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Biotransformation du glycérol brut en lipides par microorganismes hétérotrophes (levures) pour la production de biodiésel

par

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DEDICACE

Dedicated to my Family
and Teachers

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RÉSUMÉ

La recherche de nouvelles sources énergétiques représente une voie d'étude importante au regard des problèmes environnementaux liés à la forte consommation d'énergie fossiles (huiles lourdes, essence, diesel, gaz, etc.). Il s'agit aujourd'hui de mettre en œuvre des matières premières ou d'énergies renouvelables obtenues à partir de biomasse d'origine végétale, animale ou suite à de nouvelles transformations chimiques. Dans ce contexte, les huiles issues de microorganismes oléagineux produits à partir de ressources renouvelables comme le glycérol, les huiles végétales, les déchets agro-résiduels, déchets solides et liquides municipaux, les résidus forestiers et autres déchets industriels couvrant une richesse organique, pouvant être microbiologiquement assimilable, représentent des avenues potentielles. Actuellement, divers carburants renouvelables principalement le bioéthanol sont produits à partir de mélasses ou d'autres sucres fermentescibles facilement disponibles. Par ailleurs, la synthèse de lipides microbiens constitue un mécanisme biologique fondé et bien connu notamment de par la production industrielle des TAGs (Triacylglycérols). Ces microorganismes possèdent un large éventail de propriétés physiologiques et métaboliques pour l'utilisation de substrats multiples. Les microbes oléagineux accumulent des lipides intracellulaires variant de 20 à 85% (p/p) de la biomasse cellulaire totale sèche. Compte tenu de ces propriétés avantageuses, le glycérol ainsi que certains déchets agro-industriels font l'objet d'études comme sources de carbone ou substrats assimilables pour la production de lipides.

Des échantillons de sol prélevés à partir de milieux forestiers vierges et autres sols contaminés par des déversements accidentels de glycérol ont été sélectionnés et reproduits dans des milieux de cultures sélectifs supplémentées de glycérol. Des souches de bactéries, de levures et des champignons filamenteux sont apparus dans les 48h suivant l'incubation et ont été acheminés pour des analyses plus poussées. Tous les isolats ont été cultivés dans du glycérol brut (GB) enrichi avec une limitation d'azote pendant 120h. Les cellules récoltées après incubation ont été colorées avec le Soudan noir B et observées au microscope à contraste de phases. Les organismes ayant révélés des globules d'huile en observation microscopique sur lame fixe, ont été sélectionnés pour un criblage secondaire subséquent. Tous les organismes ont été initialement identifiés par Biolog suivi de séquençage (ADNr 18S). Dans le criblage secondaire, une concentration GB de 100 g/L et deux différents ratios molaires C/N ont été choisis. La fermentation a été menée jusqu'à 120h après l'inoculation des souches. Sur la base des résultats

obtenus, la souche SKY7 (teneur en lipides de 45% en p/p) a été sélectionnée pour les étapes subséquentes.

SKY7 a été identifiée comme *Yarrowia lipolytica* par la méthode biochimique et génomique via le séquençage d'ADNr 18S. Cette souche est bien connue comme oléagineuse, elle est aussi dotée de la production d'acide citrique et surtout capable d'utiliser une large gamme de substrats. Par ailleurs, les paramètres de culture ont été évalués pour *Y.lipolytica* SKY7 afin d'obtenir une production optimisée en lipides intracellulaires. Les expériences menées en erlenmeyer à petite échelle initiale ont été réalisées à différentes concentrations de glycérol, rapport molaire C/N, taille de l'inoculum, et concentrations en oligo-éléments. Les meilleurs résultats (en termes de concentrations en biomasse et de lipides accumulés) ont été enregistrés avec une concentration en glycérol de 100 g/L et un ratio C/N de 100.
Relativement aux résultats obtenus, la fermentation a été effectuée à plus grande échelle (réacteur de 15L de capacité totale avec un volume utile de 11L) en utilisant 100 g/L de glycérol, un rapport C/N molaire de 100 et 5% v/v d'inoculum et ce, sous 2 angles comparatifs : avec et sans contrôle du pH. La synthèse de lipides a été globalement observée entre 30 et 60h. Dans le cas des expériences à pH contrôlé, des concentrations en lipides (43,2% p/p) de 7,78g /L avec une biomasse de 18 g/L ont été atteintes. Même dans l'éventualité de résultats similaires en biomasse durant la fermentation, la condition de pH non contrôlé démontre des concentrations significativement plus faibles en lipides par rapport aux résultats d'expériences à pH contrôlé. Par conséquent, on conclut que le contrôle du pH pourrait améliorer la synthèse des lipides pendant la fermentation. De plus, à partir des résultats expérimentaux, on a supposé à partir de la littérature qu'*Y.lipolytica* SKY7 exigeait un rapport C/N molaire entre 350-400 pour son métabolisme en phase lipogénique. Le maintien du rapport molaire C/N dans l'intervalle indiqué étend la phase lipogénique et permet d'améliorer le rendement global de production en lipides.

La présente étude met en œuvre le potentiel de production de lipides par *Y.lipolytica* SKY7 à partir d'un milieu synthétique à base de GB. Afin de maximiser la concentration en lipides et en biomasse, la méthode de plan d'expérience Box-Behnken a été adoptée et les principaux paramètres étudiés étaient les suivants : concentration de glycérol, rapport molaire C/N et volume de l'inoculum. Des concentrations de biomasse de 29,5 g /L et 50% (p/p) de lipides ont

été enregistrés dans les conditions optimales (concentration de glycérol 82,5 g/L et de rapport C/N 75 avec un inoculum à 6,25% v/v).

Afin d'améliorer la production en lipides à partir de microorganismes hétérotrophes, une approche alternative permettant de substituer les nutriments commerciaux a été retenue. Des sources d'azote organique et inorganique ont été ainsi remplacées par des lixiviats de silos de stockage de grains et les principaux résultats ont dévoilé qu'il s'agit d'une perspective plausible pour l'utilisation de déchets à faible coût comme source nutritive pour la synthèse de lipides microbiens. Cette approche d'utilisation de déchets à faible coût permet de démontrer l'intérêt économique et environnemental de la technologie proposée en tenant compte de trois points principaux : 1) diminution de coût de procédé ; 2) contribution à la protection de l'environnement par l'élimination des nutriments à partir de flux de déchets nuisibles et ; 3) valeur ajoutée des flux de déchets a un avantage en vue de la valorisation.

En outre, les études menées à l'échelle du fermenteur (15L) avec des ajouts de lixiviats de silos (200ml/L) ont donné lieu à une biomasse et des lipides de concentrations de 25,82 et de 15,15g/L, respectivement au bout de seulement 51h de fermentation. Les rendements de la biomasse et des lipides ainsi obtenus sont de 83,45g/mol C et 53,28g/ C mol avec une teneur en lipides de 63,85% en p/p de la masse cellulaire sèche. Toutefois, le remplacement de la source nutritive seule ne peut pas rendre le processus économiquement viable. Les conditions opérationnelles influencent vigoureusement l'économie du processus. La stérilisation des réacteurs et des milieux composants est une étape exigeante en manipulations et à haute demande en énergie. Ainsi, en éliminant cette opération, on pourrait accroître l'économie globale du procédé pour une faisabilité plus probable.

