Asther, M., Carriere, F. 2011. Quantitative study of lipase secretion, extracellular lipolysis, and lipid storage in the yeast Yarrowia lipolytica grown in the presence of olive oil: analogies with lipolysis in humans. *Appl Microbiol Biotechnol*, **89**, 1947-1962.

Papanikolaou, S., Chevalot, I., Galiotou-Panayotou, M., Komaitis, M., Marc, I., Aggelis, G. 2007. Industrial derivative of tallow: a promising renewable substrate for microbial lipid, single-cell protein and lipase production by Yarrowia lipolytica. *Electron J Biotechnol,* **10**,425-35.

Papanikolaou, S., Galiotou-Panayotou, M., Fakas, S., Komaitis, M., Aggelis, G. 2008. Citric acid production by Yarrowia lipolytica cultivated on olivemill wastewater-based media. *Bioresour Technol,* **99**, 2419-2428.

Pereira-Meirelles, F., Rocha-Leão, M.H., Sant Anna, G.L.Jr. 1997. A stable lipase from Candida lipolytica: cultivation conditions and crude enzyme characteristics. *Appl Biochem Biotechnol*, **63-65**, 73-85.

Pereira-Meirelles, F.V., Rocha-Leao, M.H.M., Sant'Ana, G.L. Jr. 2000. Lipase location in Yarrowia lipolytica cells. *Biotechnol Lett*, **22**, 71-75.

Rywińska, A., Juszczyk, P., Wojtatowicz, M., Robak, M., Lazar, Z., Tomaszewska, L., Rymowicz, W. 2013. Glycerol as a promising substrate for Yarrowia lipolytica biotechnological applications. *Biomass Bioenergy*, **48**, 148-166.

Saenge, C., Cheirsilp, B., Suksaroge, T., Bourtoom, T. 2011. Potential use of oleaginous red yeast Rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochem,* **46**, 210-218.

Sharmaa, R., Chistib, Y., Banerjeea, U.C. 2001. Production, purification, characterization, and applications of lipases. *Biotechnol Adv,* **19**, 627-62.

Szczesna-Antczak, M., Antczak, T., Piotrowicz- Wasiak, M., Rzyska, M., Binkowska, N., Bielecki, S. 2006. Relationships between lipases and lipids in mycelia of two Mucor strains. *Enzym Microb Technol,* **39**,1214-1222.

Taoka, Y., Nagano, N., Okita, Y., Izumida, H., Sugimoto, S., Hayashi, M. 2011. Effect of Tween 80 on the growth, lipid accumulation and fatty acid composition of Thraustochytrium aureum ATCC 34304. *J Biosci Bioeng,* **111**, 420-424.

Volpato, G., Rodrigues, R.C., Heck, J.X., Ayub M.A.Z. 2008. Production of organic solvent tolerant lipase by Staphylococcus caseolyticus EX17 using raw glycerol as substrate. *J Chem Technol Biotechnol,* **83**, 821-828.

Wei, G., Li Y., Du, G., Chen, J. 2003. Effect of surfactants on extracellular accumulation of glutathione by Saccharomyces cerevisiae. *Process Biochem,* **38**, 1133-1138.

Yadav, K.N.S., Adsul, M.G., Bastawde, K.B., Jadhav, D.D., Thulasiram, H.V., Gokhale, D.V. 2011. Differential induction, purifi- cation and characterization of cold active lipase from Yarrowia lipolytica NCIM 3639. *Bioresour Technol*, **102**,10663-10670

Yu, M.R., Lange, S., Richter, S., Tan, T.W., Schmid, R.D. 2007. Highlevel expression of extracellular lipase Lip2 from Yarrowia lipolytica in Pichia pastoris and its purification and characterization. *Protein Expr Purif,* **53**, 255-263.

Zhang, X., Yan, S., Tyagi, R.D. 2015. Strategies of preserving lipids in microorganism after fermentation. *Bioresour Technol,* **192**, 718-25.

Tableau 6.1 Effect of surfactants on metabolites production of *Y. lipolytica* cultured in crude glycerol media

Surfactants	Biomass (g/L)	Lipid (g/L)[a]	Lipid content (%)	Lipase activity (U/mL)[b]	CA (g/L)[c]
Control	8.70	2.68	30.90	2-4	1.2
Tween 20	16.55	5.48	33.12	12.50	2.2
Tween 80	16.97	5.94	35.06	15.10	3.3
Triton X-100	14.73	5.10	34.62	14.33	3.5

a) the maximum lipid quantity was observed at 60 h.
b) the maximum lipase activity is observed at 72 h.
c) the maximum CA observed at 100 h.

Tableau 6.2 Effect of oils on metabolites production of *Y. lipolytica* cultured in crude glycerol media

Surfactants	Biomass (g/L)	Lipid (g/L)[a]	Lipid content (%)	Lipase activity (U/mL)[b]	CA (g/L)[c]
Control	8.70	2.68	30.90	2-4	1.2
Olive oil	17.12	6.13	35.80	25.10	1.7
Vegetable oil	17.06	5.64	33.06	22.45	2.3
Motor oil	10.25	3.33	32.48	5.50	0.5

a) the maximum lipid yield was observed at 60 h.
b) the maximum lipase activity is observed at 72 h.
c) the maximum CA observed at 100 h.

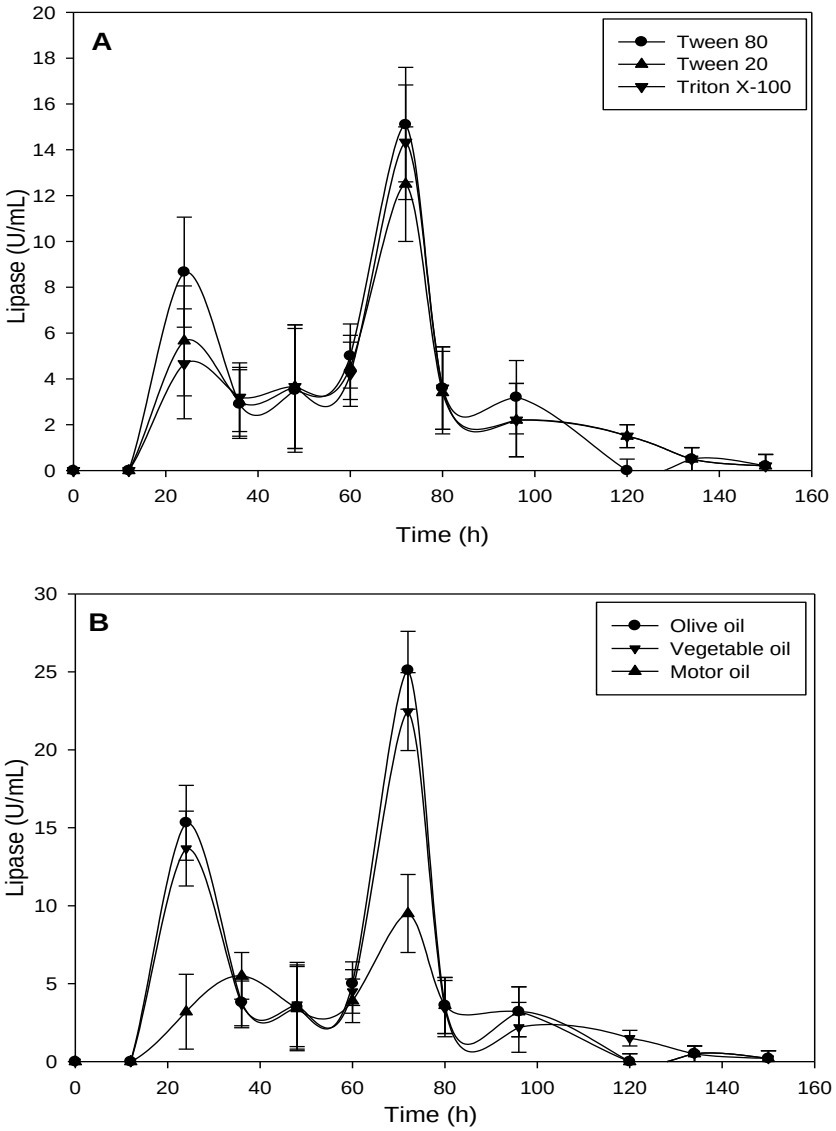


Figure 6.1 **Lipase activity in the presence of surfactants and oils as inducers (A: in the presence of surfactants, B: in the presence of oils)**

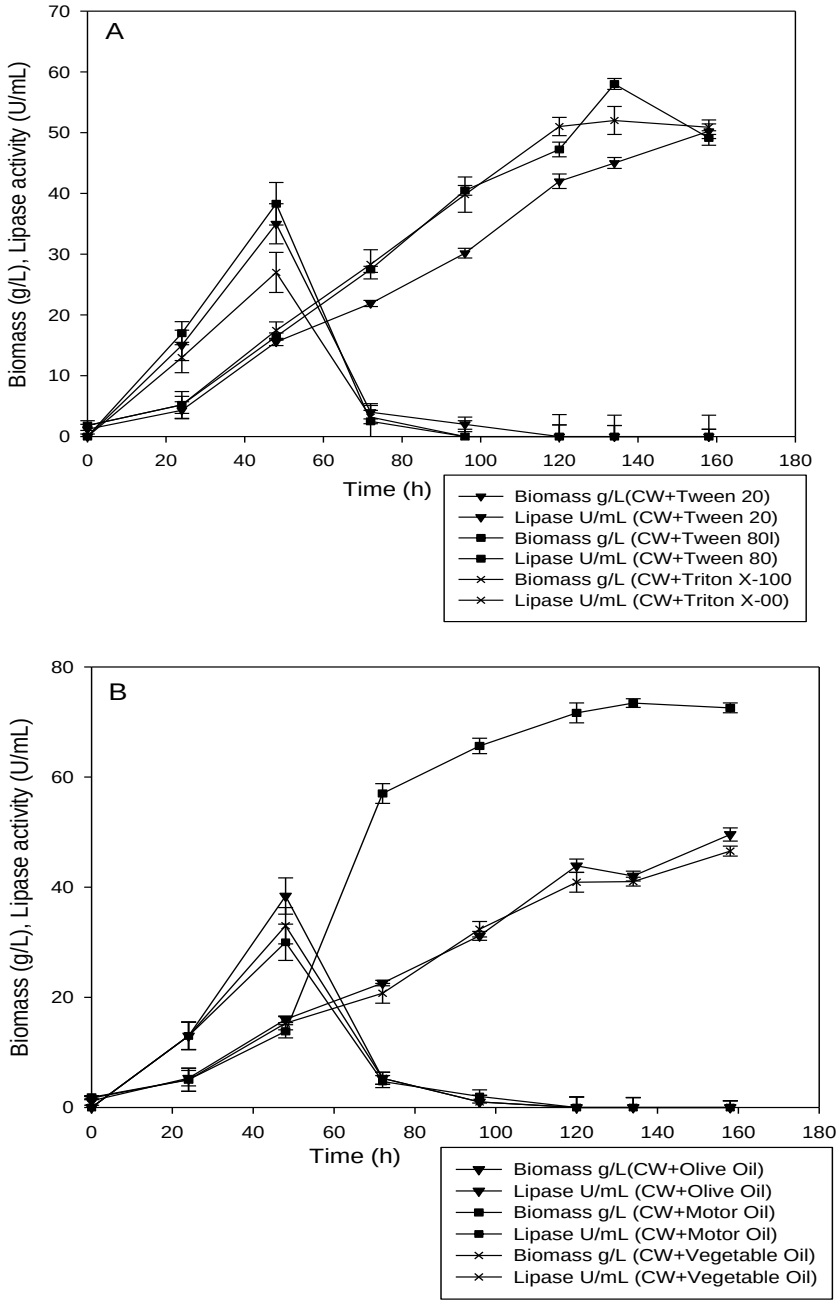
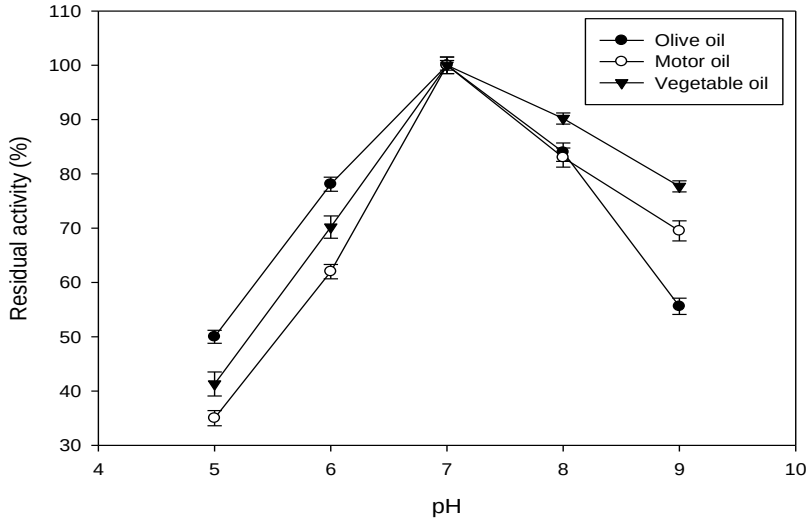


Figure 6.2 **Evolution of biomass production and lipase activity in the presence of crustacean wastes and different inducers (A: in the presence of surfactants, B: in the presence of oils)**



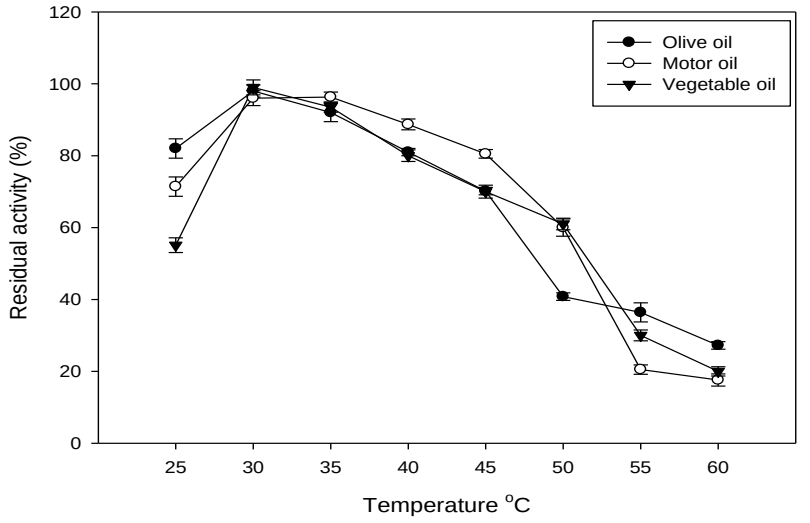


Figure 6.3 **Residual activity of crude lipase as a function of time. Residual activity is expressed as a percentage of the initial activity**

7 CHAPITRE 7: COMPARATIVE STUDY BETWEEN MICROWAVE AND ULTRASONICATION AIDED IN SITU TRANSESTERIFICATION OF MICROBIAL LIPIDS

Comparative study between microwave and ultrasonication aided *in situ* transesterification of microbial lipids

Magdouli Sara[a], Satinder Kaur Brar[b], Jean François Blais[c,*]

[a] Ph.D. student, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-4677, Fax: (418) 654-2600, email: sara.magdouli@ete.inrs.ca

[b] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-3116, Fax: (418) 654-2600, email: satinder.brar@ete.inrs.ca

[c] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-2575, Fax: (418) 654-2600, email: blaisjf@ete.inrs.ca

* Corresponding author: blaisjf@ete.inrs.ca

RSC Advances (2016) 6, 56009

7.1 Résumé

De récents travaux ont mis l'accent sur le développement d'une méthode rapide pour convertir des lipides microbiens en biodiesel. La transestérification *in situ* permet de réduire les besoins en solvants en combinant les deux étapes, l'extraction des lipides et la conversion en biodiesel, en une seule. Un plan de Box-Behnken a été utilisé pour déterminer les variables permettant d'optimiser le rendement et la conversion biodiesel. Deux méthodes de transestérification *in situ* (micro-ondes et ultrasonication) ont été comparées sur la base de leurs rendements de conversion et de leur performance. Avec l'approche des micro-ondes environ 99,0 ± 0,5% de conversion des esters méthyliques (lipides convertis/lipides totaux) a été obtenue en présence d'un ratio molaire de méthanol/lipides de 183:1 et 2% de NaOH pendant 20 min à 100°C. Les résultats avec l'approche des ultrasons ont été d'environ 95,1 ± 0,2% avec le même ratio méthanol/lipides et 3% de NaOH durant 20 min à 25°C. Le profil final des esters méthyliques était entièrement compatible avec celui du procédé classique à base de chloroforme et de méthanol et l'extraction a nécessité 12 h.

Mots-clés : transestérification *in situ*, ultrasonication, micro-ondes, esters méthyliques

7.2 Abstract

Recent trends have focused on the development of a rapid method to convert microbial lipids to biodiesel. *In situ* transesterification allowed minimizing the requirement of solvents by combining the two steps (extraction of lipid and conversion to biodiesel) to a single step. Box–Behnken design was used for optimization of the variables to optimize the biodiesel yield and conversion. Microwave and ultrasonication assisted *in-situ* transesterification methods were compared based on the conversion efficiencies and their performance. Microwave approach revealed that around 99.0 ± 0.5% of conversion of FAMEs (w lipid conversion/w total lipids) was obtained in the presence of methanol to lipid molar ratio above 183:1 and NaOH addition of 2% (w/w) lipid in 20 min at 100^{o}C. Meanwhile, the ultrasonication yielded around 95.1 ± 0.2% (w/w total lipids) in the presence of methanol to lipid molar ratio 183:1 and NaOH addition 3% (w/w) lipid in 20 min at 25^{o}C. The final profile of FAMEs was fully compatible with that of the conventional process based on chloroform and methanol extraction and required 12 h for extraction.

Keywords: In situ transesterification; Ultrasonication; Microwave; Fatty acids methyl esters (FAMEs)

7.3 Introduction

The gradual depletion of fossil fuel reserves and the continued use of petroleum-based fuels have encouraged researchers to seek viable, sustainable, and environmental friendly alternative sources of energy (Pinto et al., 2005; Chisti, 2007; Vasudevan & Birggs,2008). The exploitation of vegetable oils for biodiesel production had created numerous problems of food supplies and arable lands. Therefore, microbial oils called single cells oils (SCO) are considered to be a viable alternative since they do not have an impact on food supply and they do not require arable lands and could replace fossil fuels (Shen et al., 2009). Many technical hurdles limit the use of these renewable source on large scale, especially, harvesting and extraction processes. Lipid extraction from oleaginous microorganisms required large amounts of organic solvents. Commonly, Folch method or its variant, the Bligh and Dyer method, have been used extensively for lipid extraction and quantitation (Christie, 2013).

However, owing to the hazardous nature of extraction using flammable organic solvents, and the adverse impact of solvent on the environment, it is strongly recommended to reduce the organic solvents and time of the extraction process. Terpenes, green solvents obtained from plants have been investigated as a replacement of organic solvents, although their efficiency and high costs limit their potential uses (Tanzi et al., 2013). An ideal solution was to perform both extraction and transesterification processes simultaneously in one step thereby eliminating the solvent extraction step required to obtain the oil feedstock. In-situ transesterification refers to the direct transesterification of lipids in a biomass matrix without prior lipid extraction and offers the advantage of reducing processing units, lowering the fuel product costs and later quantifying fatty acids. Besides, process wastes and eventual pollution could also be reduced by this method (Ehimen et al., 2010). Moreover, several methods are listed in literature (e.g. solvent, enzymatic, mechanical, alkali, acid); however, not all were applicable due to their relatively high cost and equipment corrosion. Besides, there is no definitive standard method for neither lipid extraction nor quantification, nor for process development (Jacob, 1992). Current works were based essentially on lipid extraction from algal species (Suzuki et al., 1973; Jin et al., 2012; Pedersen, 1962).

Consequently, choosing a relevant method and optimizing its parameters was the main challenge. Microwave-assisted *in situ* transesterification could be an alternative to address the above concerns. This method allowed cell disruption and enhanced mass transfer rates (Kanitkar, 2010), which may result in high oils and lipids recovery. Microwave irradiation has been reported to extract oil derived biomass, soils and vegetable feedstock (Barnabas et al., 1897; Kiss et al.,

2000; Pordesimo, et al.,2004; Lucchesi et al.,2004; Hernando et al., 2007). Besides, this method allowed good quality of extracts with better target compound recovery.

A process that enables simultaneous oil extraction and transesterification is thus worthwhile to develop. Response surface methodology (RSM), a multivariate technique, was used in this work to optimize the levels of different variables (e.g. temperature, reaction time, catalyst concentration, and different methanol to lipid molar ratios.) reported highly critical in the *in situ* transesterification process. An optimum yield of FAMEs was envisaged. The analyses were performed on lyophilized biomass. Several trials were conducted to optimize the parameters related to this study. Besides, the impact of ultra-sonication aided in-situ transesterification on FAMEs composition was also investigated.

7.4 Material and methods

7.4.1 Crude glycerol, reagents and analyses

All the reagents were of analytical grade and used without further purification. Methanol, hexane and NaOH were purchased from Fisher Scientific, Canada. Crude glycerol was obtained from Rothsay in Canada. Ultra-sonication experiments were conducted with ultrasonic processor CPX 750 (Cole-Parmer Instrument, IL) at 24 kHz. Microwave trials were carried out with MARS microwave extractor, CEM Corporation, North, 155 Carolina, USA) equipped with Teflon tubes irradiated simultaneously. FAMEs were analyzed using a Gas Chromatograph linked with Mass Spectroscopy (GC–MS) (Perkin Elmer, Clarus 500). The dimensions of the column used are 30 m, 0.25 mm, with a phase thickness of 0.2 L/m. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1.3-dichlorobenzene was used as internal standard at 50 ppm.

7.4.2 Strain, culture and harvesting conditions

The strain, *Trichosporon oleaginosus* (ATCC20509) was grown in a glycerol based medium containing (per liter): 1 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.2 g yeast extract, 50 g glycerol, and minerals 0.04 g $CaCl_2.2H_2O$, 0.0055 g $FeSO_4.7H_2O$, 0.0052 g citric acid·H_2O, 0.001 g $ZnSO_4.7H_2O$, and 0.00076 g $MnSO_4.H_2O$ were added (Zheng et al., 2012). Experiment was performed in 5 L fermenter at pH 6.5 and 28°C. pH was controlled by the addition of 4N (NaOH and H_2SO_4). After 70 h, the biomass was harvested by centrifugation at 5,000 x *g* for 15 min. Biomass was washed twice with distilled water to remove the residual nutrients and glycerol. The experimental method is shown in Figure 7.1.

7.4.3 Conventional extraction and transesterification method

Extraction was carried out at room temperature using the standard chloroform and methanol extraction procedure (Folch and Sloane,1957; Vicente et al., 2009). About 0.2 g dry biomass resulting from the fermentation of *T. oleaginosus* after 72 h was mixed with 4 mL solvent mixture of chloroform and methanol (2:1 (v/v)), and then subjected to 60°C for 4 h. The mixture was then centrifuged at 5000 x *g* for 15 min and the solvent phase was withdrawn and transferred into a pre-weighed glass vial (W1). The extraction procedure was repeated two times. Afterwards, the vial containing the total volume of the supernatant collected from each extraction was subjected

to 60°C in an oven to evaporate the solvents and was then weighed (W2). The lipid amount was calculated by the difference of W2 and W1. The lipid content in the biomass is calculated as (W2-W1)/200 mg × 100%. The obtained lipid was first dissolved in hexane (25 mL hexane per gram lipid), then mixed with methanol. Lipid to methanol molar ratio is 1:6 (0.3 mL methanol for per gram lipid). Sodium hydroxide was used as catalyst with addition of 1% (w/w) (NaOH/oil). The mixture was then subjected to 55^{o}C for 2 h. After reaction, 5% (w/v) NaCl solution was added (100 mL NaCl solution per gram lipid), and then FAMEs was extracted by two times washing with hexane (100 mL per gram lipid). After phase separation by settling, the hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% sodium bicarbonate solution (20 mL per gram lipid) and the mixture was allowed to stand for 15 min for phase separation, and the top layer was collected and dried at 60 ± 1^{o}C in an oven (Halim et al., 2011).

7.4.4 Ultrasonication aided transesterification

Amounts of methanol and NaOH catalysts corresponding to mL equivalent of methanol/oil ratio (6:1, 183:1, 360:1) relating to 0.08, 2.45, 6.4 mL were added to 0.2 g of dry biomass and then reacted with a sonication probe immersed directly in the solution in a beaker placed in a water bath to control temperature at around 25^{o}C for 20 min. Thermal meter was inserted to the bath to check the temperature. The sonication time was fixed at 20 min with one pause (2 min) at every 5 min sonication, and methanol to oil ratio was set at 60:1 - 360:1 (v/w). Amount of catalyst was varied from NaOH catalyst at 1 to 5% (w/w).

7.4.5 Transesterification aided by microwave heating

Transesterification reactions were carried out in the presence of NaOH catalyst (1 to 5% (w/w) at various reaction temperatures (40-100^{o}C). The catalyst was dissolved in methanol (6:1 – 360:1) (v/w) and the resulting solution was added to the oil. This reaction was then irradiated by microwave field under reflux and heated to the desired transesterification temperature in desired time. Power output of microwave was 400 W. An aliquot of 25 mL of the hexane was added to each vessel. This reaction was then irradiated by microwave field under reflux and heated to the desired transesterification temperature.

7.4.6 *In situ* transesterification with microwave

A pre-determined mass of 0.2 g of biomass was weighed accurately into each teflon vessels. Corresponding percent of methanol and NaOH was added separately to each vessel. The microwave power was set to 400 W. The temperature was kept at ambient (± 25ºC and the time was set to an initial 15 min ramp with 15 min hold time and a final 15 min cooling time). After the transesterification, vessels were removed, 5% w/v NaCl solution was added (1 mL per gram biomass) and all samples filtered using Whatman filter paper to remove the residual biomass and the solvent was evaporated. The collected samples were allowed to stand overnight or (centrifugation (5,000 x *g*, 20 min). A small aliquot of the supernatant was siphoned off and transferred to a vial for gas chromatographic analysis.

7.4.7 Two-stage process

The extractive-transesterification experiments were conducted using microwave radiation. In the two-step production, transesterification was carried out on the lipid previously extracted from dry biomass with chloroform/methanol (e.c. conventional method), then using microwave and ultrasonicator, following the transesterification (described and presented in Figure 7.1).

7.4.8 Optimization of *in situ* transesterification by Box–Behnken design (BBD)

A 4-level 4-factor Box–Behnken design was adopted to evaluate the effects of temperature (X1), reaction time (X2), methanol to lipid molar ratios (X3), catalyst concentration (X4), and lipid conversion efficiency of *T. oleaginosus* on crude glycerol based medium. In this regard, the experimental plan contained 29 trials and the independent variables were studied at three different levels, namely low (-1), medium (0) and high (+1), whose values are shown in Table 7.1.

The effect of the three factors and their interactions were studied using the response surface methodology (Myers & Montgomery, 2002). Based on experience and economic feasibility, a three factorial subset design was employed (Gilmour, 2006). The total number of experimental runs was 29 with replications as shown in Table 7.1. The temperature, time, methanol to oil ratio and catalyst were varied in the ranges of 40-100ºC, 20-60 min, 6:1-360:1 (v/w), 1-5% (w/w) respectively. The lipid conversion efficiency was taken as the response variable (Y). The experimental design used in this work is shown in Table 7.1. The response variable was fitted by a second order model to correlate the response variables to the independent variables. The

second order polynomial coefficients were calculated and analyzed using the 'Design Expert' software (Version 7.0, Stat-Ease Inc., Minneapolis, USA). The general form of the second degree polynomial equation is:

Équation 7.1 $Y= \beta_0+ \sum \beta_i X_i+\beta_{ij}X_iX_j+\sum\beta_{ii}X_i^2$

Y: the predicted lipid conversion efficiency (% w/w lipid)

β_0: the intercept

β_i: the linear coefficient

β_{ij}: the quadratic coefficient

β_{ii}: the linear-by-linear interaction between Xi and Xj regression coefficients

Xi, Xj: input variables

Statistical analysis of the model was utilized to evaluate the analysis of variance (ANOVA). This analysis englobed Fisher's F test (overall model significance), associated probability *p (F),* correlation coefficient R and determination coefficient R^2. All parameters play role in measuring the goodness of fit of regression model. Quadratic models were used for each variable and were represented as contour plots (3D). Response surface curves were generated using Design Expert software.

7.5 Results and discussion

7.5.1 Statistical analysis of experimental design

The conventional extraction method which consisted of a mixture of chloroform/methanol (2:1 (v/v)) provided lipid content of 47.3 ± 0.9% (w/w) of dry biomass. This percentage is considered as 100% of conversion of biomass to lipid. The lipid conversion efficiency to fatty acids methyl esters (FAMEs) is calculated by determining amount of FAMEs by GC–MS and dividing this value by total lipids (g FAMEs/g total lipids). Many parameters have been reported to control the lipid efficiency including (e.g. the amount of catalyst added, reaction time, temperature and molar methanol to lipid ratio).

The statistical significance of the designs was determined by F-test for ANOVA (Table 7.3). As seen from this table, operating parameters had a significant effect on the fatty acid methyl ester content which is confirmed by the p-values of the analysis. Values of "Prob > F" are less than 0.05 which indicated that the model is significant with 98.54% confidence level. Therefore, the P-value of the lack of fit analysis was (< 0.0001) which confirmed that the model was significant and reliable for lipid production in this study. Besides, correlation coefficient, R^2 (0.989) supported the correlation between the *in situ* transesterification process parameters.

The value of adj-R^2 (0.979) suggested that the total variation of 97.99% for the lipid concentration was attributed to the independent variables and only about 3.01% of the total variation could not be explained by the model. Besides, model coefficients for each variable are also shown in Table 7.2. The larger F-value and smaller P-value suggested higher significance of the corresponding coefficient. Among the model terms, X1 (temperature), X3 (methanol/oil ratio), $X1^2$, $X3^2$ were significant. By contrast, other terms were not significant. The relationship between the response and experimental levels of each variable can be demonstrated by three-dimensional response surface plots which represented the regression equation mentioned below:

Équation 7.2 $Y= 82.5 + 17.08333\ X1 + 0.066666\ X2 + 28.316666\ X3 + 0\ X4 – 1.625\ X1X2 + 6.4\ X1X3 -2.025\ X1X4 - 0.475\ X2X3 + 0.65\ X2X4 -1.525\ X3X4 – 9\ X1^2 - 0.05\ X2^2 - 27.675\ X3^2 - 0.175\ X4^2$

where Y is the observed response (lipid conversion efficiency) for the microwave in-situ transesterification. X1, X2, X3 and X4 are the coded values of independent factors temperature, reaction time, methanol to oil molar ratio and catalyst amount, respectively.

7.5.2 Optimization of microwave process parameters with RSM

In conventional method of biodiesel synthesis, the reaction time and temperature are 30 min–12 hours and 55–65°C, respectively (Meher et al., 2006; Zhang et al., 2014; Rashid et al., 2009). Besides, Melo-Junior et al. (2009) have studied in detail the esterification of oleic acid (C18) under microwave irradiation while varying alcohol type (methanol or ethanol), temperature (150-225°C) and molar ratio of alcohol/fatty acid (3.5-20), a conversion rate up to 60% was obtained in 60 min of reaction. In this regard, present study was carried out to optimize different parameters in the microwave assisted direct transesterification; reaction temperature, time, methanol to oil molar ratio and catalyst amount were chosen as variables. To compare the temperature effect on the conversion yield, in-situ transesterification was conducted at 40, 80 and 100^{o}C. Thus, according to literature, when using a homogeneous catalyst (herein NaOH), harsher condition including high temperature (Lotero et al., 2005), is required to achieve high FAMEs yields. Besides, preliminary study has showed that only 14.5 ± 1.2% of FAMEs were obtained under low temperature at 25^{o}C. Conversely, higher conversion efficiency above (90.0 ± 1.2% (w/w)) was obtained in a lower reaction time 20 min at 100°C. Microwave effect at 100^{o}C was four fold compared to 40^{o}C which confirmed the positive role of temperature (low p value < 0.0001). At 70^{o}C, around 83.0 ± 0.6% of FAMEs (w/w) was obtained. Therefore, higher the reaction temperature, the more the reaction can be driven. This is in accordance with Im et al. (2014) who proved the positive effect of temperature on FAMEs yield, around 91.1% was obtained at 95^{o}C for 90 min. Moreover, Sunita et al. (2008) have observed that the conversion rate of oil to biodiesel increased significantly with the rise in temperature and was reported to be 73% and 97% at 180 and 200^{o}C, respectively. Moreover, a complete conversion (100%) of caprylic acid for the esterification was achieved at a higher temperature, 175–200^{o}C (Furuta et al., 2004; López et al., 2008). High temperature may lead to the formation of microzones called “hot spots”, which lead to an increase in the escalation of chemical reaction rate (Manco et al., 2012). The loss of methanol was not seen in this study compared to current studies (Zhang et al., 2014; Eevera et al., 2009; Leung & Guo, 2006), this is mainly due to nature of the closed system that resists higher temperatures. Both high temperature and thermal effect caused by the microwaves enhanced the extractive properties of methanol to extract more lipids in the biomass via diffusive extraction and extended microwave effect caused

the penetration through the cell walls and forces out the oils into the solvent mixture through disruptive extraction. Another observation to be taken in advantage from this work is the absence of emulsions and soap formation which is primarily related to the high temperature effect, thus, free fatty acids (FFA) are converted efficiently into FAMEs, which has been proven in previous studies that noted the role of microwave irradiation in the reduction of FFA content within the first 15 min (Suppalakpanya et al., 2010). Furthermore, Kamath et al. (2011) reported around 87.39% of FFA reduction during the transesterification of crude karanjja oil through microwave irradiation. No soap formation is principally due to absence of the catalytic poisoning by water formed as a result of esterification, so that microwaves and high temperature reduced the free fatty acid content and made it easier to separate biodiesel and alcohol layers. As seen in Figure 7.2, catalyst more than 3% (w/w) showed a positive effect on the in situ transesterification reaction. Herein, NaOH is used as a homogeneous, solvent-catalyst; the choice of this catalyst rather than others is related to its higher yield of biodiesel conversion rates (Refaat et al., 2008) and its ability to break chemically the molecule of the raw renewable oil into methyl or ethyl esters. Highest biodiesel conversion of 93.9 ± 0.3% was observed using 3% (w/w) of NaOH catalyst with methanol to oil ratio of 183:1. Conversely, the lower amount of catalyst (proportional to methanol ratio 6:1) may not efficiently advance the reaction and gave a yield of 24.5 ± 0.1% (w/w) of conversion rate.

Methanol to lipid ratio had a significant effect on the *in situ* transesterification, and this was confirmed with a low p value < 0.0001. Herein, methanol exhibited binary action and acted as a solvent for extraction of the microbial oils/lipids and a reactant for transesterification of esters (Mulbry et al., 2009). Thus, applying microwave irradiation during *in situ* transesterification will serve for dual purpose (e.c. rendering lipids available for reaction as well as intensification of process).

Methanol to oil molar ratio was varied from 6:1 to 360:1 in the microwave direct transesterification reaction. A lower ratio than 6:1 (v/w) does not favor the *in situ* transesterification process and a lower yield is observed. When the methanol to oil molar ratio was increased to 183:1, the maximum biodiesel conversion observed was 92.3 ± 1.0% because of the increased contact area between methanol and oil/lipid. This is in accordance with Sunita et al. (2008) who found that increasing methanol to oil ration from 10:1 to 20:1 enhance the conversion of sunflower oil to biodiesel from 30% to 90%, respectively.

Further increase of molar ratio up to 360:1 did not give significant difference. Generally, a higher amount of methanol may reduce the concentration of the catalyst in the reactant mixture and does

not give higher yield during the transesterification reaction (Zhang et al., 2010). Moreover, with a lower methanol ratio, the downstream cost can be controlled (Stiefel & Dassori, 2009).

The reaction time of around 20 min seemed to be adequate for the complete process. The reaction time had no significant effect (*p* value = 0.9510) on the FAMEs content at higher temperature and even time can be further reduced. Generally, extended reaction times allowed higher exposure of microwave irradiations to the reaction mixture which resulted in higher efficiency of extraction and biodiesel conversion.

From the above analysis, the optimum given by the model to achieve a maximum of lipid conversion efficiency was 183:1 of methanol ratio with 2% of catalyst amount (w/w) and at temperature higher than 80˚C, around (99.0 ± 0.5% w/w total lipids) in minimum time required 20 min.

7.5.3 Comparison of microwave vs. ultrasonication for in situ transesterification

As discussed earlier, the biggest issue during *in-situ* transesterification is the requirement of large volumes of solvent and longer reaction time. During microwave process, 183:1 (w/w) and 20 min was the optimum condition for lipid extraction and high biodiesel recovery. For this purpose, ultrasonification has been also tested for its efficiency regarding biodiesel conversion. Accordingly, ultrasonification has been carried out to achieve higher yields of conversion during esterification and transesterification. High conversions yields were reported for converting algal oils and vegetable oils which allowed reduction in the reaction time (Hobuss et al., 2012). This approach was highly dependent on temperature and other operating parameters. Around 97.3% was obtained during conversion of palm oil in 45 min at 60˚C with 0.3% KOH (Lima et al., 2012) and higher temperature (>60◦C) was less effective during the conversion step. In the present study, ultrasonification is carried out in an open system which results in methanol evaporation. Besides, higher temperatures during ultrasonification were reported to lower FAMEs content (Eevera et al., 2009; Leung & Guo, 2006). Although, higher temperatures are required for harsh extraction in the microwave as reported in the previous section, Parkar et al. (2012) reported that physical effects of cavitation bubble dynamics in ultrasound assisted transesterification are more pronounced at lower temperature of 15ºC, albeit the low conversion yield of 13.45%. Hence, the temperature was fixed to 25ºC (neither high nor low). Herein, *in situ* transesterification using ultrasound was optimized considering catalyst amount, methanol to oil molar ratio, and reaction time as reaction parameters. The optimisation of different variables is given in Table 7.4. The

model was highly significant (R^2 = 0.998). This indicates that model cannot explain only 0.01% of the total variations which shows that the model fits quite well. Moreover, *p* value for the model was lower than 0.05, which confirms the statistical relation between the response and selected factors. This shows that regression analysis is statistically significant. Therefore in this model, most significant factors are methanol to oil molar ratio, ($p < 0.0001$) followed by catalyst amount ($p = 0.114$) and reaction time ($p = 0.680$).

Akin to microwave approach, catalyst amount of 1, 3 and 5% (w/w) were considered. Besides, beyond 5% (w/w) catalyst, no further increase in the conversion of the oil to biodiesel could be achieved as the reaction was limited by mass transfer. Maximum biodiesel conversion of 95.0 ± 0.5% (w/w) was observed using 5% (w/w), the catalyst in the presence of high methanol ratio 183:1. As seen in Table 7.3, it can be found that the efficiency of lipid conversion via ultrasonicator equipment (20 kHz, 700 W) increased with the increase of methanol to oil ratio and catalyst amount (%). P values were around (< 0.0001) and (0.1140) for methanol to oil ratio and catalyst amount which justified their positive influence on the lipid conversion. Around 90.1 ± 2.2% (w/w total lipids) was attained in 20 min with 183:1 methanol to oil ratio (w/w). Higher conversion efficiency shown by ultrasound could be attributed to increased mass and heat transfer provided by the physical and chemical effects during intensification of reaction (Gole & Gogate, 2012). Another observation to be pointed out by the present study is the formation of emulsions due to the reaction of catalyst with methanol. NaOH leads to water formation which slows the reaction rate and causes soap formation (Saifuddin et al., 2015). Thus, the FAMEs mixture remains in emulsion for more than 12 h. For that purpose, hexane was added and the mixture was filtrated and then allowed to stand for 15 min. Thereafter, the top layer of FAMEs in hexane was collected for quantification. However, at 100^{o}C with microwave irradiation, this problem was resolved since with closed vessels (under controlled pressure and temperature), the solvent can be heated above its normal boiling point, the fact that enhanced extraction efficiency and speed (Veggi, et al., 2013). Therefore, short reaction time, cleaner reaction product, and reduced separation-purification times are the key observations in this the present study.