Un procédé dit non stérile en présence de méthanol a été étudié pour éliminer le processus de stérilisation. Les premiers résultats obtenus à partir des expériences en erlenmeyer ont démontré une accumulation de biomasse de 9,8 g/L avec une teneur en lipides de 48% p/p quand on rajoute 3% de méthanol (v/v). Cependant, lorsque des conditions analogues ont été reproduites à l'échelle de bioréacteur, la fermentation contrôlée relevant de ce procédé a échoué (contamination observée) après 18h du début de la fermentation. Par conséquent, une autre approche alternative a été formulée. Un traitement UV d'une durée de 10 minutes a été introduit pour traiter l'eau du robinet avant son ajout comme eau de procédé servant à la dilution du milieu

de culture. Grâce à ce traitement aux UV avec une addition de 3% (v/v) de méthanol, nous avons pu retarder l'apparition de la contamination jusqu'à 42h après le début de la fermentation. Il en résultait une biomasse élevée (17,57g/L) et une concentration de lipides (8,8 g/L) non négligeable via ce processus non stérile. En comparaison avec la condition expérimentale précédente (3% v/v de méthanol seul) la biomasse et la concentration des lipides ont été améliorés par des facteurs de 3 et 4, respectivement.

Afin d'améliorer l'accumulation de lipides à l'intérieur des cellules microbiennes, une approche biochimique a été expérimentée. Une série de concentrations différentes en biotine (10, 50 et 100 µg/L), ajoutée en supplément au milieu de fermentation établi (précédemment) a été étudiée.
En général, la biotine est l'un des facteurs de croissance importants et nécessaires pour la synthèse des lipides membranaires (paroi cellulaire). En outre, la biotine renferme un groupement prosthétique principal (ACC- l'acétyl-CoA carboxylase) agissant par la carboxylation de l'acétyl-CoA en malonyl-CoA pour la synthèse des TAGs. Des travaux antérieurs sur l'ajout de biotine ont été rapportés pour améliorer la production d'acides gras à longue chaîne. Dans le cas présent, l'addition de 100 µg/L de biotine a notamment amélioré la concentration de lipides dans des cultures en batch de *Y. lipolytica*. Par rapport aux conditions de contrôle nous avons remarqué une amélioration du rendement par un facteur de 0,5 sur la concentration totale de lipides totaux extraits. Les concentrations de biomasse et de lipides réelles (expérimentales) ainsi obtenues sont de 26,56g/L de biomasse avec 10,12g/L de lipides dans les conditions de contrôle et de 27,6 g/L de biomasse et de 16.16g/L de lipides pour les études incluant la biotine.

En outre, d'autres expériences réalisées sur la production de lipase et d'acide citrique chez *Y. lipolytica* ont été discutées. Le milieu de fermentation est composé de glycérol brut et de savon comme source de carbone. La production supplémentaire d'acide citrique peut être valorisée par son usage en nutrition et autres applications connexes. Dans le cas de la lipase, elle peut être extraite et utilisée dans l'industrie des détergents ou encore pour diluer le milieu de culture pour la fermentation lipidique. En recyclant le bouillon de fermentation contenant la lipase, il est possible de solubiliser les matières grasses et le savon à hautes concentrations dans le milieu de culture avant inoculation afin de faciliter la fermentation et l'homogénéisation du milieu de culture.

Une approche métagénomique basée sur Illumina Miseqe a permis d'évaluer les boues secondaires issues d'industries de pâtes et papiers. L'analyse de la communauté microbienne a révélé l'occurrence de différents produits à valeur ajoutée à partir des microbes indigènes des boues en question. En outre, sur la base de l'UTO (Unités Taxonomiques Opérationnelles) l'abondance de chaque UTO a été calculée. La classification taxonomique UTO a illustré la présence de microbiome multifonctionnel dans les boues papetières. D'après la classification taxonomique et la base de données en littérature, de multiples applications possibles (lipides, des acides organiques, des biopolymères, des enzymes et des Exopolysaccharides) ont été évaluées.

ABSTRACT

The mitigation of climatic change due to high carbon release from synthetic fuels, ever increasing demand and rapid depletion of petroleum fuel resources demands the search for alternative carbon neutral fuels. In this context, renewable oils from oleaginous microbes produced from renewable resources like glycerol, plant oils, agro residual waste, municipal solid and liquid waste, forest and other industrial waste harboring microbiologically utilizable carbon hold a promise for future energy demands. Currently, various renewable fuels mainly fuel ethanol is produced from molasses or other easily available fermentable sugars. In the meantime, lipid synthesis from microbe has a long tradition and several TAG (Triacylglycerides) of high economic importance were produced. These microbes possess a wide array of substrate utilization properties. Oil harboring microbes accumulate lipid varying from 20 to 85% (w/w) of total cell dry biomass. Taking advantage of these properties in the present study glycerol and other agro-industry wastes were evaluated for lipid production.

Soil samples collected from virgin forest soils and glycerol contaminated soils were screened on crude glycerol supplemented selection media. Bacteria, yeast, and filamentous fungal strains appeared within 48h of incubation time were selected for further screening. All the isolates were grown in crude glycerol (CG) enriched nitrogen deficient media for 120h. Harvested cells were stained with Sudan black B and observed under phase contrast microscope. Organisms that possessed oil globules were selected for secondary screening. All the organisms were initially identified by Biolog followed by (18s rDNA) sequencing. In secondary screening a CG concentration of 100 g/L and two different C/N ratios were chosen and the fermentation was conducted up to120h. Based on the results obtained the strain SKY7 (lipid content 45% w/w) was chosen for further studies.