For a conventional method, reaction time for the transesterification was assumed to be 12 h. In contrast, with the microwave and ultrasounds, the time was reduced to 20 min. Herein, microwave-assisted reactions may reduce not only the time but also eliminate the need for the catalyst, however, higher reaction temperatures are required (Geuens et al., 2008; Harun et al., 2010). During this process, microwaves interacted with triglycerides and methanol present in the mixture which resulted in increased of interfacial polarization (a combination of ionic conduction

and dipolar momentum) and ionic conduction (Kanitkar, 2010; Wei et al., 2008; Gude et al., 2013). These two reactions are the major causes of superheating phenomenon which is observed at elevated temperatures and led to a large reduction of activation energy with a high diffusivity of the solvent into the internal parts of biomass. Thus, methanol is defined to be a strong microwave absorber and the presence of an -OH group attached to biomass matrix behaves as though it was anchored to an immobile raft, so localized rotations result in localized superheating and the reaction may occur rapidly (Tierney & Lidstrom, 2005). Consequently, desorption of intracellular components (lipids droplets) from the active sites of the biomass matrix was enhanced.

When compared to microwave method, ultrasonic-assisted extraction uses cavitation process to recover oils from microbial cells. Resulting bubbles during this process collapse near cell walls so that the cell contents are released (Harun et al., 2010; Wei et al., 2008; Giroud et al., 2013). The ultrasonic waves had a significant effect on cell disruption. A cavitation process is resulted due to the higher pressure and shear on the cell walls which contributes to the formation of free radicals of reacting species (Rokhina et al., 2009). Accordingly, ultrasound permits the formation of highly reactive radicals through dissociation of entrapped vapor molecules in the bubble, which are subjected to extreme conditions generated at the collapse of the bubble. In ultrasound assisted direct transesterification, cavitational effect caused by turbulence in reaction medium and free radicals are responsible for process intensification (Gogate et al., 2011).

During two-stage of conventional transesterification, around 93.8 ± 1.3% (w lipid/w total lipids) was achieved with methanol to lipid molar ratio 6:1 in the presence of NaOH amount 1% (w/w) lipid during 2 h, however, under similar conditions, only 3.0 ± 0.2% (w lipid/w total lipids) was obtained in *in-situ* transesterification (one stage). To obtain higher efficiency, the increase of methanol to oil ratio above 360:1 and NaOH above 5% (w/w) were required, thus, more than 90.4 ± 1.5% was achieved during 12 h. It is clear that *in-situ* tranesterification required much larger amount of methanol and NaOH catalyst and far longer time to achieve similar lipid conversion yield than two stage transesterification process. These higher requirements during transesterification are due to the nature of cell wall that make barrier to solvent to access and extract lipid droplets from intracellular compartment. So more solvent is required to weaken, disrupt and penetrate into cell walls. In this regard, *in-situ* transesterification is preferable to overcome these hurdles.

In the presence of microwave irradiation, transesterification was carried out in two stages and around 98.5 ± 0.5% (w/w) was obtained at 100^{o}C in the presence of 1% (w/w) catalyst and 183:1 (% v/w) of methanol ratio. With ultrasonication method, a higher conversion efficiency of

94.1 ± 0.1% was achieved under same conditions at 25^{o}C. Therefore, transesterification carried in two stages with microwave irradiation or ultrasonication bubbles have the advantage to reduce the longer time and the large amount of catalyst.

In the present study, microwave assisted direct transesterification showed higher efficiency than ultrasound assisted in-situ transesterification. Taken together, both approaches reduce the time, catalyst amount and energy requirements (Table 7.4). However, main obstacle for commercial application of these intensification methods is their scale up challenges. More research is required for successful implementation of these methods for direct conversion of microbial biomass to biodiesel at commercial scale. Besides, possible recovery of the catalyst from the residual biomass and its reuse needs more attention from the researchers. In this regard, future direction of research ought to focus on the process improvisation, catalyst recovery and reuse.

7.5.4 Comparison of composition of FAMEs from different transesterification processes

The analysis of the FAMEs composition is presented in Table 7.6. Microwave *in situ* transesterification process with a molar ratio of 183:1 at 100 ^{o}C favored a higher content of C18:2. Similar results were observed during ultrasonication aided *in-situ* transesterification at 25 ^{o}C, in 20 min and with a methanol to oil ratio of 183:1. Meanwhile, a lower C16:0 and C18:1 was observed. In fact, a lower molar ratio favored the production of phospholipids present in cell membrane (Giroud et al., 2013). On the other hand, higher methanol: oil ratio disrupted cells and allowed more contact with lipid droplets and major FAMEs belonged to intracellular lipids. The composition of FAMEs from two stage transesterification, conventional *in-situ* transesterification, microwave *in-situ* transesterification and ultrasonication *in-situ* transesterification were almost similar.

7.6 Conclusion

The production of single cell oils and their conversion process to biodiesel are of wide interest in fuel market. Lyophilized biomass of *T. oleaginosus* was utilized for the production of biodiesel using two means of in-situ transesterification: microwave technique and ultrasonication. Among the two methods, microwave was found to give higher conversion efficiency to biodiesel amounting to 99.0 ± 0.5% (w/w) total lipids as compared to 95.0 ± 0.2% (w/w) total lipids with ultrasonication assisted technique. Another advantage of microwave assisted transesterification is the absence of emulsions during the whole process, the fact that reduce the separation time obtained (> 99% reduction in separation time), and all with a reduced energy consumption, meanwhile, a low reaction temperature (25^{o}C) was required for transesterification during ultrasonication method that will reduce the cost of production of biodiesel. Taken together, both approaches revealed that methanol: hexane efficiently converted FAMEs compared to conventional process which relied on chloroform: methanol 2:1 (v/v) and hexane mixtures and required more catalyst and more time to obtain the desired conversion efficiency. The *in-situ* transesterification process proved to be faster and easier method to produce biodiesel with lower catalyst 1% (w/w) and in short time of 20 min. Overall, microwave *in-situ* transesterification would be a promising alternative of the current two-stage transesterification process and combining the effects of the microwave and ultrasonic energy via hybrid reactor can be innovative and beneficial at large scale.

7.7 Acknowledgements

Special thanks are due to the Natural Sciences and Engineering Research Council of Canada (Grant A 4984, Strategic Grant-STPGP 412994-11, Canada Research Chair) for their financial support. The views and opinions expressed in this paper are those of the authors.

7.8 References

Barnabas, I.J., Dean, J.R., Fowlis, L.A., Owen, S.P. 1995. Extraction of polycyclic hydrocarbons from highly contaminated soils using microwave energy, *Analyst,* **120**, 1897-1904

Chisti, Y. 2007. Biodiesel from microalgae, *Biotechnol Adv*, **25**, 294-306

Christie W.W. 2013. James Hutton Institute and Mylnefield Lipid Analysis.

Dejoye Tanzi, C., Abert Vian, M., Chemat, F. 2013. New procedure for extraction of algal lipids from wet biomass: a green clean and scalable process. *Bioresour Technol*, **134**, 271-275

Eevera, T., Rajendran, K., Saradha, S. 2009. Biodiesel production process optimization and characterization to assess the suitability of the product for varied environmental conditions, *Renew Energy,* **34**, 762-765.

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

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

Furuta, S., Matsuhashi, H., Arata, K. 2004. Biodiesel fuel production with solid superacid catalysis in fixed bed reactor under atmospheric pressure, *Catal Commun*, **5**, 721-723.

Geuens, J., Kremsner, J.M., Nebel, B.A., Schober, S., Dommisse, R.A., Mittelbach, M., Tavernier, S., Kappe, C.O., Maes, B.U.W. 2008. Microwave-assisted catalyst-free transesterification of triglycerides with 1-butanol under supercritical conditions, *Energy Fuels*, **22**, 643-645.

Gilmour, S.G. 2006. Response surface designs for experiments in bioprocessing, Biometrics, **62**, 323-331.

Giroud, S., Frare, C., Strijkstra, A., Boerema, A., Arnold, W., Ruf, T. 2013. Membrane Phospholipid Fatty Acid Composition Regulates Cardiac SERCA Activity in a Hibernator, the Syrian Hamster (Mesocricetus auratus). PLoS One, **8**, 1-9.

Gole, V.L., Gogate, P.R. 2012. Intensification of synthesis of biodiesel from non-edible oils using sonochemical reactors. *Ind Eng Chem Res,* **51**, 11866-11874.

Gogate, P.R., Sutkar, V.S., Pandit, A.B. 2011. Sonochemical reactors: important design and scale up considerations with a special emphasis on heterogeneous systems *Chem Eng J*, 1**66**, 1066-1082.

Gude, V.G., Patil, P.D., Martinez-Guerra, E. Deng, S., Nirmalakhandan, N. 2013. MW energy potential for biodiesel production. *Sustain Chem Process*,**1**, 1-31.

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

Harun, R., Singh, M., Forde, G.M., Danquah, M.K. 2010. Bioprocess engineering of microalgae to produce a variety of consumer products, *Renew Sust Energ Rev*, **14**, 1037-1047.

Hernando, J., Leton, P., Matia, M.P., NovellaL, J.L., Builla-Alvarez-Builla, J. 2007. Biodiesel and FAME synthesis assisted by microwaves: homogeneous batch and flow processes, *Fuel Process Technol*, **86**, 1641-1644.

Hobuss, C.B., Venzke, D., Pacheco, B.S., Souza, A.O., Santos, M.A., Moura, S.,Quina, F.H., Fiametti, K.G.,Vladimir Oliveira, J., Pereira, C.M. 2012. Ultrasound-assisted synthesis of aliphatic acid esters at room temperature. *Ultrason Sonochem,***19**, 387-389.

Im, H. J., Lee, H. S., Park, M., Yang, S., J. W., Lee, J. W. 2014. Concurrent extraction and reaction for the production of biodiesel from wet microalgae. *Bioresour Technol*, **152**, 534-537

Jacob Z. 1992. Yeast lipids Extraction, quality analysis, and acceptability, *Crit Rev Biotechnol*, **12**, 463-491.

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

Kamath, H.V., Regupathi, I., Saidutta, M.B. 2011. Optimization of two step karanja biodiesel synthesis under microwave irradiation Fuel Process Technol, **92**, 100-105.

Kanitkar, A.V. 2010. Parameterization of Microwave Assisted Oil Extraction and its Transesterification to Biodiesel, Master's Thesis, Lousiana State University, Baton Rougue, LA., USA, (2010), 129 p.

Kiss, G.A.C., Forgacs, E., Cserhati, T., Mota, T., Morais, H., Ramos, A. 2000. Optimization of the microwave-assisted extraction of pigments from paprika (Capsicum annuum L.) powders, *J Chromatogr A*, **889**, 41-49.

Leung, D.Y.C., Guo, Y. 2006. Transesterification of neat and used frying oil: Optimization for biodiesel production, *Fuel Process Technol*, **87**, 883-890

Li, H., Pordesimo, L.O., Weiss, J., Wilhelm, L.R. 2004. Microwave and ultrasound assisted extraction of soybean oil. *Trans ASAE*, **47**, 1187-1194.

Lima, L.P., Santos, F.F.P., Costa, E., Fernandes, F.A.N. 2012. Production of free fatty acids from waste oil by application of ultrasound*, Biomass Convers Biorefin,* **2**, 309-315.

Lucchesi, M.E., Chemat, F., Smadja, J. 2004. Solvent-free microwave extraction of essential oil from aromatic herbs: comparison with conventional hydrodistillation. *J Chromatogr A,* 1**043**. 323-327.

Lotero, E., Liu, Y., Lopez, D.E., Suwannakarn, K., Bruce D.A., Goodwin, J. 2005. Synthesis of Biodiesel via Acid Catalysis. *Ind Eng Chem Res*, **44**, 5353-63.

López, D.E., Goodwin, J.G., Bruce D.A., Furuta, S. 2008. Esterification and transesterification using modified-zirconia catalysts, Appl Catal A Gen, **339**, 76-83.

Manco, I., Giordani, L., Vaccari, V., Oddone, M. 2012. Microwave technology for the biodiesel production: Analytical assessments, *Fuel*, **95**, 108-112.

Melo-Junior, C.A.R., Albuquerque, C.E.R, Fotuny, M., Dariva, C., Egues, S., Sastos, A.F. Ramos, A.L.D. 2009. Use of microwaves irradiation in the non-catalytic esterification of C18 fatty acids Energy. *Fuels*, **23**, 580-585.

Meher, L.C., Vidya, D.S.S., Naik, S.N. 2006. Optimization of alkali-catalyzed transesterification of Pongamia pinnata oil for production of biodiesel, *Bioresour Technol*, **97**, 1392-1397.

Mulbry, W., Kondrad, S., Buyer, J., Luthria, D. 2009. Optimization of an Oil Extraction Process for Algae from the Treatment of Manure Effluent, *J Am Oil Chem Soc*, **86**, 909-915.

Myers, R.H., Montgomery, D.C. 2002. Response surface methodology: process and product optimization using designed experiments. New York, Wiley (2002).

Parkar, P.A., Choudhary, H.A., Moholkar, V.S. 2012. Mechanistic and kinetic investigations in ultrasound assisted acid catalyzed biodiesel synthesis. *Chem Eng J,* **187**, 248-260.

Pedersen. T.A. 1962. Lipid Formation in Cryptcoccus terricolus – III. Extraction and Purification of Lipids. *Acta Chem Scand,* **16**, 374-382.

Pinto, A.C., Guarieiro, L.L.N., Rezende, M.J.C., Ribeiro, N.M., Lopes, W.A., Pereira, P.A.P., De Andrade, J.B. 2005. Biodiesel: An overview, J Brazil Chem Soc, **16**, 1313-1330.

Rashid, U., Anwar, F., Ansari, T.M., Arif, M., Ahmad, M. 2009. Optimization of alkaline transesterification of rice bran oil for biodiesel production using response surface methodology. *J Chem Technol Biotechnol*, **84**, 1364-1370.

Rokhina, E.V., Lens, P., Virkutyte, J. 2009. Low-frequency ultrasound in biotechnology: state of the art, *Trends Biotechnol*, **27**, 298-306.

Refaat, A.A., El Sheltawy, S.T., Sadek, K.U. 2008. Optimum reaction time, performance and exhaust emissions of biodiesel produced by microwave irradiation, *Int J Environ Sci Technol*, **5**, 315-322.

Saifuddin, N., Samiuddin, A., Kumaran, P. 2015. A Review on Processing Technology for Biodiesel Production, *Trends Appl Sci Res*, **10**, 1-37.

Shen, Y., Pei, Z., Yuan, W., Mao E. 2009. Effect of nitrogen and extraction method on algae lipid yield, *Int J Agric Biol Eng,* **2**, 51.

Stiefel, S., Dassori, G. 2009. Simulation of Biodiesel Production through Transesterification of Vegetable Oils, *Ind Eng Chem Res*, **48**, 1068-1071.

Suzuki, T., Takigawa, A., Hasegawa, K. 1973. Lipid extraction methods for Lipomyces starkeyi, *Agric Biol Chem*, **37**, 2653-2656.

Sunita, G., Devassy, B.M., Vinu, A., Sawant, D.P., Balasubramanian, V.V., Halligudi S. B. 2008. Synthesis of biodiesel over zirconia-supported isopoly and heteropoly tungstate catalysts, *Catal Commun*, **9**, 696-702.

Suppalakpanya, K., Ratanawilai, S.B., Tongurai, C. 2010. Production of ethyl ester from crude palm oil by two-step reaction with a microwave system. *Fuel*, **89**, 2140-2144.

Tierney, J.P., Lidstrom, P. Microwave Assisted Organic Synthesis, 2005. Oxford, UK: CRC Press, 2005.

Vasudevan, P.T., Birggs, M. 2008. Biodiesel production: current state of the art and challenges. J Ind Microbiol Biotechnol, **35**, 421-430.

Veggi, P.C., Martinez, J., Meireles, M.A.A. 2013. In: Food Engineering Series 4, CFaCG, editor, Springer, New York, USA, (2013), 15 p

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

Wei, F., Gao, G.Z., Wang, X.F., Dong, X.Y. 2008. Quantitative determination of oil content in small quantity of oilseed rape by ultrasound-assisted extraction combined with gas chromatography, *Ultrason Sonochem*, **15**, 938-942.

Zhang, X., Yan, S., Tyagi, R.D., Drogui, P., Surampalli, R.Y. 2014. Ultrasonication assisted lipid extraction from oleaginous microorganisms, *Bioresour Technol*, 158, 253-261.

Zhang, S., Zu, Y.G., Fu, Y.J., Luo, M., Zhang, D.Y., Efferth, T. 2010. Rapid microwave-assisted transesterification of yellow horn oil to biodiesel using a heteropolyacid solid catalyst, *Bioresour Technol*, **101**, 931-936.

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

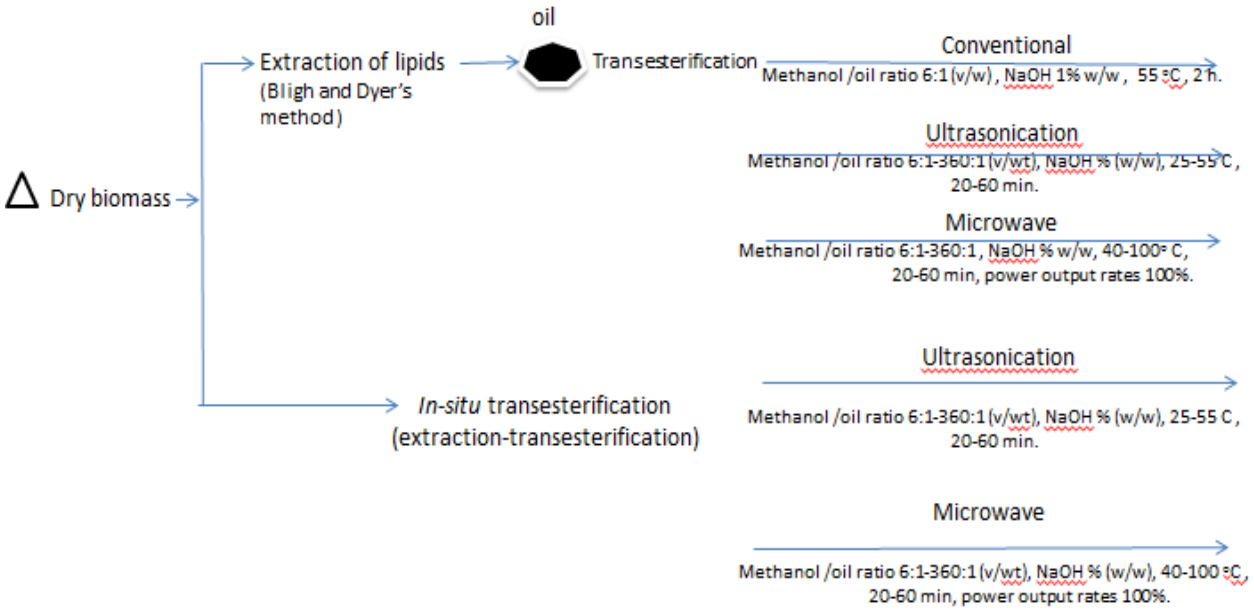


Figure 7.1 Schematic representation of different transesterification methods

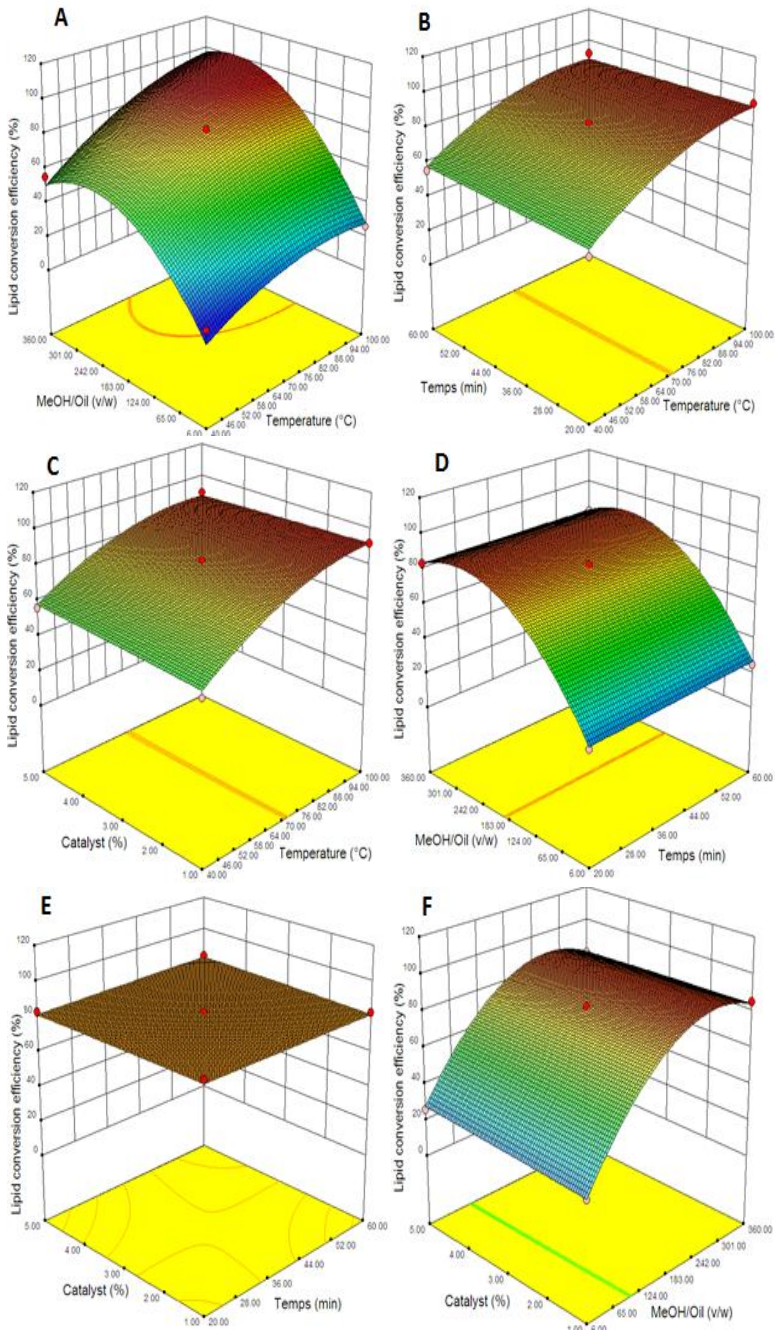


Figure 7.2 **Response surface plots showing binary interaction of different variables. The interaction between: (A) methanol/oil ratio (% v/w) and temperature (ºC); (B) temperature (ºC) and time (min); (C) catalyst amount (%) and temperature (ºC); (D) methanol/oil (% v/w)**

Tableau 7.1 Coding and levels of experiment factors

Factor	Symbol	Code level		
		-1	**0**	**+1**
Temperature(°C)	X_1	40	70	100
Time (min)	X_2	20	40	60
Methanol to oil ratio (v/w)	X_3	6:1	183:1	360:1
Catalyst (%) w/w)	X_4	1	3	5

Tableau 7.2 Box–Behnken design arrangement

Run	Code level			
	X1	X2	X3	X4
1	0	-1	1	0
2	-1	1	0	0
3	1	0	0	1
4	1	0	-1	0
5	0	0	0	0
6	1	-1	0	0
7	0	1	0	1
8	0	1	0	-1
9	-1	-1	0	0
10	0	0	0	0
11	0	0	1	-1
12	0	0	1	1
13	0	0	0	0
14	-1	0	-1	0
15	0	-1	0	-1
16	1	1	0	0
17	-1	0	0	-1
18	0	1	1	0
19	0	0	0	0
20	-1	0	1	0
21	0	0	-1	-1
22	0	-1	0	1
23	1	0	1	0
24	1	0	0	-1
25	0	0	0	0
26	-1	0	0	1
27	0	1	-1	0
28	0	-1	-1	0
29	0	0	-1	1

Tableau 7.3 Analysis of variance (ANOVA) for response surface quadratic model for the FAME conversion

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	18787.6921	14	1341.978	98.5454606	< 0.0001
X1	3502.08333	1	3502.08333	257.168459	< 0.0001
X2	0.05333333	1	0.05333333	0.00391643	0.9510
X3	9622.00333	1	9622.00333	706.572498	< 0.0001
X4	0	1	0	0	1.0000
X1X2	10.5625	1	10.5625	0.77563598	0.3933
X1X3	163,84	1	163.84	12.0312615	0.0038
X1X4	16.4025	1	16.4025	1.20448466	0.2909
X2X3	0.9025	1	0.9025	0.06627328	0.8006
X2X4	1.69	1	1.69	0.12410176	0.7299
X3X4	9.3025	1	9.3025	0.68311041	0.4224
$X1^2$	525.405405	1	525.405405	38.5820911	< 0.0001
$X2^2$	0.01621622	1	0.01621622	0.00119081	0.9730
$X3^2$	4968.03649	1	4968.03649	364.817786	< 0.0001
$X4^2$	0.19864865	1	0.19864865	0.01458736	0.9056
Residual	190.65	14	13.6178571		
Lack of Fit	190.21	10	19.021	172.918182	< 0.0001
Pure Error	0.44	4	0.11		
Cor Total	18978.3421	28			

Tableau 7.4 Results of the optimisation of different variables

Runs	Time (min)	Catalyst (%)	Methanol/oil ratio (w/w)	Lipid conversion efficiency (%)
1	60	3	6	25.1
2	40	3	183	92.3
3	40	3	183	93.0
4	40	5	6	25.8
5	20	3	6	28.9
6	40	5	360	95.9
7	40	1	6	25.9
8	40	3	183	93.9
9	60	5	183	94.1
10	40	1	360	93.9
11	20	1	183	90.1
12	40	3	183	92.1
13	60	1	183	93.4
14	20	3	360	92.2
15	20	5	183	95.5
16	60	3	360	92.2
17	40	3	183	94.2

R-Squared = 0.998, Adj R-Squared = 0.997 Pred R-Squared = 0.983.

Tableau 7.5 Comparative study of in situ transesterification methods

	Conventional	Ultra-sonication	Microwave
Time	12 h	20 min	20 min
Temperature (°C)	60	25	100
Power requirements	-	700 W	400 W
Differences	- Easy separation - Longer time - Higher methanol content	- Difficulty of separation (12 h) - Emulsification and saponification - Reduced time	- Separation and purification steps not required (5 min) - No emulsification - Reduced time - Lower catalyst and methanol amount

Tableau 7.6 Comparison of fatty acid profiles of biodiesel produced using transesterification methods

Fatty acids	Conventional transesterification			Microwave *in-situ* transesterification			Ultrasonication *in-situ* transesterification		
	6:1	183:1	360:1	6:1	183:1	360:1	6:1	183:1	360:1
C14:0	ND	0.5	ND	ND	0.5	0.5	ND	0.5	0.5
C15:0	ND	0.5	0.5	0.5	0.5	0.5	0.4	0.5	0.5
C16:0	22.1	26.5	28.4	25.9	28.2	28.5	25.7	28.5	28.7
C16:1	0.4	0.9	0.7	1.1	1.0	1.1	1.1	1	1
C18:0	9.0	9.9	10.5	9.2	9.9	10.1	9.3	10.1	10.2
C18:1	39.4	48.0	48.5	44.4	49.3	46.7	44.1	49.2	49.3
C18:2	28.5	11.8	10.3	19.0	8.9	9.0	18.1	8.1	8.9
C20:0	0.6	1	1.1	1.2	1.2	0.9	1.3	1	1.1
C22 :0	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27
C24 :0	0.29	0.30	0.31	0.30	0.31	0.30	0.29	0.3	0.3

8 CHAPITRE 8: CO-CULTURE FOR LIPID PRODUCTION: ADVANCES AND CHALLENGES

Co-culture for lipid production: advances and challenges

Magdouli S.[a], S.K. Brar[b] and J.F. Blais[c]*

[a] Ph.D. student, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-4677, Fax: (418) 654-2600, email: sara.magdouli@ete.inrs.ca

[b] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-3116, Fax: (418) 654-2600, email: satinder.brar@ete.inrs.ca

[c] Professor, Institut national de la recherche scientifique (Centre Eau, Terre et Environnement), Université du Québec, 490 rue de la Couronne, Québec, Qc, Canada, G1K 9A9, Phone: (418) 654-2575, Fax: (418) 654-2600, email: jean-francois.blais@ete.inrs.ca

* Corresponding author

Email: jean-francois.blais@ete.inrs.ca

Biomass and Bioenergy (2016) 92, 20-30

8.1 Résumé

L’utilisation de communautés microbiennes pour la production de biocarburants est devenue une approche importante parmi les procédés biochimiques courants. La co-culture a été amplement étudiée dans le but de répondre aux limitations de l'utilisation du substrat par des souches individuelles pour obtenir d'autres bioproduits. Les effets de cette stratégie sur la productivité de lipides ne sont toutefois pas bien connus. Malgré les nombreuses recherches effectuées sur la production de lipides par des microorganismes oléagineux, la stratégie de co-culture a été bien examinée seulement chez certaines algues et la plupart des études se sont attardées sur les différents modes, par exemple hétérotrophe et mixotrophe. La littérature livre peu d'informations sur les stratégies d'amélioration de la production de lipides avec d'autres espèces que les micro-algues. Cette revue met en évidence plusieurs systèmes de co-culture existants pour améliorer la productivité de biomasses et de lipides chez d’autres espèces. Un aperçu des stratégies de culture des micro-algues pour la production de lipides est d’abord présenté. Par la suite, un résumé des autres stratégies rapportées dans la littérature pour d’autres espèces est exposé. Enfin, les avantages et les inconvénients de cette approche, ainsi que les possibilités de surmonter les difficultés sont examinés.

Mots-clés : production de lipides, co-culture, biodiesel, microorganismes oléagineux, défis

8.2 Abstract

The exploration of microbial communities to efficiently produce biofuels has become a highly critical approach among biochemical processes. Co-cultures have been widely studied to address the limitations in substrate utilization by individual strains for the production of other bioproducts. Accordingly, many concerns have arisen about the effects of this strategy on lipid productivity. Despite the extensive research on lipid production by oleaginous microorganisms, co-culture strategy has been well-reviewed in algal species and most of the reports have been concentrated on the different nutritional growth modes (e.g. heterotrophic and mixotrophic). Much original research regarding the various microalgal cultivation strategies has been reported in an attempt to promote microalgal biofuel production. However, current literature indicates the scarce information regarding strategies for the improvement of lipid production with other species rather than microalgae. From a systematic perspective, this review will highlight several co-culture systems existing for the achievement of improved biomass and lipid productivity among other species. It first discloses the current state of microalgal assemblies and their strategies for lipid production, giving general introduction to the topic. Subsequently, it summarizes other assemblies found in the literature that aimed to lipid production. Finally, it discusses the relative advantages and disadvantages and the possibilities to overcome the different challenges.

Keywords: Lipid production, co-culture, biofuel, oleaginous microorganisms, challenges

8.3 Introduction

Intense utilization of fossil fuels for energy production has resulted in global environmental pollution as well as resource depletion, and climate change. This requires an urgent research for new attractive substrates with pre-determined patterns for energy production (Hong et al., 2014). Hence, the necessity of developing an environmental friendly and sustainable energy source that can meet the global requirements and satisfy the existing quality standards has become a pressing challenge. An alternative way to produce biodiesel is the use of microbial oils through the cultivation of microbial communities. These microbes in turn can present many advantages, such as short-life cycle, higher production rate, less labor requirement and easy scale-up (Ma, 2006; Yi & Zheng, 2006). Oil-rich microbes, called single cell oils (SCO), are mainly produced by oleaginous microorganisms known by their high lipid productivity and oil content that exceeded 20% of biomass weight (Meng et al., 2009). The most common studies on microbial oils have been carried out on single cell cultures. However, recent concerns in this field have been oriented towards co-culture system for biodiesel production. This system has been recognized as an efficient model due to the existing interactions between different cultures in most natural environments. Commonly, microbes; clusters of microbial cells, are found in close association, they grow and survive in the same biocoenosis as long as the nutrient sources are available, either in mutualistic relationship or as antagonists. Therefore, microbial interactions (e.g. physical and biological) existing among cultures could be exploited and reproduced at laboratory scale. This concept is not new and for decades, mixed cultures have had primary role in treating wastewaters (Ji et al., 2013; Su et al., 2011) producing biomass and bioactive compounds (O'Reilly & Scott, 1995) and degrading halogens and hydrocarbons (Abed, 2010; Subashchandrabose et al., 2011). Others purposes are illustrated in Table 8.1. Hereby, the use of co-culture system for value-added products (VAP) production is challenging, thus, many works have been done for the production of polyhydroxyalkanoates (PHA) and biohydrogen through the co-culture of *Enterobacter aerogenes* and/or *Rhodobacter spheroides* and *Rhodopseudomonas* BHU01 (Arumugam et al., 2014; Rai et al., 2012).

The objective of the mixed culture was mainly based on the mixture of more than one species in such a way that one strain possesses an enzymatic activity that the other is lacking. Among successful trials, the mixture of amylolytic microorganism with a non-amylolytic producer strain to hydrolyze starch as carbon source was very representative. *Saccharomycopsis* (*Endomycopsis*) *fibuliger* was used as the amylolytic microorganism either in combination with bacteria or yeasts to produce many metabolites, such as single cell protein (SCP) (Jarl, 1969), lactic acid

(Haggstrom & Dostalek, 1981) and ethanol (Dostalek & Haggstrom, 1983), extracellular polymeric substances (EPS) through the co-culture of microalgae/cyanobacteria and macromycetes (Angelis et al., 2012).

Currently, co-cultivation system aimed to overcome the contradiction existing between biomass productivity and lipid content in order to obtain significantly higher lipid productivity. In fact, high lipid content is often offset by lower growth rates and the increase in lipid content dose not result in increased lipid productivity, however, it leads to lower biomass and lipid productivity. So far, many studies have focused to increase the lipid productivity through promoting the accumulation of both total biomass and lipid yield via co-immobilization technique with various bacterial species. Much original research regarding this approach has been reported in algal species. For instance, the assembly between *Chlorella vulgaris* and *C. sorokiniana* co-immobilized with *Azospirillum brasilense* has resulted to an increase in the lipid content of the cells more than 350 ug/g dw. Not only, was lipid content increased, but also, a remarkable variety of fatty acids increased from five to eight different fatty acids in microalgae co–immobilized (de-Bashan et al., 2002). Additionally, coupling algal growth with other microbial species, either, algae, yeast or fungi has been reported for many other species such as *Monoraphidium sp* FXY10 (Cheirsilp et al., 2011; Zhao et al., 2014), *Rhodotorula glutinis*, *Ambrosiozyma cicatricose* (Cai et al., 2007; Cheirsilp et al., 2012; Cheirsilp et al., 2011; Xue et al., 2010)**.** More deeply details will be presented in the coming sections. In this way, co-culture system between different microorganisms either algae, yeast or bacteria will be challenging to address the lipid production issue.

This review, thus, aims to highlight the different approaches of co-culture systems designed for lipid production and to identify the key studies to overcome some of the technical challenges associated with such systems. Furthermore, the relative advantages and disadvantages will be also summarized in the review. Finally, the processes or technologies currently available on lipid accumulation in co-culture systems to overcome the challenges inherent to this field of work will be also discussed.

8.4 Microalgae for lipid production

Microalgae have been well-reviewed as a potential factory for lipid production for biofuel production (Gong et al., 2011). It is presumed that achieving high yields and titers for industrial production might require improvement of algal strains through genetic engineering or recombinant DNA technologies (Miao & Wu, 2004; Rasala et al., 2013). Previous studies on lipid accumulation

via multispecies microbial consortia were limited. Accordingly, relations existing between algal species, beneficial or antagonistic, mutualistic or symbiotic are being studied and further research is currently underway to exploit them. Besides, microbial consortia are able to perform more complex tasks than mono-cultures and can carry out difficult functions impossible for individual strains or species (Brenner et al., 2008). Numerous studies have focused on *Chlorophyta*, which have higher oil contents, and easily cultivated, particularly *Chlorella* species (Miao & Wu, 2004; Xiong et al., 2008). In nature, many assemblages between microbial communities of microalgae and other species have been cited. The present section reports the existing interactions which are dedicated to lipid production.

8.5 Microalgae-microalgae interactions for lipid production

To reduce the cost of raw materials for biodiesel production, the co-culture system presented a proficient and safer alternative. Recently, *Chlorella sp. U4341* and *Monoraphidium sp. FXY-10*, potential feedstock for biodiesel production (Bogen et al., 2013; Liang et al., 2009; Yu et al., 2011) were tested in co-culture system for lipid production under photoautotrophic conditions. This combination was advantageous and permits to *Monoraphidium sp.* to increase their lipid productivity 20-fold. Although the mixture was feasible, the difficulty was residing on the step of harvesting and separation. The challenge was to release lipids in an economical way with low energy cost and with possible recovery of high-value products after lipid extraction. Harvesting accounts for up to 50% of the total cost of biodiesel production (Wrede et al., 2014) and having an extract without contamination by cellular components such as chlorophyll was a key requirement. Recent trends focused on screening of valuable approaches based principally on selective decomposition of cell wall with low cost. Due to small micro algal cells (2–20 μm) and their colloidal stability in suspension (Vandamme et al., 2013), sedimentation was reported to be not efficient; this fact restricts their potential use. Further, the increased energy requirements and the addition of chemicals delimit this process. Novel approaches were reviewed in literature, such as centrifugation, filtration, flocculation, and flotation (Gultom & Hu, 2013; Milledge & Heaven, 2013; Pragya et al., 2013; Sharma et al., 2013). Despite higher efficiency (e.g. 90% recovery), the higher energy input cost of centrifugation, especially with a low value product such as biofuel (Leite et al., 2013), reduced its efficacy and lowered its economical viability. Moreover, filtration has been long investigated with specific species, *Coelastrum proboscideum* and *Spirulina platensis*. This strategy was found successful; however, it was restricted to multicellular microalgae (Gultom & Hu, 2013; Pragya et al., 2013), added to the slowness of this process,

which makes it uneconomical. The supplying of flocculants has been investigated to increase processing speed (Uduman et al., 2010). This process is performed through the use of chemical flocculants (inorganic and organic) (Christenson & Sims, 2011), biological organisms or an electrical impulse (Vandamme et al., 2013), and addressed to improve algal harvesting. All of these methods still need works as they are not universally successful and do not work for all microalgae strains. Further, the use of chemicals resulted to higher concentrations of residual in the biomass residue after extraction of lipids (Rwehumbiza et al., 2012). The use of bio-flocculants has also been proposed to reduce the total energy calculations and offer more sustainability and renewability. New trends rely on bacterial bioflocculants isolated from bacterial species sharing higher auto-flocculation capacities, such as *Scenedesmus spp, Solibacillus silvestris* and *Bacillus sp* (Powell & Hill, 2013; Wan et al., 2013). Besides, filamentous fungi represent attractive bio-flocculating agents due to their self-pelletization and higher microalgal trapping efficiencies. Moreover, an important factor affecting oil production in algal species is the higher cost of the organic carbon and process control that needed to be optimized. Thus, the strategy of co-culture has been tested to minimize the cost of operating system. Different culture models have been investigated in the quest for lipid production. Under heterotrophic or mixotrophic conditions, the co-culture of *Chlorella sp.* U4341 and *Monoraphidium sp*. FXY-10 led to 93.4 - 223.42 and 21.23 mg/L/d of lipid productivity, respectively. However, in photoautotrophic condition, the lipid productivity was around 29.52 mg/L/d (Zhao et al., 2014). These results were explained by the synergistic effects between the two microalgae and the nutrient-starvation, which is directly related to the increased lipid yield under co-cultivation conditions. Besides, the resulting biodiesel from this assembly presented an acceptable iodine value (IV), cetane number (CN), and cold filter plugging point (CFPP) (Zhao et al., 2014). During the co-culture of algae for lipid production, dense culture occurs and velocity growth is relatively high. Naturally, microalgae produce EPS and in case of co-culture systems, more interaction lead to more large amounts of EPS, as a metabolic strategy to grow in unfavorable conditions (nitrogen deficiency required for lipid synthesis). EPS production may eventually limit the mass transfer which makes the supplying culture with nutrients and CO_2 very difficult. So, the choice of micro algal-micro algal assemblies for lipid production may be limited and not applicable at large scale. Accordingly, the micro-algal co-culture assembly is still a complex consortium to study due to the strong interaction between micro-organisms co-cultivated, biological tasks (development and respiration) and physics (hydrodynamics). To elucidate and understand their behaviour under nutrient limitation and during lipid synthesis, many predictive models can be exploited. These models must take into account different parameters from single and dual mixture of species which would help to predict outcomes

of this mixture experiment rather than lipid production. It has been noticed that the green microalga *Parietochloris incise*, enhanced not only its TAG production under nitrogen depletion, but also the production of arachidonic acid, a valuable nutraceutical, in TAGs (Solovchenko et al., 2008). From co-culture model, the feasibility of obtaining other platform chemicals will help to establish other promising lines for future research.