SKY7 was identified as *Yarrowia lipolytica* by biolog and 18s rDNA sequencing method. This strain is a well-known for lipid and citric acid production and capable of utilizing a wide range of substrates. Further, the culture parameters were evaluated for *Y. lipolytica* SKY7 in order to achieve high lipid production. Initial shake flask experiments were carried out at different glycerol concentrations, C/N molar ratio, inoculum size, and trace element concentrations. The results showed a glycerol concentration of 100g/L with a C/N ratio of 100 is optimal for biomass and lipid production. Based on the results, fermentation was carried out (15L reactor with

working volume of 11L) with 100g/L glycerol, C/N molar ratio 100 and 5% v/v inoculum without the addition of trace elements and with and without controlling the pH. The obtained results indicate that lipid synthesis was observed between 30 to 60h. In the case of pH controlled experiments, a lipid concentration of (43.2%w/w) 7.78g/L with a biomass concentration of 18g/L was achieved. Even though, the similar amount of biomass concentration was observed in fermentation under uncontrolled pH conditions lipid concentration was significantly low (15%) compared to pH controlled experiments. Hence, it is concluded that controlling pH will improve the lipid synthesis. Moreover from the experimental results, it was assumed that *Y.lipolytica* SKY7 required a C/N molar ratio between 350 to 400 when it enters into lipogenic phase. Maintaining the C/N molar ratio at the reported range extends the lipogenic phase and improves the overall lipid production. The above study demonstrates the potential of lipid production by *Y.lipolytica* SKY7 from crude glycerol-based synthetic media. In order to maximize the lipid and biomass concentration, a Box-Behnken method was employed and the principle parameters in lipid production (glycerol concentration, C/N molar ratio and inoculum volume) were investigated. The statistically optimized cultivation condition (glycerol concentration 82.5g/L and C/N ratio 75 with inoculum volume 6.25v/v) resulted in 29.5g/L biomass with 50%w/w lipid accumulation.

To improve the lipid production from heterotrophic microbes, an alternative approach in terms of replacing the commercial nutrients sources was attempted. Based on the studies organic and inorganic nitrogen sources were replaced by silos leachate and the initial results showed a prospect of utilizing low-cost waste material as a nutrient source for lipid synthesis. This adds to the process in the following ways: 1) makes it economically feasible 2) environmental protection by nutrient removal from waste streams, thereby reducing the mitigation of local climate change and 3) value addition of waste stream. Further, the studies conducted in fermenter scale (15L) with the supplementation of silos leachate (200mL/L) resulted in a biomass and lipid concentrations of 25.82 and 15.15g/L, respectively within 51h of fermentation. The biomass and lipid yield thus obtained was 83.45g/C mole and 53.28g/C mole with a lipid content of 63.85%w/w on dry cell mass. However, replacing the nutrient source alone cannot make the process economically feasible. The operational conditions also influence greatly in the process economy. The sterilization of reactor and media components is a high energy demanding process. Thus, eliminating the sterilization step could boost the overall process economy. A

methanol-based non-sterile process was investigated to eradicate the sterilization process. Initial results obtained from the shake flask experiments showed a biomass accumulation of 9.8g/L with a lipid content of 48% w/w with a supplementation of 3% methanol. However, when the similar conditions were replicated on controlled fermentation in fermenter the process failed (observed contamination) at 18h of the fermentation process. Hence, yet another alternative approach was formulated. A 10 minutes UV treatment was introduced to treat the tap water before it was added to dilute the media. Due to the UV treatment, an addition of 3%v/v methanol the occurrence of contamination was prolonged to 42h. This resulted in a high biomass (17.57g/L) and lipid (8.8g/L) accumulation in a non-sterile condition. When it was compared with the previous experimental condition (3%v/v methanol alone) the biomass and lipid concentrations were improved 3 and 4 times, respectively.

In order to improve the lipid accumulation in cells, a biochemical approach was attempted. A series of biotin (10, 50 and 10µg/L) supplementation to the fermentation media was studied. In general biotin is one of the important growth factor, which is required for cell wall lipid synthesis. Moreover, biotin is known as the main prosthetic group (ACC- Acetyl CoA Carboxylase) in the carboxylation of Acetyl CoA to malonyl CoA in TAG synthesis. Earlier reports on biotin supplementation were reported to improve the long chain fatty acid production. In the present case, the addition of 100µg/L biotin was notably improved the lipid concentration in batch cultures of *Y.lipolytica*. Compared to the control conditions a half fold increase in lipid concentration was observed. The actual biomass and lipid concentration thus obtained was 26.56 g/L biomass with 10.12 g/L lipid in the case of control and 27.6 g/L biomass and 16.16 g/L lipid in the case of biotin supplemented studies.

Furthermore, studies with *Y.lipolytica* results in citric acid and lipase production in the media with glycerol and soap as a carbon source. The additional production of citric acid can be extracted and used for food and other for applications. In the case of lipase, it can be extracted and used in detergent industry or it can be re-utilized to dilute the media. Using the fermentation broth containing lipase will help to solubilize the fat and soap prior to inoculation to aid the fermentation.

A metagenomics approach based on Illumina MiSeq was attempted to evaluate the pulp and paper industry sludge. The microbial community analysis revealed the capacity of different value added products from the microbes originated in the sludge. Further, based on the OUT's (operational taxonomic units) the abundance of each OUT was calculated. The taxonomic classification of OUT revealed the presence of multifunctional microbiome in pulp and paper sludge. Based on the taxonomic classification and the available literature the possible applications (lipid, organic acids, biopolymer, enzymes, and exopolysaccharides) were assessed.

AVANT-PROPOS

PUBLICATIONS/MANUSCRIPTS DANS LA THÈSE

1. **M. Kuttiraja** & Shyam Krishna & Ayed Dhouha & Rajeshwar Dayal Tyagi (2015). A Substrate-Based Approach for the Selection of Oil-Bearing Heterotrophs from Nitrogen-Deficient Soil for Lipid Production. *Appl Biochem Biotechnol* 175(4):1926-37.
2. **Mathiazhakan Kuttiraja**, Ayed Douha, Jose R Valero, and Rajeswar Dayal Tyagi (2016). Elucidating the effect of glycerol concentration and C/N ratio on Lipid Production using *Yarrowia lipolytica* - SKY7. *Applied Biochem Biotechnol* (Soumis)
3. **Mathiazhakan Kuttiraja**, Ayed Dhouha, Rajeswar Dayal Tyagi (2016). Harnessing the effect of pH on lipid production in batch cultures of *Yarrowia lipolytica* – SKY7. *Energy* (Soumis)
4. **Mathiazhakan Kuttiraja**; Ayed Dhouha; Rajeshwar D. Tyagi. (2016). Optimization of culture parameters for lipid production using response surface methodology and elucidating the kinetics of lipid production of new isolate *Y.lipolytica* SKY7. *Biomass and Bioenergy* (Soumis).
5. **Mathiazhakan Kuttiraja** and Rajeswar Dayal Tyagi (2016).The recent trends in carbon capturing from classical carbon sources and waste by-products for lipid production- A review (Soumis).
6. **Mathiazhakan Kuttiraja**, Jose Valero, and Rajeswar Dayal Tyagi (2016). Replacement of commercial nutrients sources by silo liquidate for biomass and lipid production using crude glycerol as a sole carbon source by *Y.lipolytica* SKY7(À soumettre).
7. **Mathiazhakan Kuttiraja**, Jose R Valero, and Rajeshwar Dyal Tyagi (2016). Studies on the influence of methanol in biomass and lipid production and development of a non-sterile fermentation process for lipid production from crude glycerol obtained from biodiesel industry (À soumettre).
8. **Mathiazhakan Kuttiraja** and Rajeshwar Dyal Tyagi (2016). A biochemical approach to improving the biomass and lipid production using biotin and intermediate supply of Diammonium sulfate (À soumettre)
9. **Mathiazhakan Kuttiraja**, Khdhiri Mondher, Lalit Kumar Goswami, Ram Saurabh Kumar, Yadav Jayshankar, Tyagi.Rajeswar Dayal, Constant, Philippe (2016). Characterization of Microbial Ecosystem in Sludge of Different Pulp and Paper Industrial Wastewater (À soumettre)