8.6 Interactions between microalgae and bacteria

Among the inter-algal associations cited in literature, the assembly between microalgae–bacteria is very advantageous at laboratory scale and it was investigated for many purposes, including waste water treatment, metal, and nutrient pollutants removal (Ji et al., 2013; Su et al., 2011; Subashchandrabose et al., 2011). Among successful assemblies, the assembly between *Chlorella* sp., cyanobacteria, and bacteria is well-studied. Nevertheless, a better understanding on *Chlorella* sp-bacteria is desired since various species were unknown. Bacteria have a rapid growth rate than algae, so even small inoculum can lead to population explosion of bacterial numbers and a concomitant collapse of the algae. At the same time, various algae are cobalamin auxotrophic (Croft et al., 2005), due to their loss of vitamin B12 independent form of the enzyme. Thus, algal metabolism is ultimately dependent on vitamin B12 which is implicated in methionine synthesis. For instance, *Pseudomonas sp*. and *Rhizobium sp.* are found in the biocenosis of *Botryococcus braunii*, where *Rhizobium sp*., acted as a probiotic bacterium boosting the growth of *B. braunii* (Rivas et al., 2010). More recently, Kazamia et al. (2012) studied the benefits of cultivating *Lobomonas rostrata* with *Mesorhizobium loti*, rhizobial bacteria. In this assembly, the bacterium provides vitamin B12 to algae sustaining its growth. Meanwhile, algae provide photosynthetic products, organic exudates and toxic metabolites inhibiting bacterial growth (Riquelme & Avendaño-Herrera, 2003), confirming mutual symbiosis. Moreover, bacteria were found to prevent other bacteria from invading it, as that ecological niche is already occupied. This phenomenon is due to the competitive exclusion principle of community ecology, as referred by different authors (Kazamia et al., 2012). This strategy, namely “synthetic ecology” is beneficial at large scale, especially when sterile environment is not allowed. This approach is required to overcome the limitations of the culture of algal species in open photo-bioreactors. Taken all together, these advantages permit the improvement of growth and lipid productivity. Hence, bacteria were considered as microalgae-growth promoters and their effects on microalgae were related to their own metabolites (Figure 8.1), especially indol 3-acetic acid that promoted the lipid productivity. Likewise, many experiments were carried out to prove that indol 3-acetic acid

promoted growth and enhanced the interaction between microalgae and bacteria (de-Bashan et al., 2008). Besides, heterotrophic bacteria were found to use and accumulate organic compounds produced by algae and whose accumulation in the growth media is reported to inhibit photosynthesis (Subashchandrabose et al., 2011). Subashchandrabose et al. (2011) have reported that along algae-bacteria assembly, bacteria consume dissolved oxygen, evolved from photosynthesis; which may increase the photosynthetic efficiency Although the benefits are shown during this assembly between algae-bacteria, for others, benefits are not completely known.

In recent studies, B12-producing rhizobia improved the growth rate of *C. reinhardtii* during high temperature stress (Xie et al., 2013a). Accordingly, these assemblies revealed higher production of ω3 fatty acids of *Ankistrodesmus sp. strain SP2-15* (linolenic acid (c18:3ω3) and stearidonic acid (c18:4ω3) (DoNascimento et al., 2012). Beneficial association is also observed in the case of *C. vlgaris* and *C. sorokiniana* co-cultured with a growth-promoting bacterium, *Azospirillum brasilens*e increasing the lipid yield as well as the chlorophyll a and b, lutein, and β-carotene violoaxanthin contents (de-Bashan et al., 2002). A strong evidence of the influence of bacteria on microalgal metabolism was noticed and there was a need to optimize the bacterial–algal consortium. The co-culture of bacterial strains isolated from long-term laboratory microalgal cultures had resulted in better growth than axenic microalgae (Subashchandrabose et al., 2011). Oh et al. (2014) patented the combination of *Rhizobium sp. KB10* and *Bolryococcus braunii, Rhizobium sp. KB10*. This association increased growth of the algae by nine times and also enhanced the content of C18 (i.e., oleate). Along with higher biodiesel production, problems of contamination in this mixed culture were also solved. Earlier, only highly selective conditions were tested to guarantee uncontaminated culture. This strategy was performed with specific conditions for *D. salina* in highly saline media and *Spirulina platensis* at high pH, however such conditions are not available for all species. Consequently, this approach of algal-bacterium association has gained much attention (Wrede et al., 2014).

A cooperative approach using a mixed bacterial culture (activated sludge) and microalgae, *C. protothecoides* (UTEX-1806) and *Ettlia sp.* YC001 was investigated. This cooperative algal-bacterial system resulted in higher lipid productivity under photoheterotrophic conditions compared to photoautotrophic conditions (e.g. 28.7- and 17.3-fold higher), respectively. This system was evaluated as performing to treat and degrade thiocyanate (Ryu et al., 2014).

Other works were also in agreement with this concept, thus, Wu et al. (2012) showed that co-cultivation of *Bradyrhizobium japonicum* with *C. reinhardtii* strain cc849, increased the micro algal

growth , around 3.9 x 10^7 mL^{-1} which accounts for 26%, higher as compared with cultivation of the algal strain alone under the same conditions. This higher growth was associated with the increase of respiration rate in the co-cultures, around 7.71 µmol O_2 mg^{-1} chl h^{-1} (Wu et al., 2012). The rapid respiration rate led to a rapid O_2 consumption and prompt formation of anaerobic conditions in the co-cultures, which might have resulted in lower consumption of endogenous reserves consumed by aerobic respiration metabolism, higher Fe-hydrogenase activity, and more H_2 production.

Accordingly, DoNascimento et al. (2013) have revealed that the inoculation of the microalgae, *Ankistrodesmus sp.* strain SP2-15 with the bacterium, *Rhizobium* strain 10II led to up of 30% in chlorophyll, biomass and lipids accumulation of *Ankistrodesmus sp* accompanied with higher lipid productivity of up to 112 mg L^{-1} d^{-1} at day 6 of culture air-bubbled bottles.

These studies were approved by Choi et al. (2012) who concluded that the co-immobilization of *A. brasilense* with *Chlorella spp*. contributed to higher affinity to carbon source and led to higher volumetric productivity (1.02 mg d^{-1} and 0.73 mg 100 mL^{-1} d^{-1}) compared to *Chlorella* spp. immobilized alone. This approach of immobilization with *A. brasilense* promotes again the accumulation of starch in Chlorella spp. when grown heterotrophically. This could happens because of the bacterium changes the metabolic pathways of *Chlorella* spp. (de-Bashan et al., 2008), which induces accumulation of carbohydrates.

Bai & Gutierrez (2014) reported that the co-cultivation of microalgae and cyanobacteria under mixotrophic condition with sodium acetate as carbon source resulted in higher biomass productivity. Nevertheless, this assembly between microalgae and cyanobacteria has been reported to have negative effects. For instance, in the presence of a bacterial community, many variations in chemical composition of microalgae occurred. The bacteria could inhibit the growth of microalgae by switching their stoichiometry (Danger et al., 2007; Daufresne & Loreau, 2001). Previous studies have proved that bacteria were able slow algal growth (Therien et al., 2014; Wang et al., 2015) and degrade cell wall through cell to cell attachment (Chen et al., 2012; Furusawa et al., 2003). Thus, many strategies were tested to prevent micro algal cells lysis and many explanations were given to understand this phenomenon. Among them, the production of antibiotic substances (Rooney-Varga et al., 2005), synthesis of ecto enzymes (Zoppini et al., 2005) and the occurrence of nutrient competition (Trabelsi & Rassoulzadegan, 2011; Wang et al., 2015) were few reasons. It is hence important to develop metabolic models, which are validated to understand growth conditions and can take into account the position of the cell in its cycle.

The auto-flocculation approach was not restricted to microalgae-fungi, but, it was investigated during the culture of bacteria-microalgae. This approach was explored through coupling of wastewater treatment with micro algal culture due to the higher cost of micro algal culture medium. This combination is efficient for chemical oxygen demand (COD) removal. Despite their efficiency, the COD removal of microalgae is still lower and the resulting effluent is usually unsatisfactory. Besides, heterotrophic cultivation of microalgae under axenic conditions is difficult and the frequency of contamination is higher. Despite these limitations, co-culture of microalgae and bio-flocculant-producing bacteria in synthetic wastewater presented numerous advantages. In the presence of bacteria, COD removal is improved and the degradation of complex pollutants is favored. Besides, this strategy is advantageous since it affected the energy balance as the residual biomass generated from the above culture could be recycled and nutrients from the downstream processing could be used for algal growth which helped to minimize the energy costs. Bio-flocculant producing microbes can overcome the negative effects of traditional flocculants, and also promote the metabolic degradation and transformation of some recalcitrant compounds, allowing them to be easily assimilated by algae (Zhang et al., 2012). These results were in agreement with Wang et al. (2015), who found that the bio-flocculant-producing bacteria *Rhizobium radiobacter* enhanced microalgae harvesting efficiency to 40.0–50.0% which is more relevant in comparison with axenic *C. vulgaris* with a harvesting efficiency of 0.2%.

Many issues are still under study and the intense competition between the algae and bio-flocculant-producing bacteria remains a big question. Perez-Garcia et al. (2011) elucidated that an intense competition between the algae and bacteria is existing, where bacteria probably repress the growth of algae, which may affect the cell density and lipid content of algae. Therefore, optimizing co-culture conditions is a prerequisite to weaken the negative competition between the microalgae and bioflocculant-producing bacteria. Therien et al. (2014) investigated the co-culture of the cyanobacterium, *Synechococcus sp*. PCC 7002 to produce acetate which is required for lipid production of *Chlamydomonas reinhartdii.* This approach is an encouraging practice and a photosynthetic alternative to provide exogenous acetate into growth media for microalgae grown chemotrophically or photomixotrophically. This practice provides a strong proof of concept for modulating costly medium components through co-cultivation. Thus, acetic acid, or any exogenous carbon source can externally be provided from an organic acid producing strain to enhance lipid production. This approach presented economical and practical challenge for large-scale production. Other than enhancing growth and lipid production of *C. reinhartdii,* the use of *Synechococcus sp*. PCC 7002 encapsulated in alginate beads was found to be a potential mechanism for separation and harvesting and later for recycling of the *Synechococcus sp.* cells

(Therien et al., 2014). Concurrently, encapsulation has been shown to positively modulate the overgrowth of *Synechococcus sp* and eventually to avoid that a culture took over the co-culture during lipid synthesis. In spite of recent progress on algal co-culture, more studies have to be conducted, particularly, on life-cycle analyses. Without careful assessment of the energy balance and environmental impacts, co-culture system cannot have the desired success. There is a pressing need to conduct pilot studies.

8.7 Interactions between microalgae yeast and molds

Symbiosis of yeast and algae was well studied by many authors (Cai et al., 2007; Santos et al., 2011; Santos et al., 2013) and reviews elucidated the benefits of microbial association of two species to improve the growth rate and biomass concentration (Cheirsilp et al., 2012; Cheirsilp et al., 2011). Successful combinations between microalgae and yeasts were given in Table 8.2. Naturally, microalgae are potential candidates for nitrogen and phosphorus removal, carbon dioxide uptake and oxygen production. However, they are not very performant in organic matter removal from wastewater because of their slower growth and longer cultivation time (Ji et al., 2013; Su et al., 2011). On the other hand, these microalgae provided higher oxygen levels, widely required by heterotrophic yeast for their growth. Hence, algal species acted as an oxygen generator for the yeast, whereas yeast provided CO_2 for the microalga and produced lipids. For fungi-algae symbiosis, fungi consume the carbon provided by the algae through photosynthesis. Meanwhile, fungi provide protection to the algae by retaining water and serve as a niche for mineral nutrients (Zoller & Lutzoni, 2003). Among fungal species, lichen was reported as an efficient partner and a self-sufficient symbiotic association. Lichen offered the advantage of lignin and cellulose degradation. These saprophytic activities are essential when photosynthetic activity is limited (Beckett et al., 2013; Wrede et al., 2014). Among the culture systems reported, the co-cultivation of microalga (*Isochrysis galbana* 8701) and yeast (*Ambrosiozyma cicatricose*) led to a productivity of around 20.71 mg/L (Cai et al., 2007). Cheirsilp et al. (2012) investigated the mixed culture between *R. glutinis* and *C. vulgaris* and its performance to enhance lipid production from industrial wastes.

Likewise, the co-cultivation of *Spirulina platensis* and *R. glutinis* increased significantly the accumulation of total biomass and total lipid yield (Xue et al., 2010), with a concomitant capacity of chemical oxygen demand and nitrogen removal, 73% and 35%, respectively. In another report, mixed cultures of *Chlorella sp*. KKU S2 and oleaginous yeast, *Torulaspora maleeae* Y30 or *Torulaspora globosa* YU5/2, in the presence of sugarcane juice as organic carbon source,

resulted in improved growth and hence higher microbial oil production (Papone et al., 2012). An increase of 96% in lipid yield in the mixed culture was obtained as compared with the monoculture. The microalga may react as an O_2 producer, enhancing the growth of the yeast, whereas the latter produced CO_2 that can be used by the microalgae under photoautotrophic cultivation. Of particular importance in the mixed culture was the optimization of operating parameters such as culture volume ratio (Cheirsilp et al., 2012; Cheirsilp et al., 2011; Papone et al., 2012), CO_2 uptake and mode of culture. They affected relatively the growth rate of heterotrophs and autotrophs. The inoculum proportion of autotroph to heterotroph was considered as an important parameter and each proportion was relative for each study (Cheirsilp et al., 2011; Papone et al., 2012). Different strategies were investigated to optimize the culture conditions. In the case of heterotrophic and photoautotrophic microorganisms in a separate photobioreactor, respective cultures produced gases while growing. Autotrophically grown *C. protothecoides* improved biomass productivity (0.015 g/L/h) and lipid productivity (2.2 mg/L/h) when aerated with the off-gas from *R. toruloides*, in comparison with the same culture aerated entirely with air and without exchanging gases (Santos et al., 2013). The improvement of lipid productivity of *C. protothecoides* grown autotrophically and aerated with the CO_2-enriched air from its heterotrophic culture was above 55% for biomass and lipid yield when compared with the bioreactor aerated only with air. With heterotrophically grown *C. protothecoides,* biomass and lipid productivity reached around 0.052 g/L/h and 28.6 mg/L/h, respectively, when aerated with O_2-enriched air originated from the autotrophic *C. protothecoides* culture in bubble columns as compared with aeration using ambient air (Santos et al., 2011). Likewise, the supply of CO_2 produced from *T. maleeae* Y30 to *Chlorella sp*. KKU-S2 resulted in higher lipid (0.223 g/L/h) and biomass productivity (0.48 g/L/h) (Puangbut & Leesing, 2012).

Recently, oleaginous yeast, *T. spathulata* JU4-57 was co-cultivated with many species of *Chlorella* to study the advantage of co-culture system on lipid production (Kitcha & Cheirsilp, 2014; Zoller & Lutzoni, 2003). Among different combinations, the co-culture of *T. spathulata,* with *C. vulgaris var. vulgaris* TISTR 8261 yielded highest biomass of 12.2 g/L with a higher lipid content of 47% (w/w) (Kitcha & Cheirsilp, 2014).

Cai et al. (2007) also reported that lipids resulted from the mixed culture of the microalgae, *Isochrysis galbana* 8701 and the yeast, *Ambrosiozyma cicatricose* was rich in saturated fatty acids compared to individual cultures. In this study, the fatty acid profile was similar to that of plant oil which consisted of palmitic and oleic acids. The microbial lipid obtained from this co-culture could be used as a biodiesel feedstock. Further improvement of co-culture system is essential in

addressing the technical hurdles. Finally, the symbiosis between fungi-algae was not investigated solely for lipid production, but, it was designed for assisted flocculation. This approach offered the advantage of pelletization through combination of biomass and lipids as partners. This approach revealed higher utilization of the cell wall carbohydrates and secretion of cell-wall degrading cellulases (Xie et al., 2013b). Self-pelletization of filamentous fungi, explained by coagulative and non-coagulative mechanisms (Luo et al., 2013; Xia et al., 2014), does not require the addition of chemicals and has demonstrated a potential reduction of energy input with *C. vulgaris* (Gultom & Hu, 2013; Wrede et al., 2014; Xie et al., 2013b; Zhang & Hu, 2012). This assisted harvesting approach can be commercially applied particularly when freshwater or seawater algal species will be used for biodiesel production, it may resolve problems associated with the energy-intensive and costly harvesting processes.