PUBLICATION EN DEHORS DE LA THÈSE

1. **M.Kuttiraja**, R.D.Tyagi (2016). Lignocellulosic biomass-The future renewable low-cost carbon source of bio-oil production Book chapter for ASCE publisher (À soumettre)
2. Raveendran Sindhu · Mathiazhakan Kuttiraja · Thunoli Payyanvalappil Prabisha · Parameswaran Binod · Rajeev K. Sukumaran · Ashok Pandey (2016). Development of a combined pretreatment and hydrolysis strategy of rice straw for the production of bioethanol and biopolymer. *Bioresource Technology* (Article en presse)
3. Raveendran Sindhu · Mathiazhakan Kuttiraja · Parameswaran Binod · Rajeev Kumar Sukumaran · Ashok Pandey (2014). Physico – chemical characterization of alkali pretreated sugarcane tops and optimization of enzymatic saccharification using response surface methodology. *Renewable Energy*, 62. 362-368
4. Raveendran Sindhu · Mathiazhakan Kuttiraja · Parameswaran Binod · Rajeev K. Sukumaran · Ashok Pandey. (2014). Bioethanol production from dilute acid pretreated Indian bamboo variety (Dendrocalamus sp.) by separate hydrolysis and fermentation. *Industrial Crops and Products*, 52:169–176.
5. .M Kuttiraja · Sindhu Raveendran · Preeti Elizabeth Varghese ·Sandhya Soolankandath Variem · Binod Parameswaran · Vani Sankar · Ashok Pandey · Rajeev Kumar Sukumaran (2014). Bioethanol production from bamboo (Dendrocalamus sp.) process waste. *Biomass and Bioenergy*, 59, 142-150.

CONGRÈS ET CONFÉRENCES

1. **Mathiazhakan Kuttiraja,** Ayed Dhouha, and Rajeshwar Dayal Tyagi (2015). Elucidating the effect of glycerol concentration and C/N ratio on Lipid Production using *Yarrowia lipolytica* - SKY7. *International Meeting on Advanced Materials and Processes for Environment, Energy and Health (PAEES), 14th October 2015, INRS-ETE, Quebec, Canada.*
2. Saurabh Kumar Ram, Lalit Kumar, **Mathiazhakan Kuttiraja**, R. D. Tyagi (2016). Real-Time Microbial Community analysis of Activated Sludge at various operational conditions of fermentation. 51st CAWQ Conference, 23 February 2016, Ryerson University, Toronto, Ontario.

RAPPORTS CONFIDENTIELS

1. **Mathiazhakan Kuttiraja** & Rajeshwar.Dayal. Tyagi (2013). PRODUCTION OF LIPID USING CRUDE (purified) GLYCEROL. Rapport soumis à l'Institut National de la Recherche Scientifique- Eau, Terre et Environnement, Québec, Canada.

2. L. Kumar, J. S. S. Yadav, **M. Kuttiraja**, S. K. Ram,S. Yan, R. D. Tyagi (2015). Study of variation in activated sludge composition, sludge microbial community and their impact on Polyhydroxyalkanoate (PHAs) production. Rapport soumis à l'Institut National de la Recherche Scientifique- Eau, Terre et Environnement, Québec, Canada.

SÉQUENCES D'ADN SOUMISES

1. Kuttiraja,M., Krishna,S. and Tyagi,R.D. Gene Bank: KF908249.1, Candida membranifaciens isolate A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. 2014. GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/KF908249).
2. Kuttiraja,M., Krishna,S. and Tyagi,R.D. GenBank: KF908250.1 Yarrowia lipolytica isolate B internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. 2014. GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/KF908250).
3. Kuttiraja,M., Krishna,S. and Tyagi,R.D. GenBank: KF908251.1 Yarrowia lipolytica isolate B internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. 2014. GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/KF908251).
4. Kuttiraja,M., Krishna,S. and Tyagi,R.D. GenBank: KF908252.1, Candida membranifaciens isolate D internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. 2014.GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/KF908252).
5. Kuttiraja,M., Krishna,S. and Tyagi,R.D.GenBank: KF908253.1,Candida membranifaciens isolate E 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. 2014. GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/KF908253).
6. Kuttiraja,M., Krishna,S. and Tyagi,R.D.GenBank: KF908254.1, Candida membranifaciens isolate F 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. 2014. GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/KF908254).
7. Kuttiraja,M., Krishna,S. and Tyagi,R.D.GenBank: KF908255.1, Candida membranifaciens isolate G internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. GenBank database. 2014. (http://www.ncbi.nlm.nih.gov/nuccore/KF908255).
8. Kuttiraja,M., Krishna,S. and Tyagi,R.D.GenBank: KF908256.1 Yarrowia lipolytica internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. 2104. GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/KF908256).
9. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894693, *Wautersiella falsenii* strain SK10 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludge during PHA production. 2014. GenBank database http://www.ncbi.nlm.nih.gov/nuccore/KF894693.1
10. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894692.1, *Elizabethkingia miricola* strain SK9 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludge

during PHA production. 2014. GenBank database *http://www.ncbi.nlm.nih.gov/nuccore/KF894692.1*

11. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894691.1, *Pseudomonas otitidis* strain SK8 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludge during PHA production. 2014. GenBank database http://www.ncbi.nlm.nih.gov/nuccore/KF894691.1

12. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894690.1, *Raoultella planticola* strain SK7 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludge during PHA production. 2014. GenBank database http://www.ncbi.nlm.nih.gov/nuccore/KF894690.1

13. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894689, *Staphylococcus pasteuri* strain SK6 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludge during PHA production. 2014. GenBank database http://www.ncbi.nlm.nih.gov/nuccore/KF894689.1

14. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894688, *Chryseobacterium indologenes* strain SK5 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludg during PHA production. GenBank database http://www.ncbi.nlm.nih.gov/nuccore/KF894688.1

15. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894687, *Acinetobacter beijerinckii* SK4 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludge during PHA production. 2014. GenBank database http://www.ncbi.nlm.nih.gov/nuccore/KF894687.1

16. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894686, *Acinetobacter lwoffii* strain SK3 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludge during PHA production. 2014. GenBank database http://www.ncbi.nlm.nih.gov/nuccore/KF894686.1

17. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894685, *Bacillus cereus* strain SK2 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludge during PHA production. 2014. GenBank database http://www.ncbi.nlm.nih.gov/nuccore/KF894685.1

18. Krishna,S., Kuttiraja,M., Kumar,L. and Tyagi,R.D. Gene Bank: KF894684, *Pseudomonas otitidis* strain SK1 16S ribosomal RNA gene, partial sequence. Culture-dependent dominant bacterial community analysis of pulp and paper secondary sludge during PHA production. 2014. GenBank database http://www.ncbi.nlm.nih.gov/nuccore/KF894684.1

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ABRÉVIATIONS

^{o}C	Degree Celsius
ACC	Acetyl-coA carboxylase
ACL	ATP citrate lyase
ADP	Adenosine diphosphate
AMP	Adenosine mono phosphate
ATP	Adenosine tri phosphate
BOD	Biological oxygen demand
C/N	Carbon : nitrogen
C mole	Carbon moles
CDW	Cell dry weight
CO_2	Carbon di oxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
FAMES	Fatty acid methyl esters
FFA	Free fatty acid
FID	Flame ionization detector
GC-FID	Gas chromatography-Flame ionization detector
GEM	Glycerol enriched medium
GLA	Gama lenolinic acid
GRAS	Generally recognized as safe
ITS	Internal transcribes spacer region
L	Liter
ME	Malic enzyme
mL	Milli liters
mM	Millimol
MSA	Multiple sequence alignment

MSS	Municipal sewage sludge
NCBI	National center for biotechnology information
OD	Optical density
PCR	Polymerase chain reaction
Rpm	Revolution per minute
SCO	Single cell oil
SS	Suspended solids
SKY	Shyam Krishna yeast
TSS	Total suspended solids
TAG	Triacylglycerids
YPD	Yeast extract peptone dextrose media
YPG	Yeast extract peptone glycerol media
Yx/s	Yield of biomass/gram of substrate consumed
Yp/s	Yield of product/gram of substrate consumed
Yp/x	Yield of product/gram of biomass
μ	Specific growth rate
Ʊ	Specific lipid production rate

CHAPTER-I

SYNTHESIS

Chapter I- Revue de littérature

1. Introduction

L'idée de Rudolph Diesel d'utiliser l'huile végétale comme carburant dans le moteur diesel a changé le domaine du transport dans les années 1890. Consécutivement, l'huile d'arachides a été démontrée dans l'exposition universelle organisée par le gouvernement français en 1900 comme un carburant diesel. Dans un premier temps, l'huile végétale pure était utilisée pour générer de l'énergie pour les locomotives dans les pays ruraux africains (Huang et al., 2009). La découverte de pétrole brut a conduit à l'industrialisation et a changé le sort de l'économie mondiale. Depuis la découverte du pétrole brut, il s'est avéré comme la principale marchandise répondant aux besoins énergétiques du secteur des transports et de l'énergie jusqu'à ce jour. Bien que les sources d'énergie alternatives (bio-éthanol, hydrogène) aient été explorées, une alternative absolue pour le mazout et l'essence n'est seulement explorée qu'à échelle de démonstration et de développement. L'industrialisation rapide et l'urbanisation ont conduit à une demande croissante pour le pétrole comme source majeure d'énergie. Par contre l'utilisation incontrôlée des hydrocarbures a mené à la libération de gaz à effet de serre, entraînant finalement le réchauffement climatique. Pour ces raisons préjudiciables, la libération de gaz à effet de serre dans l'environnement est strictement réglementée par différentes organisations mondiales. Pendant ce temps, l'utilisation de carburant d'origine fossile est inévitable car elle joue un rôle crucial dans le monde actuel. La demande croissante de combustibles fossiles non renouvelables provoque son épuisement et pose une source de préoccupation dans le monde entier pour la substitution du pétrole par des alternatives renouvelables efficaces (Beligon et al., 2015). Ces alternatives renouvelables aux combustibles fossiles doivent nécessairement remédier à la problématique d'épuisement rapide des réserves de pétrole et également à l'impact négatif sur l'environnement. D'un point de vue énergétique et environnemental, les combustibles de 2ème et de 3ème générations à partir de ressources renouvelables et durables sont prometteuses.

Une des approches largement débattues pour relever ces défis est la bioconversion des biosolides ou des déchets industriels en lipides par fermentation microbienne (Cheirsilp & Louhasakul, 2013). L'un des principaux problèmes lors de l'utilisation des sous-produits industriels pour la bioconversion est la découverte d'hôtes microbiens capables de tolérer des variations de lot à lot (batch to batch) et les impuretés présentes dans ce coproduit (Choi et al., 2011; Fontanille et al., 2012). S'il s'agit de dépistage de souches microbiennes appropriées pour la bioconversion de

matières premières industrielles, une approche à base de substrat plutôt qu'une approche axée sur les produits a été proposée comme moyen d'améliorer la réussite du processus (Rumbold et al., 2009). En utilisant la même stratégie de dépistage, des microorganismes ayant des capacités naturelles et utilisant la matière première brute peuvent être identifiés. Ceci permettra d'éviter la nécessité de concevoir des microorganismes performants par modification génétique pour les adapter à utiliser ces coproduits.

Cependant, plusieurs souches microbiennes ont déjà été identifiées comme candidats potentiels pour la production de lipides afin de produire du biodiesel, mais des études de recherche de premier degré seulement ont été menées surtout à l'échelle industrielle (Beligon et al., 2015). Tout comme pour les autres générations de biocarburants, la bioconversion de carbone en lipides héberge des défis insaisissables. Parmi ces obstacles on retrouve l'identification d'une souche performante pour l'accumulation de lipides, le coût de substrats utilisés, le remplacement des sources d'azote par d'autres à faible ou zéro coût et finalement l'efficacité des méthodes d'extraction de lipides utilisées qui devraient être examinés avec plus d'indulgence.

En effet, les réactions biochimiques catalysées par ACL (ATP citrate lyase) jouent un rôle crucial dans la biosynthèse des lipides chez les microorganismes. Le rôle de l'ACL a été bien étudié par divers auteurs (Ratledge & Wynn, 2002), mais très peu de travaux basés sur la recherche biochimique ont été effectués pour augmenter l'activité enzymatique associée, à savoir ACL, ACC (acétyl-CoA carboxylase) et de la déshydrogénase malique dans les microorganismes oléagineux potentiellement sélectionnés pour la production de biodiesel.

Techniquement, le biodiesel est défini comme un mélange d'esters méthyliques d'acides gras (EMAG) produits par la réaction catalytique de l'huile avec des alcools à chaîne courte. Les alcools à chaîne mono comme (méthanol ou éthanol) sont préférables. Le biodiesel est une alternative appropriée pour le carburant pétrodiesel acceptable et respectueux de l'environnement, facilement disponible et aisément transportable pour être utilisé directement dans les moteurs diesel sans aucune modification tout en contribuant à la réduction des gaz à effet de serre (GES) (Carrareto et al., 2004; Demirbas, 2009; Li et al., 2008).