8.8 Co-culture in yeast and molds

Many mold species have been reported for lipid storage and various fungal species were explored for the production of polyunsaturated fatty acids namely, docohexaenoic acid (DHA), eicosapentaenoic acid (EPA), ω-linolenic acid (GLA) and arachidonic acid (ARA). Only few studies on the utilization of fungal oils for biodiesel production were reported (Yi & Zheng, 2006). Besides, majority of studies are based on monoculture system. Dostalek has reported that through the co-culture of *Sps. fibuligera* and *R. toruloides* using starch media, a higher lipid concentration (36.5%) was achieved, however, during monoculture system, a feed feedback product inhibition occurs and *Sps. Fibuligera* was able to accumulate reducing sugars without using them (Dostalek & Haggstrom, 1983). The co-culture model was performed to avoid catabolic repression and enhance the lipid production. Thus, this relationship may be resilient to perform metabolite production, the fact that may help to predict and direct community dynamics. White rot fungi exhibited different delignification patterns and their higher enzymatic activities may draw a propitious future play, especially, when matching fungal co-cultures for bio-lipid production. Coupling lipid fermentation with degradation of lignocellulosic biomass via co-culture system is a key to improve lipid yield and reduce the production cost. As referred by Liu et al. (2014), combining coprophilous fungi, such as *Cladosporium sp. F1* and *Byssochlamys sp. F52* improved the reducing sugar production. This resulting pool of sugar can be converted into lipids under controlled environment. Consequently, the conversion of lingocellulosic biomass to useful reducing sugars, which can be further converted into desired end products, such as lipids is very challenging (Chen et al., 2013; Yu et al., 2011). Moreover, oleaginous microorganisms are known

to quickly accumulate oils, such as intracellular lipids with high lipid titer in the presence of carbohydrates (Liu et al., 2012). Accordingly, Dommisse observed that a co-culture of *Aspergillus flavipes* and *Pycnoporus sanguineus* ameliorated the pulping properties of *Eucalyptus grandis* and caused cellulose degradation (VanHeerden et al., 2008). Bootten et al. (2011) suggested that the co-culture of *Piromyces communis*, *Neocallimastic frontalis,* and *Caecomyces communis*, three rumen fungi, enhanced alfalfa hay, a complex structure of xylan, cellulose, lignin, conversion. The major obstacle to widespread utilization of this potential resource is the absence of feasible technologies that converted lignocellulosic materials to useful lipid products (Sanderson, 2011; Weng et al., 2008; Yousuf, 2012). Further, most of pretreatments designed for lignocellulose conversion generated various inhibitors, such as acetic acid, furfural, hydroxymethylfurfural (HMF), neutral and acidic phenolics (Palmqvist & Hahn-Hagerdal, 2000). The occurrence of these side-products and their tolerance by microorganisms is a technical roadblock. Thus, managing inhibitors and screening partners tolerating these factors is a potential area of research to be explored. As previewed before, filamentous fungi can be used also to harvest microalgae owing to their self or induced flocculation activities. This innovative approach was investigated successfully with *Aspergillus fumigatus* (Al-Hothaly et al., 2015; Wrede et al., 2014). The ability of fungi to flocculate microalgal species was widely investigated for waste waters treatment and Wrede et al. (2014) have conclusively demonstrated that bio-flocculation tends to enhance biomass and lipid production. Exploration of these properties of filamentous fungus for the treatment of wastewater and development of biofloculation's processes with enhanced lipid productivities has the potential to improve *in situ* bioremediation and energy production. Nevertheless, the suitability of bio-flocculation approach as a method for microalgal harvest has never been conclusively studied especially at large scale. Only few studies have all been conducted at a laboratory scale.

8.9 Co-culture in bacteria

It has been known for decades that bacterial assembly was recognized advantageous and microorganisms together are able to withstand shear forces, nutrient deprivation and pH changes. Moreover, EPS produced along bacterial cultures confers more mechanical strength (Davey & O'Toole, 2000; Jefferson, 2004), Currently, fewer data is available about lipid accumulation in bacterium and the most abundant class of neutral lipids found are PHAs serving as intracellular carbon and energy storage compounds (Steinbüchel, 1991). Actinomycetes, such as *Mycobacterium* (Barksdale & Kim, 1977), *Nocardia, Rhodococcus* (Alvarez et al., 1997),

Streptomyces (Olukoshi & Packter, 1994) and *Gordonia* (Gouda et al., 2008), have been reported for TAG accumlation. However, no available data was related to both mixed bacterial culture and lipid production. Meanwhile, the symbiosis of two bacteria still exists, but not for lipid production purposes. For instance, *Lactolus helveticus* was mixed with *R. glutinis;* this latter took the benefit of the ability of *L. helvecticus* to grow on lactose substrates (cheese whey) to produce carotenoids and exopolysaccharides (Frengova et al., 1997). The lipid content obtained was above 4.15% of the total carotenoids produced. Generally, co-culture system has been explored for the degradation of hydrocarbons. For example, mixed system of *Pseudomonas putida*, *Acinetobacter venetianus,* and *Alcaligenes faecalis,* was efficient to oxidize *n*-alkanes and *n*-alkanols to alkanoates, respectively (Pepi et al., 2003), giving rise to fatty acids (the last oxidized products of *n*-alkanes degradation) and also an ideal carbon source to promote lipid production. This approach may open new horizons of lipid production via alkane pathway (Peralta-Yahya & Keasling, 2010; Schirmer et al., 2010). As discussed previously, during lipid production, the role of bacterium in co-culture model aimed, particularly, to induce flocculation. Among reported species, *Solibacillus silvestris* and *Bacillus sp* were found to induce a high flocculation efficiency of up to 90% in the presence of *Nannochloropsis oceanica* during harvesting process in the presence of algal species (Powell & Hill, 2013; Wan et al., 2013). Besides, other bacterial capacities, such as emulsification can be directed to lipid production. As already known, bacterial strains, known for their production of surfactants, are able to alter the cell surface hydrophobicity which would affect the direct substrate uptake and later lipid accumulation. This strategy needs to be considered for successful biofuel production.

8.10 Advantages and challenges

Owing to the close mutually beneficial physical and physiological interactions along microbial communities, the exploitation of co-culture model holds great promise as strategy for lipid production. In fact, the co-culture of microorganisms at laboratory scale and their fatty acids production can be optimized and controlled as required.

Additionally, current research is focusing on engineering multiple biosynthesis pathways, into a single organism. This approach resulted in conversion inefficiency due to many bottlenecks in metabolic pathways (Pickens et al., 2011). Consequently, a mixed culture approach allowed the selection of microbes suitable to perform one task of the overall conversion process (Alper & Stephanopoulos, 2009) and deviated the engineering focus from introducing new functionalities

to improving existing metabolic pathways. Therefore, understanding how microbial communities react during lipid production opens the door on a new world of exploration and insight and reliable method for renewable and sustainable energy production. In this respect, many concerns on co-culture system have arisen. This novel approach in which microbes are grown together allowed triggering of specific biosynthetic pathways, mainly associated with defense and permitted the discovery of new leads. Hence, the synchronization of two species for mutual exploitation of medium and complementary metabolic pathways to grow, reproduce and survive will be well-understood. Other beneficial effects have also been cited since the co-culture system would play a role in pH adjustment, gas, substance exchange (Gen et al., 2014). Additionally, this technology led to more biomass with different characteristics and can later be processed separately to have two or more products. Mixed cultures generate a single biomass type that has an equal composition to the weighted average of each one of the species that originated from it, and could be used for biodiesel production. Moreover, individual cultures required an intensive energy for sterilization. Owing to these disadvantages, mixed cultures is highly suggested as it does not require sterile conditions. Howbeit, pure culture is still preferred as it usually leads to more reproducible results. An ideal co-culture system should be designed to provide structural support and mimic the natural biological characteristics of each partner in order to enhance cellular interactions.

Besides, co-culture model was reported to lead to higher free fatty acids (FFA) release of into the extracellular medium (DellaGreca et al., 2010; Fergola et al., 2007; Tate et al., 2013). The abundance of FFA is considered to be a potential precursor for lipid biosynthesis and it may stimulate the transcription of downstream genes (Coleman et al., 2002; Ohlrogge & Jaworski, 1997). Likewise, the nitrogen starvation is observed to occur rapidly in mixed cultures, the fact, that enhances the lipid biogenesis via *in vivo* pathway (Sukenik & Livne, 1991; Takagi et al., 2000). Experiments have shown that nitrogen depletion started early in co-culture system compared to individual culture and enhanced the production of various metabolites, such as peptides, amino acids, and nitric oxide (Mendes & Vermelho, 2013). Consequently, co-culture approach is a prominent inducer for lipid biosynthesis and different metabolites. Other advantage of co-culture to be cited is the shortening of culture time in co-culture notably as the nitrogen depletion and lipid accumulation occurs very fast (Zhao et al., 2014). Accordingly, many explanations have been presented to understand the functionality of such system. One of them is the priority taken by one strain over others. For example, during the culture of *Monoraphidium sp*. FXY-10 and *Chlorella sp.* U4341, a competition pressure was observed and U4341 produced more C18 fatty acids e.g. linolenic acid that inhibited the growth of FXY-10. On the other side,

FXY-10 generated fatty acids as competitive arms known by low tolerance capacity. Conversely, U4341 is reported to tolerate the toxicity against high concentration of C18 fatty acids and the growth was almost unchanged or only slightly inhibited (Zhao et al., 2014). These findings are obedient with previous studies showing the ability of *C. vulgaris* to release inhibitory compounds when co-cultivated with other microorganisms (Pratt & Fong, 1940). Major compounds were reported to be a mixture of fatty acids, such as linolenic acid, followed by linoleic, oleic, and palmitic acids (Chiang et al., 2004; DellaGreca et al., 2010) that showed a higher toxicity. However, this approach is not restricted to competitive relation and toxic compounds secretion. Combination can be symbiotic and one strain can synthesize some compounds that other strain lacks. So far, the mixture of *R. toruloides* 21167 and *Saccharomycopsis fibuligera* A11 was synergistic due to the impotence of *R. toruloides* to produce inulases and amylases that *S. fibuligera* was able to produce. The production of these enzymes can actively hydrolyze cassava starch and the resulting product can be converted into single cell oil. The immobilization of the yeast has the role to increase amylase production above 325 U/mL within 72 h of incubation. During the co-culture, the lipid yield of *R. toruloides* 21167 reached 64.9% (w/w) from a cell mass of 20.1 g/L produced from cassava starch (6.0% w/v). Major fatty acids synthetized were C16:0, C18:0, C18:1 and C18:2. These latters presented over 96% of the fatty acids commonly used for biodiesel production (Gen et al., 2014). Recently, Zhao et al. (2011) reported that *R. mucilaginosa* TJY15a has around 53.2% (w/w) of oil content from a cell mass of 12.24 g/L when co-cultivated with immobilized cells of *P. guilliermondii* strain 1 in a medium containing 2.0% of inulin (Zhao et al., 2011). Accordingly, other benefits of mixed culture systems have to be listed including the tunability and high resistance to environmental stress (Eiteman et al., 2009; Fazzini et al., 2010).

To date, more research is being carried out to develop a new technology aimed to expand biofuel production from biomass. **Consolidated bioprocessing (CBP)** is a new approach used recently for bioethanol production from cellulosic biomass. This approach consists on co-cultivating two strains, one ethanologenic and the other cellulolytic (Park et al., 2012). Such system combining an oleaginous microorganism with a cellulolytic strain can be a considerable step in the biotechnology of biodiesel production from cellulosic biomass.

Meng et al. (2009) observed that *R. glutinis* can produce a maximum lipid amount of 72% of dry weight. When, it is co-cultivated with *Spirulina platensis*, the biomass and the lipid accumulation are boosted (Xue et al., 2010). In fact, *R. glutinis* provided CO_2 to the microalgae, whereas this latter acted as an oxygen generator. Kazamia et al. (2012) reported a stable equilibrium between population densities, in both batch and chemostat conditions. This behavior is due to a regulation

system (Smith & Douglas, 1987), a phenomenon that is unusual for symbiotic species. Moreover, it may be essential to select strains with high lipid productivities, coming from complementary niches and having identical nutrient profiles, the fact, that may help to minimize the competition between species. In this regard, choosing a consortium of variable lipid-producing strains is again required to have a clear idea on their rheology and lipogenic capacities.

Both mixed-culture and pure culture had similar environmental impacts that permit to confront the global energy demand and pollution problems through an integrated and sustainable approach. Despite the numerous advantages of mixed-culture, the main objective is to get high lipid production with higher biomass productivity. Considerable technical hurdles, largely due to different factors e.g strain selection, operating parameters cultivation and recovery processes have to be overcome.

Unlike contamination risks associated with mixed cultures are minimal. Biotechnology is still an expensive industry leading to expensive bio-based biofuels. Therefore, considerable efforts to identify other alternative carbon sources with low prices are required. Firstly, the utilization of low value substrates, such as agro-industrial wastes and various by-products to reduce the production cost of raw materials is promising. This approach will allow a lower investment and operating costs for the whole global process. Secondly, the screening and the choice of microbial consortia is again primordial in any biotechnological process. The selections of species with similar nutritional and metabolic requirements lead to a successful process. Besides, synthetic biology systems allowed the construction of super lipid production strains that satisfy technology demands. Thirdly, the optimization of culture medium and conditions (e.g. establishing the right cell ratios, the proper medium composition), the proper bioreactor design and the best integration bioprocess development of the lipid purification process remains challenging. Possible solutions to limit these drawbacks are presented in Table 8.3.

Mixed cultures presented higher power densities with multiple metabolic reactions supporting the mass transfer losses and limiting the diffusion process. Hence, the enhancement of mass transfer is one of the major challenges improving production yields. Thus, oxygen is highly required for lipid synthesis. For this purpose, Vega et al. (1990) demonstrated a strong connection between mass transfer, kinetics, cell growth, and production of the desired products. Because of high cell densities in mixed culture, growth will depend on the mass transfer rate (Slivka et al., 2011). Therefore, diffusion limitations at high cell densities resulted to lower overall productivity. Higher mass transfer rates, lower operation costs, and easier scale-up are fundamental parameters to develop an efficient bioreactor system (Munasinghe & Khanal, 2010). For this purpose, an

adequate design of bioreactors that can provide a higher mass transfer coefficient (Munasinghe & Khanal, 2010), is required. Several designs of reactor have been proposed to enhance the mass transfer rate. The increase of the agitation speed and/or the modification of the impellor design were investigated to improve the mass transfer efficiency (Bredwell et al., 1999). The parameters should be controlled to avoid cells damage and growth inhibition caused by high shear rates and excessive agitation (Kadic, 2010). However, the higher power demand of agitation greatly reduces the economic viability in large-scale (Munasinghe & Khanal, 2010; Ungerman & Heindel, 2007). Recently, the use of nanoparticles with high functional groups during fermentation process has the role to increase the mass transfer efficiency (Zhu et al., 2010; Zhu et al., 2009; Zhu et al., 2008). Added to these technical goals, other parameters have to be considered such as the commercial viability of the medium e.g. cost contributions and carbon footprint. This issue can be addressed through controlling monoculture conditions, meanwhile in a pure culture system; strains are grown under identical conditions (e.g. medium type, operating conditions, culture substrate, etc.) to try to mimic the same biological environment.

During the current process, a high competitiveness between cultures occurred and conducted to growth inhibition; two or more species compete for one or more growth factors. This type of interaction affects both partners in a negative way. However, a temporary increase in the abundance of one interacting partner over the other is observed, which can alter lipogenesis process. Besides, the unknown contribution of each microorganism versus microorganism-lipid interactions during lipogenesis is lacking. To surmount this difficulty, knowing and understanding the function of the genes involved in microbial lipid assimilation, accumulation and processing are imperative. In particular, co-culture systems involve multiple interactions that can be not easy to distinguish from one another without the use of proper experimental design.

Knowledge of strain capabilities, kinetics and biological mechanisms has become a focus of interest for researchers, which aimed principally to avoid the misinterpretation of results. Cross-feeding and metabolite exchange are some of these mechanisms that may serve this goal to better understand cell–cell communication for monitoring population density. Recently, numerous studies have determined the co-existence of trading metabolites and molecular signals along microbial communities and a growing number of microscopic and molecular methods (e.g. high performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (NMR)) have facilitated their analysis. Eventually, rRNA (metagenomic) sequencing is of pronounced importance for understanding and steering the functionality of microbial communities. On the other hand, proteomic approaches helped to describe the cellular regulation at metabolic

level and draw a simplified microenvironment of mixed culture. Taken together, metabolic profiling and targeted growth assays offered a great opportunity to inquire microbe-microbe interactions in such complex environment. Finally, the downstream processing remains challenging due to its high cost. For instance, a continuous centrifugation process to separate lipogenic cells from the fermentation broth is mainly required. However, the separation process via continuous centrifugation is a long and energy intensive process due to the high cell densities. It is therefore imperative to construct inducible cell flocculation to perform the precipitation process. Inducible cell lysis is vital to release the intracellular lipid bodies (Martinez et al., 2011), following the cells separation from the broth. Nevertheless, the differences of gradient densities between mixed culture cells could have implications on the effectiveness of extractions. Currently, solvent extraction of microbial lipids is well reviewed (Bligh & Dyer, 1959; Greenwell et al., 2010; Halim et al., 2012) and osmotic pressure has been cited to afford a high recovery of lipids, especially from algal biomass (Yoo et al., 2012). However, chemical treatment (acid and/or base hydrolysis) has been proposed to enhance high lipid extractability of biomass (Sathish & Sims, 2012). Additionally, concerns about cost of these methods given below, are considerable since they rely on dry biomass. However, the energy requirement and the high cost for drying the biomass make this process very costly. New trends ought to be placed on the development of a liquid or wet extraction system to replace the dry extraction one. Moreover, the high cell densities resulting from extraction process generate disposal's problems. One of the current methods to resolve this problem is the use of this residual biomass as slurry (fertilizer). In spite of the availability of nutrients and minerals in residual biomass, various chemical treatments steps will be required. Secondly, the minimization of contamination risks associated with mixed cultures could not hide the high cost of the whole process which makes the biotechnology of bio based biofuels an expensive industry. Owing to this reason, several approaches aimed to lower the investment and the operating costs for the global process. Among them, the use of mixed cultures combined with the utilization of low value substrates, as by-products and agro-industrial wastes. Although, the biotechnology entwines an image of the effects of the co-culture, e.g. competition or symbiosis, it is quite difficult to meet the ideal requirements for each cell type. In fact, numerous bottlenecks need to be overcome before moving to industrial scale.

8.11 Conclusion

Symbiosis is widespread in nature because of its advantages over the individual living of organisms. Exploiting this relation has tremendous potential due to advantageous symbiotic interactions over the single culture. Recently, most of the research studies focused on the interaction between microalgae and yeasts. They are chosen to be ideal partners in a synthetic consortium. Hence, the use of bacteria in co-culture with algae is advantageous and could promote the photoautotrophic micro algal culture especially in open bioreactors. Exploring interactions between complementary metabolism microorganisms, through their physical separation, in different bioreactors, opens a new perspective that permits to add the advantages of mixed cultures without losing the physical separation of biomass. Accordingly, symbiotic interactions have been cited to improve targeted operational variables, such as productivity and operational costs. Not only does the co-culture offers an improvement in biomass and lipid productivity, but also lead to potential by-products that could be exploited, such as pigments, biofuels and polyunsaturated fatty acids. As listed earlier, various natural resources could be efficiently utilized during the process in turn generating lower carbon dioxide and greenhouse gases (GHG) emissions.

The symbiotic interactions in the microbial consortium remain an attractive line for further research, development and demonstration in the future to come, and challenges to move from proof of concept to scale up.

8.12 Acknowledgements

The authors are thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A 4984, and Canada Research Chair) for their financial support.

8.13 List of abbreviations

ARA	Arachidonic acid
CBP	Consolidated bioprocessing
CFPP	Cold filter plugging point
CN	Cetane number
COD	Chemical oxygen demand
DHA	Docohexaenoic acid
DNA	Desoxy-ribonucleic acid
EPA	Eicosapentaenoic acid
EPS	Extracellular polymeric substances
FFA	Free fatty acids
GHG	Greenhouse gases
GLA	ω-linolenic acid
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
IV	Iodine value
NMR	Nuclear magnetic resonance spectroscopy
PHA	Polyhydroxyalkanoates
SCO	Single cell oils
SCP	Single cell protein
VAP	Value-added products

8.14 References

Abed, R.M.M. 2010. Interaction between cyanobacteria and aerobic heterotrophic bacteria in the degradation of hydrocarbons. *Int Biodeterior Biodegrad*, **64**, 58-64.

Al-Hothaly, K.A., Adetutu, E.M., Taha, M., Fabbri, D., Lorenzetti, C., Conti, R., May, B.H., Shar, S.S., Bayoumi, R.A., Bali, A.S. 2015. Bio-harvesting and pyrolysis of the microalgae Botryococcus braunii. *Bioresour Technol*, **191**, 117-123.

Alper, H., Stephanopoulos, G. 2009. Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential?. *Nat Rev Micro*, 7, 715-723.

Alvarez, H.M., Kalscheuer, R., Steinbuchel, A. 1997. Accumulation of storage lipids in species of Rhodococcus and Nocardia and effect of inhibitors and polyethylene glycol. *Fett Lipid*, **99**, 239-246.