Le produit substitut doit aussi être conforme aux critères de biodégradabilité, aucun risque potentiel (moins volatile) et non toxique (Carraretto et al., 2004; Demirbas, 2009; Li et al., 2008).

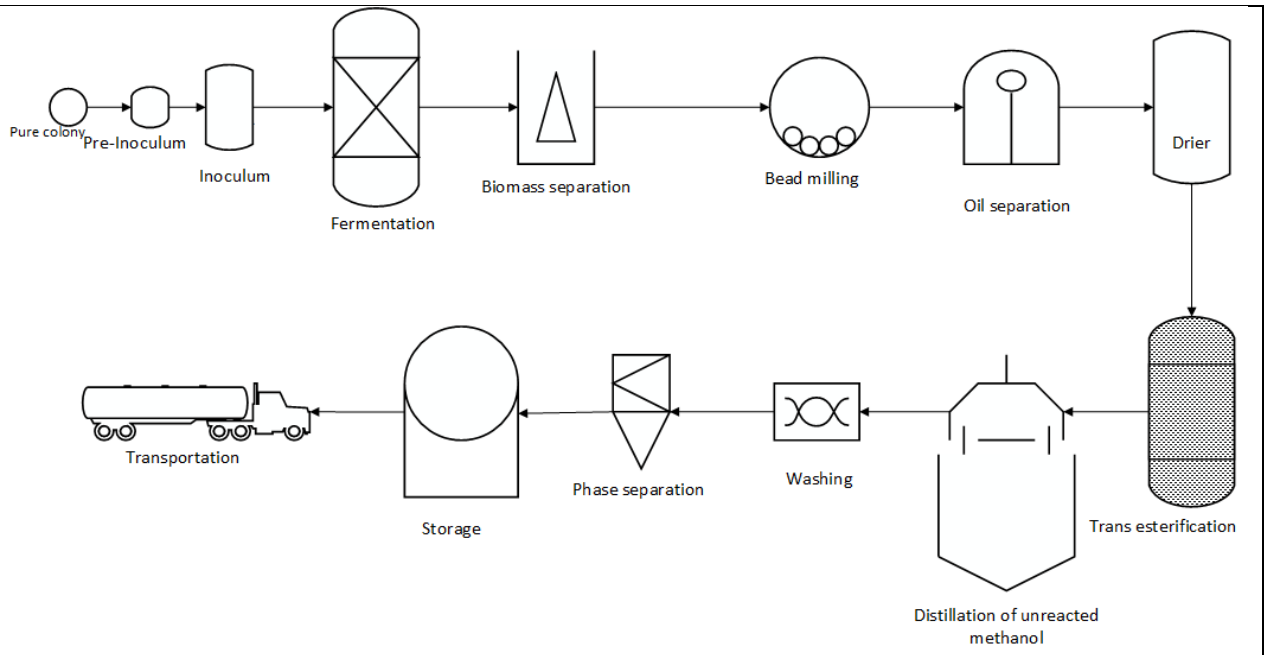


Figure 1Aperçu général d'un procédé de production de biodiesel à partir de lipides microbiens

2. Les microorganismes oléagineux

Les lipides sont une des composantes indispensables de la paroi cellulaire microbienne. Tous les microorganismes synthétisent différentes fractions de lipides dites de structure et autres de fonction. Ceux capables d'accumuler plus de 20% du poids cellulaire sec en lipides, sont connus sous le nom de microorganismes oléagineux (Wynn & Ratledge, 2005). Ils ont la particularité de produire et d'accumuler des lipides en présence de hautes concentrations en carbone accompagnées de faible apport nutritif dans leur milieu environnant. Ces lipides sont stockés sous forme triacylglycérides (TAGs) dans des organelles nommées corps lipidiques (Papanikolaou & Aggelis, 2011; Ratledge & Wynn, 2002). Bien qu'ils soient reconnus ainsi, un processus de fermentation approprié pour du biodiesel d'origine microbienne n'a pas encore vu le jour en application industrielle. Par ailleurs, les huiles végétales, les graisses animales, les lipides microalgals et les huiles usées de cuisson sont les seules alternatives actuellement exploitées pour l'obtention du biodiesel. L'utilisation d'huiles végétales ou à destinée alimentaire a un effet négatif d'un point de vue socio-économique. De plus, produire de l'énergie en exploitant des superficies allouées pour les terres arables en vue de production alimentaire a créé un conflit de priorité aliment vs énergie (Damen; Xu et al., 2012). Il y a plusieurs problèmes associés à l'utilisation de graisses animales et de l'huile usée de cuisson pour la production de biodiesel, à savoir : la teneur élevée en acides gras libres (AGL), la teneur en soufre, la matière

en suspension, la collecte et transport, le stockage, etc. Par conséquent, le besoin de l'industrie de produire de plus grandes réserves en biodiesel a suscité l'intérêt des chercheurs à se tourner vers les ressources renouvelables et l'énergie durable.

Comparativement à ces matières huileuses, l'huile végétale et d'autres ressources en pétrole, l'huile microbienne est idéale pour la production de biodiesel pour une composition similaire aux TAGs et à forte valeur ajoutée des acides carboxyliques tels que l'acide gamma-linoléique (GLA). Bien que toutes les catégories de microbes accumulent les lipides, dans ce travail. La levure a été soulignée en raison de sa croissance rapide et la capacité d'accumulation élevée en lipides.

Bien que plusieurs microorganismes hétérotrophes, y compris les levures et les champignons, ont été identifiés pour accumuler la quantité comparable de lipides, seulement 30 sur 600 organismes ont été signalés pour stocker plus de 20% de TAGs (Ratledge & Wynn, 2002). Ceci varie de 20 à 85% dans les différentes classes d'organismes (Wynn & Ratledge, 2005). Les genres microbiens répertoriés comme oléagineux dans la littérature incluent les *Basidiomycota* (*Trichosporan*), *Ascomycota* (*Y. lipolytica*) et *Zygomycota* (*Mortirella*). Le tableau 1&2 dresse des exemples d'organismes à haut potentiel oléagineux ayant une croissance remarquable à l'envol de la littérature à ce sujet.

Un organisme oléagineux est caractérisé par la particularité de son métabolisme secondaire pouvant convertir l'atome de carbone simple en TAGs par une série de réactions biochimiques. La principale différence entre les organismes oléagineux et ceux qui ne le sont pas est l'occurrence d'ACL (ATP citrate lyase) (Beopoulos et al., 2009b). Toutefois, certaines espèces microbiennes sont dotées de cette enzyme sans qu'elles aient des réserves lipidiques intracellulaires. L'absence d'autres enzymes consécutivement impliquées dans le métabolisme en question rend la lipogenèse impossible. Lorsque d'autres enzymes comme ACC (acétyl-CoA carboxylase) et enzyme malique (EM) sont inactives, les organismes synthétisent (accumulent) de l'acide citrique.

Table 1 Les microorganismes lipogéniques et leurs pourcentages de lipides correspondants

Organisme	**Substrat**	**La biomasse (g/L)**	**Lipide% (p / p)**	**Référence**
Y.lipolytica	glycérol industriel	8,1	43	(Papanikolaou & Aggelis, 2002)
R toruloides	Glucose	11,2	62,2	(Dennis Y.C. Leung et al., 2010)
C curvatus	glycérol brut	39,2	52	(Liang et al., 2010)
R toruloides	Glucose	15,5	57,3	(Lin et al., 2011)
L starkeyi	Cellobiose + xylose	25,2	52	(Gong et al., 2012)
P guilliermondii	Inuline	20,4	60,6	(Wang et al., 2012)
R toruloides	glycérol brut	35,3	46	(Uckun Kiran et al., 2013)
R toruloides	Levoglucosan	6,8	39,7	(Chang et al., 2013)
M isabellina	Xylose	28,8	64,2	(Gao et al., 2013)
T fermentans	vigne de patate douce + glycérol	17,9	41,5	(Jin et al., 2013)
R glutinis	Glucose	86,2	26,7	(Liu et al., 2015)
L starkeyi	Glucose + Xylose	61,9	29,3	(Anschau et al., 2014)
L starkeyi	Glucose + mannose	22	58,3	(Yang et al., 2014)
Y.lipolytica	Glycérol	4,92	30,1	(Poli et al., 2014)
R babjevae	glycérol brut	9,4	34,9	(Munch et al., 2015)
R diobovatum	glycérol brut	12,0	63,7	
C curvatus	Acétate	80	60	(Beligon et al., 2015)
C curvatus	Les acides gras	13,3 à 17,1	65 à 75,3	(Yang et al., 2015)
C ehinulata	Glucose	15	46	(Makri et al., 2010)
M isabellina	Glucose	27	44,6	(Makri et al., 2010)
A awamori	Glucose	12,8	35,4	(Mohan, 2014)
A Niger	glycérol brut	5,2	10,0	(André et al., 2010)
M isabellina	Xylose	28,8	64,2	(Gao et al., 2013)
L.starkeyi	Glucose	104,6	64,9	(Lin et al., 2011)

Table 2 Les espèces bactériennes oléagineuses

Organisme	Substrat	Biomasse (g/L)	Lipide% (p / p)	Référence
R erythropolis	Glucose	4.7	42,5	(Sriwongchai, 2014)
R opacus	Glucose	77,6	38	(Kurosawa et al., 2010)
Nocardia	NA	NA	NA	(Alvarez & Steinbuchel, 2002)

Le schéma typique de l'accumulation des lipides est présenté dans la figure 2. Au début de la déplétion en élément azote, la lipogenèse est déclenchée par l'activation d'un pool d'enzymes spécifiques de ce métabolisme. La phase initiale de croissance dure environ 20 heures, puis l'organisme subit une phase de transition où l'excès de carbone peut être converti en citrate, ce qui se traduit alors par la synthèse des lipides. Entre-temps la production d'acide citrique dépend aussi de la composition du milieu et le rapport C/N au cours de la fermentation. Cette dernière peut se limiter à la formation de l'acide citrique et non la production de lipides selon les conditions favorables à l'un ou l'autre processus enzymatique. Dans les étapes initiales, tandis que l'enzyme iso-citrate déshydrogénase est inactivé, AMP désaminase est contrairement activé. Cela entraîne une diminution rapide de l'AMP intracellulaire. L'AMP désaminase catalyse la réaction détaillée par l'**Équation 1**(Beopoulos et al., 2009b; Ratledge & Wynn, 2002; Wynn & Ratledge, 2005). L'activation de l'AMP désaminase est due à un piégeage de l'AMP intracellulaire qui à son tour affecte l'activation de l'IDH (isocitrate déshydrogénase). En conséquence, le cycle de Krebs ou de l'acide citrique est réprimé au niveau de l'isocitrate et équilibré par l'aconitase à l'acide citrique. NH_4^+.

Équation 1 : $$AMP \rightarrow IMP + NH_4^+$$

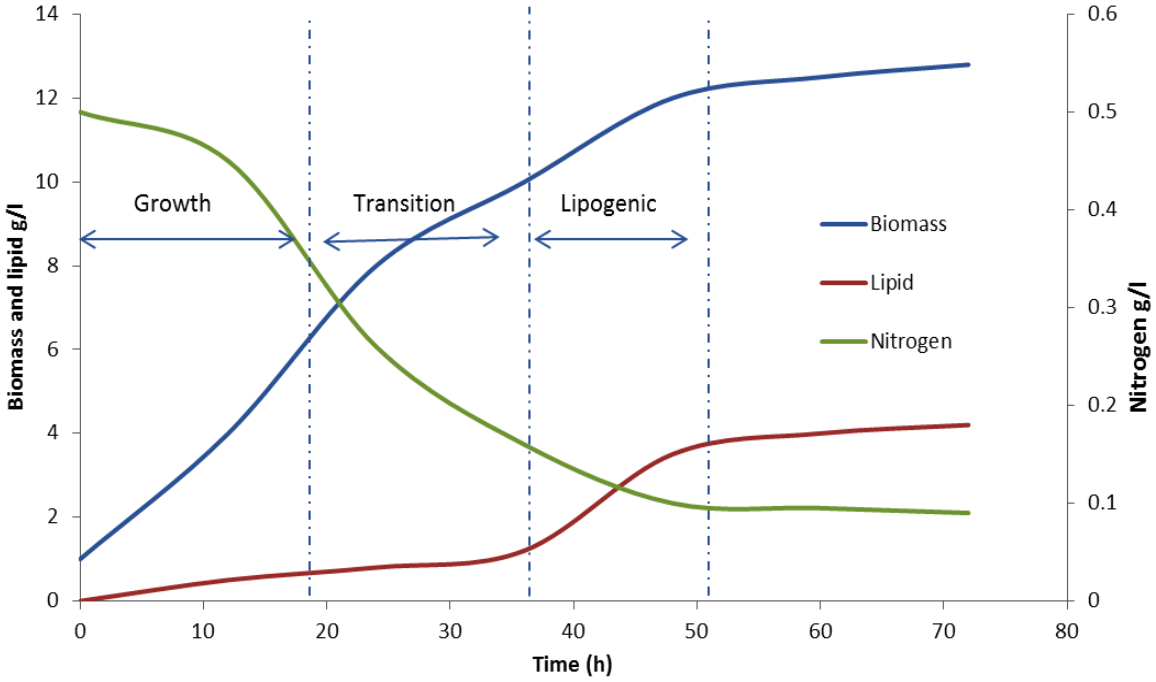


Figure 2 Les différentes étapes de l'accumulation des lipides (Kuttiraja et al., 2015