Angelis, S., Novak, A.C., Sydney, E.B., Soccol, V.T., Carvalho, J.C., Pandey, A., Noseda, M.D., Tholozan, J.L., Lorquin, J., Soccol, C.R. 2012. Co-Culture of Microalgae, Cyanobacteria, and Macromycetes for Exopolysaccharides Production: Process Preliminary Optimization and Partial Characterization. *Appl Biochem Biotechnol*, **167**, 1092-1106.

Arumugam, A., Sandhya, M., Ponnusam, V. 2014. Biohydrogen and polyhydroxyalkanoate co-production by Enterobacter aerogenes and Rhodobacter sphaeroides from Calophyllum inophyllum oil cake. *Bioresour Technol*, **164**, 170-176.

Bai, R., Gutierrez, M.T. 2014. Effect of organic carbon, C:N ratio and light on the growth and lipid productivity of microalgae/cyanobacteria co-culture. *Eng Life Sci*, **14**, 47-56.

Barksdale, L., Kim, K.S. 1977. Mycobacterium. *Bacteriol Rev*, **41**, 217-372.

Beckett, R.P., Zavarzina, A.G., Liers, C. 2013. Oxidoreductases and cellulases in lichens: Possible roles in lichen biology and soil organic matter turnove. *Fungal Biology*, **117**, 431-438.

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

Bogen, C., Klassen, V., Wichmann, J., Russa, M.L., Doebbe, A., Grundmann, M.M., Uronen, P., Kruse, O., Mussgnug, J.H. 2013. Identification of Monoraphidium contortum as a promising species for liquid biofuel production. *Bioresour Technol*, **133**, 622-626.

Bootten, T.J., Joblin, K.N., McArdle, B.H., Harris, P.J. 2011. Degradation of lignified secondary cell walls of lucerne (Medicago sativa L.) by rumen fungi growing in methanogenic co-culture. *J Appl Microbiol*, **111**, 1086-1096.

Bredwell, M.D., Srivastava, P., Worden, R.M. 1999. Reactor design issues for synthesis-gas fermentations. *Biotechnol Prog*, **15**, 834-44.

Brenner, K., You, L., Arnold, F.H. 2008. Engineering microbial consortia: a new frontier in synthetic biology. *Trends Biotechnol*, **26**, 483-489.

Cai, S.Q., Hu, C.Q., Du, S.B. 2007. Comparisons of growth and biochemical composition between mixed culture of alga and yeast and monocultures. *J Biosci Bioeng*, **104**, 391-397.

Cheirsilp, B., Kitcha, S., Torpee, S. 2012. Co-culture of an oleaginous yeast Rhodotorula glutinis and a microalga Chlorella vulgaris for biomass and lipid production using pure and crude glycerol as a sole carbon source. *Ann Microbiol*, **62**, 987-993.

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

Chen, C.L., Qi, W., Wang, J.Y. 2013. Microbial cocktail for bioconversion of green waste to reducing sugars. *J Biosci Bioeng*, **115**, 82-85.

Chen, W., Sheu, F.S., Sheu, S.Y. 2012. Aquimarina salinaria sp. nov., a novel algicidal bacterium isolated from a saltpan. *Arch Microbiol*, **194**, 103-112.

Chiang, I.Z., Huang, W.Y., Wu, J.T. 2004. Allelochemicals of Botryococcus braunii (Chlorophyceae). *J Phycol*, **40**, 474-480.

Choix, F.J., de-Bashan, L.E., Bashan, Y. 2012. Enhanced accumulation of starch and total carbohydrates in alginate-immobilized Chlorella spp. induced by Azospirillum brasilense: II. Heterotrophic conditions. *Enzyme Microb Technol*, **51**, 300-309.

Christenson, L., Sims, R. 2011. Production and harvesting of microalgae for wastewater treatment, biofuels,and bioproducts. *Biotechnol Adv*, **29**, 686-702.

Coleman, R.A., Lewin, T.M., Horn, C.G.V., Gonzalez-Baró, M.R. 2002. Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic versus degradative pathways? *J Nutr*, **132**, 2123-2126

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G. 2005 November 3 Algae acquire vitamin B12 through a symbiotic relation ship with bacteria. *Nature*, **438**, 90-93.

Danger, M., Oumarou, C., Benest, D. 2007. Bacteria can control stoichiometry and nutrient limitation of phytoplankton. *Funct Ecol*, **21**, 202-210.

Daufresne, T., Loreau, M. 2001. Plant-herbivore interactions and ecological stoichiometry: when do herbivores determine plant nutrient limitation? *Ecology Lett*, **4**, 196-206.

Davey, M.E., O'Toole, G.A. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev*, **64**, 847-867.

de-Bashan, L.E., Antoun, H., Bashan, Y. 2008. Involvement of indole-3-acetic-acid produced by the growth-promoting bacterium Azospirillum spp. in promoting growth of Chlorella vulgaris. *J Phycol*, **44**, 938-947.

de-Bashan, L.E., Bashan, Y., Moreno, M., Lebsky, V.K., Bustillos, J.J. 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae Chlorella spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium Azospirillum brasilense. *Can J Microbiol*, **48**, 514-521.

DellaGreca, M., Zarrelli, A., Fergola, P., Cerasuolo, M., Pollio, A., Pinto, G. 2010. Fatty acids released by Chlorella vulgaris and their role in interference with Pseudokirchneriella subcapitata: experiments and modelling. *J Chem Ecol*, **36**, 339-349.

DoNascimento, M., Dublan, M.A., Marquez, J.C.F.O., Curatti, L. 2013. High lipid productivity of an Ankistrodesmus-Rhizobium artificial consortium. *Bioresour Technol*, **146**, 400-407.

DoNascimento, M., Ortiz-Marquez, J.C.F., Sanchez-Rizza, L., Echarte, M.M., Curatti, L. 2012. Bioprospecting for fast growing and biomass characterization of oleaginous microalgae from South-Eastern Buenos Aires, Argentina. *Bioresour Technol*, **125**, 283-290.

Dostalek, M., Haggstrom, M.H. 1983. Mixed culture of Saccharomycopsis fibuliger and Zymomonas mobilis on starch-use of oxygen as a regulator. *Eur J Appl Microbiol Biotechnol*, **17**, 269-274.

Eiteman, M.A., Lee, S.A., Altman, R., Altman, E. 2009. A substrate-selective co fermentation strategy with Escherichia coli produces lactate by simultaneously consuming xylose and glucose. *Biotechnol Bioeng*, **102**, 822-827.

Fazzini, R.A.B., Preto, M.J., Quintas, A.C.P., Bielecka, A., Timmis, K.N., Santos, V.A.P.M.d. 2010. Consortia modulation of the stress response: proteomic analysis of single strain versus mixed culture. *Environ Microbiol*, **12**, 2436-2449.

Fergola, P., Cerasuolo, M., Pollio, A., Pinto, G., DellaGreca, M. 2007. Allelopathy and competition between Chlorella vulgaris and Pseudokirchneriella sub capitata: experiments and mathematical model. *Ecol Model*, **208**, 205-214.

Frengova, G., Simova, E., Beshkova, D. 1997. Caroteno-protein and exopolysaccharide production by co cultures of Rhodotorula glutinis and Lactobacillus helveticus. *J Indus Microbio Biotechnol*, **18**, 272-277.

Furusawa, G., Yoshikawa, T., Yasuda, A. 2003. Algicidal activity and gliding motility of Saprospira sp. SS98-5. *Can J Microbiol*, **49**, 92-100.

Gen, Q., Wang, Q., Chi, Z.M. 2014. Direct conversion of cassava starch into single cell oil by co-cultures of the oleaginous yeast Rhodosporidium toruloides and immobilized amylases-producing yeast Saccharomycopsis fibuligera. *Renew Energ*, **62**, 522-526.

Gong, Y., Hu, H., Gao, Y., Xu, X., Gao, H. 2011. Microalgae as platforms for production of recombinant proteins and valuable compounds: progress and prospects. *J Ind Microbiol Biotechnol*, **38**, 1879-1890.

Gouda, M.K., Omar, S.H., Aouad, L.M. 2008. Single cell oil production by Gordonia sp. DG using agro-industrial wastes. *World J Microbiol Biotechnol,* **24**, 1703-1711.

Greenwell, H., Laurens, L., Shields, R., Lovitt, R., Flynn, K. 2010. Placing microalgae on the biofuels priority list: a review of the technological challenges. *J R Soc Interface*, **7**, 703-726.

Gultom, S.O., Hu, B. 2013. Review of Microalgae Harvesting via Co-Pelletization with Filamentous Fungus. *Energies*, **6**, 5921-5939.

Haggstrom, M.H., Dostalek, M. 1981. Regulation of a mixed co-culture of Streptococcus lactis and Saccharomycopsis fibuliger. *Eur J Appl Microbiol Biotechnol*, **12**, 216-219.

Halim, R., Danquah, M.K., Webley, P.A. 2012. Extraction of oil from microalgae for biodiesel production: a review. *Biotechnol Adv*, **30**, 709-732.

Hong, T., Koo, C., Kwak, T., Park, H.S. 2014. An economic and environmental assessment for selecting the optimum new renewable energy system for educational facility. *Renew Sustain Energy Rev*, **29**, 286-300.

Jarl, K. 1969. Production of microbial food from low cost starch materials and purification of industry's waste starch effluents through the Symba process. *Food Technol*, **23**, 1009-1012.

Jefferson, K.K. 2004. What drives bacteria to produce a biofilm? *FEMS Microbiol Lett,* **236**, 163-173.

Ji, M.K., Kim, H.C., Sapireddy, V.R., Yun, H.S., Abou-Shanab, R.A.I., Choi, J., Lee, W., Timmes, T.C., Jeon, B.H. 2013. Simultaneous nutrient removal and lipid production from pretreated piggery wastewater by Chlorella vulgaris YSW-04. *Appl Microbiol Biotechnol*, **97**, 2701-2710.

Kadic, E. 2010. Survey of gas-liquid mass transfer in bioreactors,Lowa State University,.

Kazamia, E., Czesnick, H., Nguyen, T.T.V., Croft, M.T., Sherwood, E., Sasso, S., Hodson, S.J., Warren, M.J., Smith, A.G. 2012. Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation. *Environ Microbiol*, **14**, 1466-76.

Kitcha, S., Cheirsilp, B. 2014. Enhanced Lipid Production by Co-cultivation and Co-encapsulation of Oleaginous Yeast Trichosporonoides spathulata with Microalgae in Alginate Gel Beads. *Appl Biochem Biotechnol*, **173**, 522-534.

Leite, G.B., Abdelaziz, A.E.M., Hallenbeck, P.C. 2013. Algal biofuels: Challenges and opportunities. *Bioresour Technol*, **145**, 134-141.

Liang, Y., Sarkany, N., Cui, Y. 2009. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnol Lett*, **31**, 1043-1049.

Liu, W., Wang, Y., Yu, Z., Bao, J. 2012. Simultaneous saccharification and microbial lipid fermentation of corn stover by oleaginous yeast Trichosporon cutaneum. *Bioresour Technol*, **118**, 13-18.

Liu, Y., Tan, H., Deng, Q., Cao, L. 2014. Characterization of fibrolytic and lipid accumulating fungi isolated from fresh cattle feces. *Environ Sci Pollut Res* **21**, 9228-9233

Luo, S.S., Dong, Z.J., Wu, X.D., Liu, Y.H., Ruan, R. 2013. Pelletization Behavior of Fungal Chlorella Sp Symbiosis System. *Resear. J. Biotechnol.*, **8**, 56-59.

Ma, Y.L. 2006. Microbial oils and its research advance. *Chin J Bioprocess Eng*, **4**, 7-11.

Martinez, V., Garcia, P., Garcia, J.L., Prieto, M.A. 2011. Controlled autolysis facilitates the polyhydroxyalkanoate recovery in Pseudomonas putida KT2440. *Microbial Biotechnol*, **4**, 533-547.

Mendes, L.B.B., Vermelho, A.B. 2013. Allelopathy as a potential strategy to improve microalgae cultivation. *Biotechnol Biofuels*, **6**, 152-166.

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

Miao, X.L., Wu, Q.Y.B. 2004. Bio-oil fuel production from microalgae after heterotrophic growth. *Renew Energy*, **4**, 41-44.

Milledge, J., Heaven, S. 2013. A review of the harvesting of micro-algae for biofuel production. *Rev Environ Sci Biotechnol*, **12**, 165-178.

Munasinghe, P.C., Khanal, S.K. 2010. Biomass-derived syngas fermentation into biofuels:opportunities and challenges. *Bioresour Technol*, **101**, 5013-22.

O'Reilly, A.M., Scott, J.A. 1995. Defined coimmobilization of mixed microorganism cultures. *Enzyme Microb Technol*, **17**, 636-640.

Oh, H.M., Ahn, C.Y., Lee, Y.K., KO, S.R., Kim, H.S. 2014 March. Novel microorganism Rhizobium sp. Publication calssifcation KB 10 having properties of promoting growth of Botryococcus Braunii and increased fatty acid content. United States patent US20140087420.

Ohlrogge, J.B., Jaworski, J.G. 1997. Regulation of fatty acid synthesis. *Annu Rev Plant Physiol Plant Mol Biol*, **48**, 109-136

Olukoshi, E.R., Packter, N.M. 1994. Importance of stored triacylglycerols in Streptomyces: possible carbon source for antibiotics. *Microbiology*, **140**, 931-943.

Palmqvist, E., Hahn-Hagerdal, B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresour Technol*, **74**, 25-3.

Papone, T., Kookkhunthod, S., Leesing, R. 2012. Microbial oil production by monoculture and mixed cultures of microalgae and oleaginous yeasts using sugarcane juice as substrate. *World Acad Sci Eng Technol,* **64**, 1127-1131.

Park, E.Y., Naruse, K., Kato, T. 2012. One-pot bioethanol production from cellulose by co-culture of Acremonium cellulolyticus and Saccharomyces cerevisiae. *Biotechnol Biofuels*, **5**, 64.

Pepi, M., Minacci, A., Cello, F.D., Baldi, F., Fani, R. 2003. Long-term analysis of diesel fuel consumption in a co-culture of Acinetobacter venetianus, Pseudomonas putida and Alcaligenes faecalis. *Antonie van Leeuwenhoek*, **83**, 3-9.

Peralta-Yahya, P.P., Keasling, J.D. 2010. Advanced biofuel production in microbes. *Biotechnol J*, **5**, 147-62.

Perez-Garcia, O., Escalante, F.M., de-Bashan, L.E., Bashan, Y. 2011. Heterotrophic cultures of microalgae: metabolism and potential products. *Water Res*, **45**, 11-36.

Pickens, L.B., Tang, Y., Chooi, Y.H. 2011. Metabolic Engineering for the Production of Natural Products. *Ann Rev Chem Biomol Eng*, **2**, 211-236.

Powell, R.J., Hill, R.T. 2013. Rapid Aggregation of Biofuel-Producing Algae by the Bacterium Bacillus sp Strain RP1137. *Appl Environ Microbiol*, **79**, 6093-6101.

Pragya, N., Pandey, K.K., Sahoo, P.K. 2013. A review on harvesting, oil extraction and biofuels production technologies from microalgae. *Renew Sustain Energy Rev*, **24**, 159-171.

Pratt, R., Fong, J. 1940. Studies on Chlorella vulgaris II. Further evidence that Chlorella cells form a growth-inhibiting substance. *Am J Bot*, **27**, 431-436.

Puangbut, M., Leesing, R. 2012. Integrated cultivation technique formicrobial lipid production by photosynthetic microalgae and locally oleaginous yeast. *World Acad Sci Eng Technol*, **6**, 975-979.

Rai, P.K., Singh, S.P., Asthana, R.K. 2012. Biohydrogen production from cheese whey wastewater in a two-step anaerobic process. *Appl Biochem Biotechnol*, **167**, 1540-1549.

Rasala, B.A., Gimpel, J.A., Tran, M., Hannon, M.J., Miyake-Stoner, S.J., Specht, E.A., Mayfield, S.P. 2013. *Genetic engineering to improve algal biofuels production. In: Algae for Biofuels and Energy*.

Riquelme, C.E., Avendaño-Herrera, R.E. 2003. Interacción bacteria-microalga en elambiente marino y uso potencial en acuicultura. *Revista Chilena Historia Natural,* **76**, 725-736.

Rivas, M.O., Vargas, P., Riquelme, C.E. 2010. Interactions of Botryococcus braunii cultures with bacterial biofilms. *Microb Ecol,* **60**, 628-635.

Rooney-Varga, J.N., Giewat, M.W., Savin, M.C., Sood, S., LeGresley, M., Martin, J.L. 2005. Links between Phytoplankton and bacterial community dynamics in a coastal marine environment. *Microb. Ecol.*, **49**, 163-175.

Rwehumbiza, V.M., Harrison, R., Thomsen, L. 2012. Alum-induced flocculation of preconcentrated Nannochloropsis salina: Residual aluminium in the biomass, FAMEs and its effects on microalgae growth upon media recycling. *Chem. Eng. J.*, **200-202**, 168-175.

Ryu, B.G., Kim, J., Farooq, W., Han, J.I., Yang, J.W., Kim, W. 2014. Algal-bacterial process for the simultaneous detoxification of thiocyanate-containing wastewater and maximized lipid production under photoautotrophic/photoheterotrophic conditions. *Bioresour Technol*, **162**, 70-79.

Sanderson, K. 2011. Lignocellulosic: a chewy problem. *Nature*, **474**, 12-14.

Santos, C.A., Ferreira, M.E., Silva, T.L.d., Gouveia, L., Novais, J.M., Reis, A. 2011. A symbiotic gas exchange between bioreactors enhances microalgal biomass and lipid productivities: taking advantage of complementary nutritional modes. *J Ind Microbiol Biotechnol*, **38**, 909-917.

Santos, C.A., Sliva, M.L.C.L., Novais, J.M., Reis, A. 2013. Enhanced lipidic algae biomass production using gas transfer from a fermentative Rhodosporidium toruloides culture to an autotrophic Chlorella protothecoides culture. *Bioresour Technol*, **138**, 48-54.

Sathish, A., Sims, R.C. 2012. Biodiesel from mixed culture algae via a wet lipid extraction procedure. *Bioresour Technol*, **118**, 643-647.

Schirmer, A., Rude, M.A., Li, X., Popova, E., Cardayre, S.B.d. 2010. Microbial biosynthesis of alkanes. *Science*, **329**, 559-62.

Sharma, K.K., Garg, S., Li, Y., Malekizadeh, A., Schenk, P.M. 2013. Critical analysis of current microalgae dewatering techniques. *Biofuels*, **4**, 397-407.

Slivka, R.M., Chinn, M.S., Grunden, A.M. 2011. Gasification and synthesis gas fermentation: an alternative route to biofuel production. *Biofuels* **2**, 405-419.

Smith, D.C., Douglas, A.E. 1987. *The Biology of Symbiosis, Edward Arnold, London, UK,*.

Solovchenko, A.E., Khozin-Goldberg, I., Didi-Cohen, S., Cohen, Z., Merzlyak, M.N. 2008. Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga Parietochloris incisa. *J Appl Phycol*, **20**, 245-251.

Steinbüchel, A. 1991. Polyhydroxyalkanoic acids. *Biomaterials*, 123-213.

Su, H.Y., Zhang, Y.L., Zhang, C.M., Zhou, X.F., Li, J.P. 2011. Cultivation of Chlorella pyrenoidosa in soybean processing wastewater. *Bioresour Technol*, **102**, 9884-9890.

Subashchandrabose, S.R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., Naidu, R. 2011. Consortia of cyanobacteria/ microalgae and bacteria: biotechnological potential. *Biotechnol Adv*, **29**, 896-907.

Sukenik, A., Livne, A. 1991. Variations in lipid and fatty acid content in relation to acetyl CoA carboxylase in the marine prymnesiophyte Isochrysis galbana. *Plant Cell Physiol*, **32**, 371-378.

Takagi, M., Watanabe, K., Yamaberi, K., Yoshida, T. 2000. Limited feeding of potassium nitrate for intracellular lipid and triglyceride accumulation of Nannochloris sp.UTEX LB1999. *Appl Microbiol Biotechnol*, **54**, 112-117.

Tate, J.J., Wing, M.T.G., Rusch, K.A., Benton, M.G. 2013. The effects of plant growth substances and mixed cultures on growth and metabolite production of green algae Chlorella sp.: a review. *J Plant Growth Regul*, **32**, 417-428.

Therien, J.B., Zadvornyy, O.A., Posewitz, M.C., Bryant, D.A., Peters, J.W. 2014. Growth of Chlamydomonas reinhardtii in acetate-free medium when co-cultured with alginate-encapsulated, acetate-producing strains of Synechococcus sp. PCC 7002. *Biotechnol Biofuels*, **7**, 154.

Trabelsi, A., Rassoulzadegan, F. 2011. Inorganic nutrient control of dissolved organic carbon (DOC) dynamics in NW Mediterranean waters: An experimental approach. *Mar Biol Res,* **7**, 667-676.

Uduman, N., Qi, Y., Danquah, M.K., Hoadley, A.F.A. 2010. Marine microalgae flocculation and focused beam reflectance measurement. *Chem En J*, **162**, 935-940.

Ungerman, A.J., Heindel, T.J. 2007. Carbon monoxide mass transfer for syngas fermentation in a stirred tank reactor with dual impeller configurations. *Biotechnol Prog*, **23**, 613-20.

Vandamme, D., Foubert, I., Muylaert, K. 2013. Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends Biotechnol*, **31**, 233-239.

VanHeerden, A., Roux, N.J.L., Swart, J., Gardner-Lubbe, S., Botha, A. 2008. Assessment of wood degradation by Pycnoporus sanguineus when co-cultured with selected fungi. *World J Microbiol Biotechnol*, **24**, 2489-2497.

Vega, J., Clausen, E., Gaddy, J. 1990. Design of bioreactors for coal synthesis gas fermentations. *Resour Conserv Recycl*, **3**, 149-60.

Wan, C., Zhao, X.-Q., Guo, S.-L., Alam, M.A., Bai, F.-W. 2013. Bioflocculant production from Solibacillus silvestris W01 and its application in cost-effective harvest of marine microalga Nannochloropsis oceanica by flocculation. *Bioresour Technol*, **135**, 207-212.

Wang, Y., Yang, Y., Ma, F., Xuan, L., Xu, Y., Huo, H., Zhou, D., Dong, S. 2015. Optimization of Chlorella vulgaris and bioflocculant-producing bacteria co-culture: enhancing microalgae harvesting and lipid content. *Lett Appl Microbiol*, **60**, 497-503.

Weng, J.K., Li, X., Bonawitz, N.D., Chapple, C. 2008. Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. *Curr Opin Biotechnol,* **19**, 166-172.

Wrede, D., Taha, M., Miranda, A.F., Kadali, K., Stevenson, T., Ball, A.S., Mouradov, A. 2014. Co-Cultivation of Fungal and Microalgal Cells as an Efficient System for Harvesting Microalgal Cells, Lipid Production and Wastewater Treatment. *PLOS ONE*, **9**.

Wu S., Li X. , Yu J., Wang Q. . 2012. Increased hydrogen production in co-culture of Chlamydomonas reinhardtii and Bradyrhizobium japonicum. *Bioresour Technol*, **123** 184-188.

Xia, C.J., Wei, W., Hu, B. 2014. Statistical Analysis and Modeling of Pelletized Cultivation of Mucor circinelloides for Microbial Lipid Accumulation. *Appl Biochem Biotechnol*, **172**, 3502-3512.

Xie, B., Bishop, S., Stessman, D., Wright, D., Spalding, M.H., Halverson, L.J. 2013a. Chlamydomonas reinhardtii thermal tolerance enhancement mediated by a mutualistic interaction with vitamin B12-producing bacteria. *ISME J*, **7**, 1544-1555.

Xie, S.X., Sun, S., Dai, Y., Yuan, J.S. 2013b. Efficient coagulation of microalgae in cultures with filamentous fungi. *Algal Research-Biomass Biofuels and Bioproducts*, **2**, 28-33.

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

Xue, F., Miao, J., Zhang, X., Tan, T. 2010. A new strategy for lipid production by mix cultivation of Spirulina platensis and Rhodotorula glutinis. *Appl Biochem Biotechnol*, **160**, 498-503.

Yi, S.J., Zheng, Y.P. 2006. Research and application of oleaginous microorganism. *China Foreign Energy*, **11**, 90-94.

Yoo, G., Park, W.K., Kim, C.W., Choi, Y.E., Yang, J.W. 2012. Direct lipid extraction from wet Chlamydomonas reinhardtii biomass using osmotic shock. *Bioresour Technol*, **123**, 717-722.

Yousuf, A. 2012. Biodiesel from lignocellulosic biomass-prospects and challenges. *Waste Manage,* **32**, 2061-2067.

Yu, X., Zheng, Y., Dorgan, K.M., Chen, S. 2011. Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. *Bioresour Technol*, **102**, 6134-6140.

Zhang, J., Hu, B. 2012. A novel method to harvest microalgae via co-culture of filamentous fungi to form cell pellets. *Bioresour Technol*, **114**, 529-35.

Zhang, Y., Su, H., Zhong, Y., Zhang, C., Shen, Z., Sang, W., G.Yan, Zhou, X. 2012. The effect of bacterial contamination on the heterotrophic cultivation of Chlorella pyrenoidosa in wastewater from the production of soybean products. *Water Res,* **46**, 5509-5516.

Zhao, C.H., Chi, Z., Zhang, F., Guo, F.J., Li, M., Song, W.B. 2011. Direct conversion of inulin and extract of tubers of Jerusalem artichoke into single cell oil by co-cultures of Rhodotorula mucilaginosa TJY15a and immobilized inulinase producing yeast cells. *Bioresour Technol*, **102**, 6128-6133.

Zhao, P., Yu, X., Li, J., Tang, X., Huang, Z. 2014. Enhancing lipid productivity by co-cultivation of Chlorella sp. U4341 and Monoraphidium sp. FXY-10. *J Biosci Bioeng*, **118**, 1-6.

Zhu, H., Shanks, B.H., Choi, D.W., Heindel, T.J. 2010. Effect of functionalized MCM41 nanoparticles on syngas fermentation. *Biomass Bioenergy*, **34**, 1624-7.

Zhu, H., Shanks, B.H., Heindel, T.J. 2009. Effect of electrolytes on CO-water mass transfer. *Ind Eng Chem Res*, **48**, 3206-3210.

Zhu, H., Shanks, B.H., Heindel, T.J. 2008. Enhancing CO-water mass transfer by functionalized MCM41 nanoparticles. *Ind Eng Chem Res*, **47**, 7881-7.

Zoller, S., Lutzoni, F. 2003. Slow algae, fast fungi: exceptionally high nucleotide substitution rate differences between lichenized fungi Omphalina and their symbiotic green algae Coccomyxa. *Mol Phylog Evol*, **29**, 629-640.

Zoppini, A., Puddu, A., Fazi, S., Rosati, M., Sist, P. 2005. Extracellular enzyme activity and dynamics of bacterial community in mucilaginous aggregates of the northern Adriatic Sea. *Sci Total Environ*, **353**, 270-286.

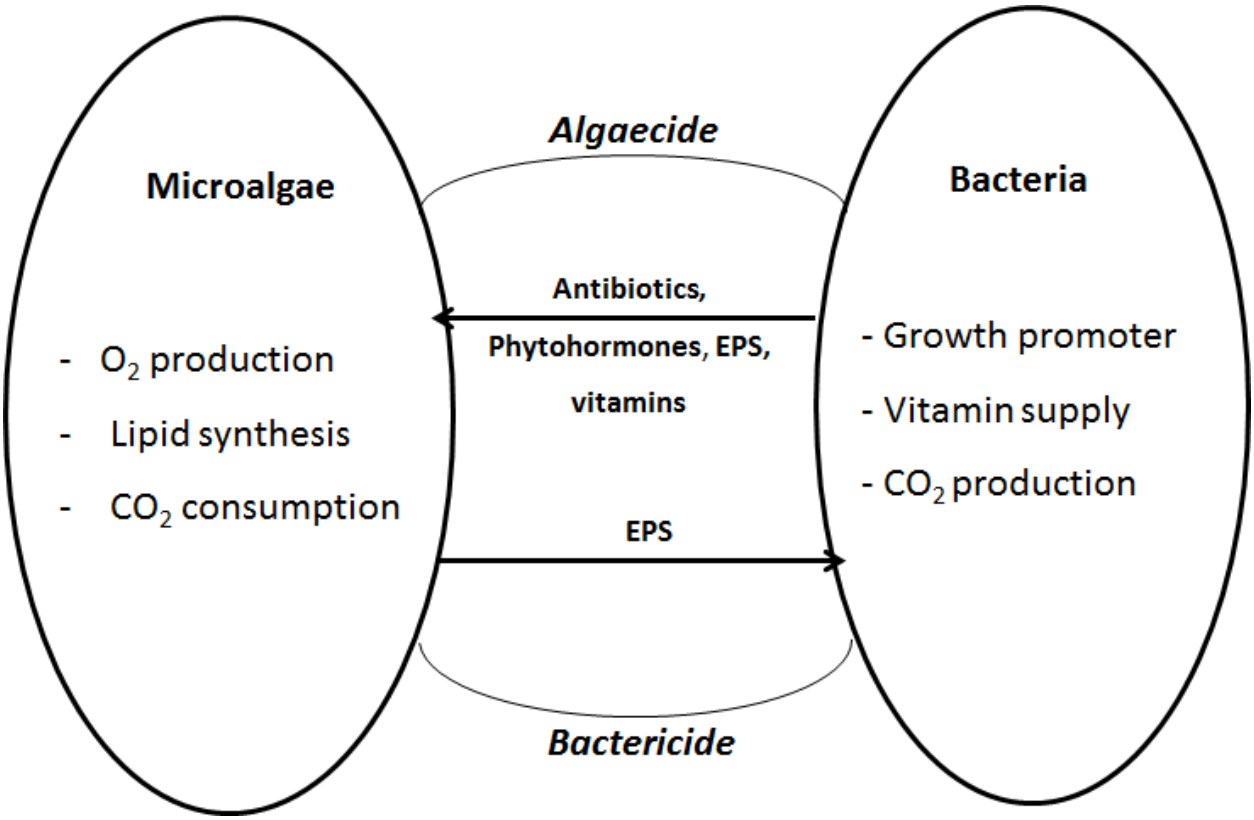


Figure 8.1 **Interactions between microalgae growth-promoting bacterium**

Tableau 8.1 Biotechnological potential of mixed cultures

Purposes	References
Harvesting bioflocculants	(Rai et al., 2012; Subashchandrabose et al., 2011)
Wastewater Treatment	(Arumugam et al., 2014; Jarl, 1969)
Production of EPS	(Haggstrom & Dostalek, 1981)
Single cell protein	(Angelis et al., 2012; de-Bashan et al., 2002; Dostalek & Haggstrom, 1983)
Flocculation process	(Cheirsilp et al., 2011; Xue et al., 2010)
Electricity generation	(Gong et al., 2011)
PHA production	(Abed, 2010; Miao & Wu, 2004; O'Reilly & Scott, 1995)
Organic acids production	(Brenner et al., 2008; Rasala et al., 2013)
Heavy metals removal	(Xiong et al., 2008)
Biohydrogen production	(Abed, 2010; Liang et al., 2009; O'Reilly & Scott, 1995)
Ethanol production	(Bogen et al., 2013; Vandamme et al., 2013; Wrede et al., 2014; Yu et al., 2011)
Growth promotion and Lipid production	(Arumugam et al., 2014; Milledge & Heaven, 2013; Pragya et al., 2013; Sharma et al., 2013)

Tableau 8.2 Lipid production by mixed culture of oleaginous yeasts and microalgae

Algae	Yeast	Substrate	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%w/w)	References
C. vulgaris	*R. glutinis*	3% pure glycerol and urea	3.2	1.1	34.4	(de-Bashan et al., 2008)
C. vulgaris	*R. glutinis*	Seafood processing effluent	4.63	2.88	62.2	(Dostalek & Haggstrom, 1983)
C. pyrenoidosa	*R. glutinis*	Distillery wastewater	7.25	95.3	63.5	(Oh et al., 2014 March)
S. platensis	*R. glutinis*	Monosodium glutamate wastewater	1.6	0.47	13.8	(Angelis et al., 2012)
Isochrysis galbana 8701	*Ambrosiozyma cicatricose*	Seawater supplemented with glucose	1.32	0.15	11.2	(Rivas et al., 2010)
Chlorella spp.	*Trichosporonoides spathulata*	Crude glycerol based medium	12.2	5.74	47.0	(Ryu et al., 2014)
Chlorella sp. KKU-S2	*Torulaspora maleeae Y30*	Synthetic medium	7.33	0.81	26.8	(Wu S. et al., 2012)
Scenedesmus obliquus	*R. glutinis*	Glucose	10.0	2.4	24.0	(DoNascimento et al., 2013)

Tableau 8.3 Challenges of co-culture in lipid production and approaches to overcome the same

Challenges	Approaches
High price of raw materials	Exploiting agro-industrial waste and by-products
Selection of suitable microbial consortia	Natural selection Synthetic biology system
Knowledge of the composition of microbial communities	rRNA (metagenomic) sequencing NMR spectroscopy High performance liquid chromatography (HPLC) Proteomic approaches
Complexity of microbe-microbe interactions	Combining metabolic profiling and targeted growth assays
Downstream processing long and costly	Harvesting via flocculation to ease the precipitation process Inducible cell lysis
Lower mass transfer	Enhancement of mass transfer (use of nanoparticules, controlling agitation parameter during fermentation) Bioreactor design
Lipid extraction	Solvent extraction Osmotic pressure Chemical treatment Liquid or wet extraction system
Generating high cell density	Use the generating biomass as fertilizer

9 ANNEXES

ANNEXE I

PUBLICATIONS EN DEHORS DE CETTE THÈSE

1. **Sarra Magdouli,** Rimeh Daghrir, Satinder Kaur Brar, Rajeshwar Dayal Tyagi (2013) Di 2-ethylhexylphtalate in the aquatic and terrestrial environment: a critical review. *Journal of Environmental Management*, 127, 36-49.
2. **Sarra Magdouli**, Tarek Rouissi, Satinder Kaur Brar, Jean-Francois Blais (2016) Propylene glycol: an industrially important C3 platform chemical. Livre intitulé "*Platform chemical biorefinery– future green industry*". Elsevier, Amsterdam, Pays-Bas, pp. 77–100.
3. **Sarra Magdouli**, Tarek Rouissi, Satinder Kaur Brar, Jean-François Blais (2016) Life cycle analysis of potential substrates of sustainable biorefiery. Livre intitulé "*Platform chemical biorefinery – future green industry*". Elsevier, Amsterdam, Pays-Bas, pp. 285–306.
4. **Sarra Magdouli**, Satinder Kaur Brar, Jean-François Blais (2016) Production of drop-in and novel bio-based platform chemicals. Livre intitulé "*Platform chemical biorefinery – future green industry*". Elsevier, Amsterdam, Pays-Bas, pp. 249–283.
5. C. Marques, Tarek Rouissi, **Sarra Magdouli**, Satinder Kaur Brar (2016) Sorbitol production from biomass and its global market. Livre intitulé "*Platform chemical biorefinery – future green industry*". Elsevier, Amsterdam, Pays-Bas, pp. 217–227.
6. Kadri Tayssir, **Sarra Magdouli**, Satinder Kaur Brar, Jean-Francois Blais (2016) Surface water and ground water remediation – integrated approaches. (Article en préparation).
7. **Sarra Magdouli**, Satinder Kaur Brar, Jean-Francois Blais (2016) Practical aspects and case studies of industrial scale fermentation. Livre intitulé "*Microbial sensing in fermentation*".
8. Tayssir Kadri, Tarek Rouissi, Satinder Kaur Brar, **Sarra Magdouli,** Rimeh Daghrir, Jean-Marc Lauzon (2016) Production and characterization of novel extracellular alkane hydroxylase, lipase and esterase produced by *A. borkumensis* for biodegradation of hydrocarbons. Article en préparation

ANNEXE II

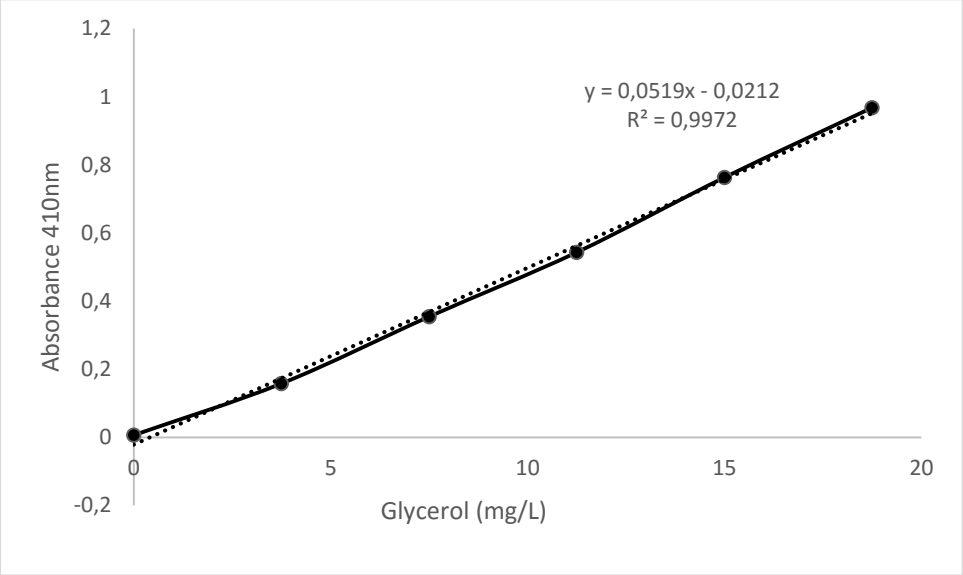


Figure 9.1 Courbe standard pour la determination de la concentration du glycérol

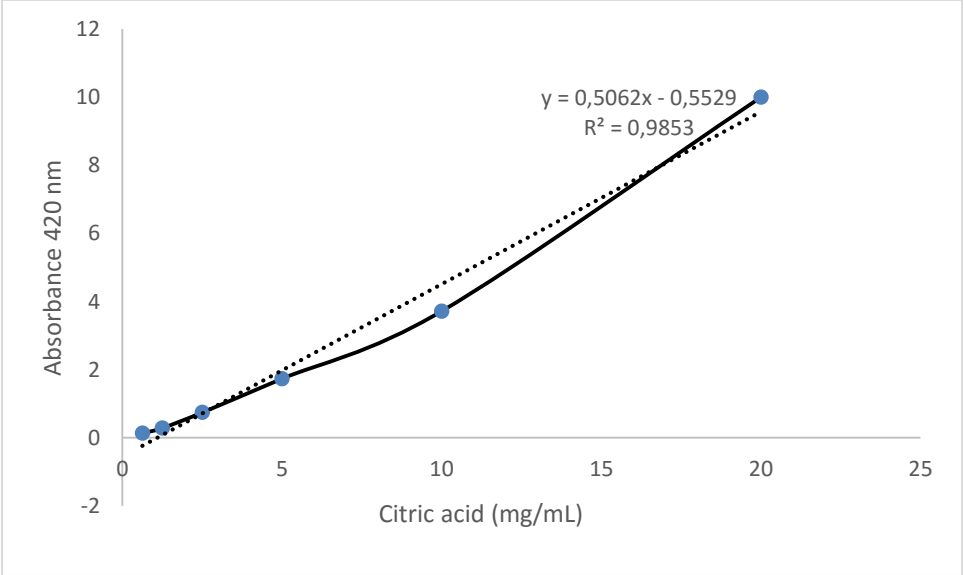


Figure 9.2 Courbe standard pour la determination de la concentration de l'acide citrique