3. Les enzymes impliquées dans la synthèse de TAGs

Comprendre la biochimie de synthèse des TAGs (dans différentes conditions de culture) est la clé pour développer un procédé économiquement viable pour la production microbienne des lipides. Il détermine la quantité de substrat qui peut être métabolisé par l'organisme pour sa croissance et la synthèse conséquente de TAGS. Les enzymes clés impliquées dans la synthèse des TAGs sont, ATP Citrate Lyase(ACL), acétyl-CoA carboxylase (ACC), et les enzymes maliques (EM). Les autres enzymes associées sont AMP désaminase, acyl-CoA oxydase, la FA élongase, etc. (Ratledge & Wynn, 2002; Wynn & Ratledge, 2005). La synthèse de TAGs commence lorsque l'activité AMP désaminase augmente. En raison du déficit en azote pour le métabolisme cellulaire; l'AMP est clivée par l'AMP désaminase pour fournir de l'azote pour le métabolisme cellulaire dans la mitochondrie. Ceci perturbe la synthèse de l'acide alpha-cétoglutarique dans le cycle de l'acide citrique. Lorsque l'iso-citrate déshydrogénase est perturbée, le niveau d'iso-citrate dans la mitochondrie est élevé. Ceci est à la suite équilibré par

l'action de l'enzyme aconitase qui favorise la conversion d'iso-citrate en citrate (ou acide citrique). L'acide citrique est en outre transporté vers le cytoplasme où il s'accumule.

3.1. ACL (ATP citrate lyase)

EC 2.3.3.8 ATP citrate lyase est une des enzymes impliquées dans le cycle AC inverse. Au début de l'épuisement de l'azote, l'isocitrate est équilibré par l'intermédiaire de l'aconitase et transporté vers le cytosol par la citrate/malate translocase. L'ATP citrate lyase est une enzyme cytosolique présente chez une large gamme de microbes et l'une des principales enzymes de microorganismes oléagineux (Ratledge & Wynn, 2002). Le citrate est ensuite transformé en acétyl-CoA par l'ACL au détriment d'une molécule d'ATP (Équation 2).

Équation 2 : $\boldsymbol{Citrate + CoA + ATP \rightarrow Ac\acute{e}tyl\ CoA + oxaloac\acute{e}tate + ADP + Pi}$

Certains organismes détiennent l'ACL mais ils accumulent moins de 20% de lipides par poids sec des cellules. Elle est tout de même considérée comme l'enzyme précurseur de l'AcétylCoA, enzyme clé de la lipogenèse. La biosynthèse des lipides commence lorsque l'acétyl-CoA réductase est réduite au malonyl-CoA par la synthèse des acide gras (Wynn & Ratledge, 2005).

3.2. Les enzyme maliques (EM)

L'enzyme malique est la deuxième enzyme clé dans la lipogenèse. Les deux composés carbonés (acétyl-Co-A) sont ajoutés à la chaîne d'acide gras par l'enzyme synthase d'acide gras ou FA synthase. Cela nécessite deux molécules de NADPH pour assurer l'élongation des TAGs et la réaction est catalysée essentiellement par l'EM. Bien qu'il existe plusieurs autres voies de formation du NADPH chez les microorganismes en général, L'EM reste indispensable en biosynthèse lipidique. La réaction impliquée dans la libération de NADPH est la suivante :

Équation 3 : $\boldsymbol{L - malate + NADP^{*} \rightarrow Pyruvate + NADPH + CO_2 + H^{+}}$

L'enzyme malique est essentielle à la synthèse des lipides, même si elle ne contribue que pour fournir le NADPH nécessaire au le métabolisme cellulaire total. L'inactivation complète ou l'inhibition de l'EM peut bloquer totalement la lipogenèse (Ratledge & Wynn, 2002). Ceci indique que l'activité prolongée d'EM peut améliorer la durée de biosynthèse des lipides chez les oléagineux. Auparavant, on pensait que la lipogenèse est contrôlée par l'ACL, mais il a été montré que la présence d'ACL seule n'est pas corrélée avec l'accumulation de lipides intracellulaires (Wynn et al., 1999). L'enzyme malique est étroitement régulée par la machinerie

génétique de l'organisme et l'alimentation continue de cette enzyme (EM) augmente la production de lipides, ce qui indique de grandes concentrations de lipides chez les souches les plus oléagènes. Cependant, dans la plupart des cas, l'activité de l'EM est bloquée suite à l'épuisement de l'azote dans le milieu de culture (Ratledge & Wynn, 2002). Les résultats obtenus par Beopolous et ses collègues suggèrent que lorsque l'organisme est en phase lipogénique l'azote serait essentiel au maintien du mécanisme cellulaire (rapport C /N > 300) (Beopoulos et al., 2009b).

3.3. Acétylcoenzyme Carboxylase (ACC)

ACC joue un rôle clé dans la synthèse et la dégradation des acides gras. C'est une enzyme à multiples sous-unités chez les procaryotes tandis qu'elle est biotine-dépendante chez les eucaryotes. Cette enzyme catalyse la première carboxylation de l'acétyl-CoA pour générer la malonyl-CoA réductase, qui est considérée comme la première étape dans la synthèse d'acides gras à longue chaîne. Dans la première étape, la biotine carboxylase dépendante effectue la carboxylation du groupe prosthétique lié de manière covalente à la protéine de liaison porteuse du carboxyle biotine. Dans l'étape suivante la carboxyl-transférase (CT) catalyse le transfert d'un groupe carboxyle de la biotine à l'acétyl-CoA (Berg et al., 2002).

L'activité de carboxylation de l'ACC dépend du contrôle de trois signaux différents chez les eucaryotes : glucagen, l'épinéphrine, et l'insuline. Parmi ceux-ci, l'insuline active la carboxylase et favorise la synthèse d'acide gras tandis que les deux autres ont un impact négatif sur la synthèse des acides gras. Le niveau de citrate, de palmityl CoA et d'AMP ont également un effet sur l'ACC. Le niveau de citrate dans la cellule active directement l'ACC tandis que la palmityl CoA et de l'AMP inhibent la synthèse d'acide gras (Berg et al., 2002). L'activité de carboxylation de l'ACC nécessite la biotine comme groupe prothétique. Chez les levures, la biotine est transportée via un transport actif (Fisher et al., 2012). En outre, chez de nombreux organismes, il s'agit d'une vitamine essentielle. La réaction catalysée par l'ACC est représentée ci-dessous. Ainsi, la biotine est essentielle pour la synthèse des acides gras à longue chaîne (Carol et al., 1999; Streit & Entcheva, 2003).

Équation 4

$$HCO_3^- + Mg - ATP + Biotine - ACC \leftrightarrow Biotine - ACC - CO_2 + Mg - ADP + Pi$$

Équation 5

$$Biotine - ACC - CO_2 + Acétyl\ CoA \leftrightarrow Biotine - ACC + Malonyl\ CoA$$

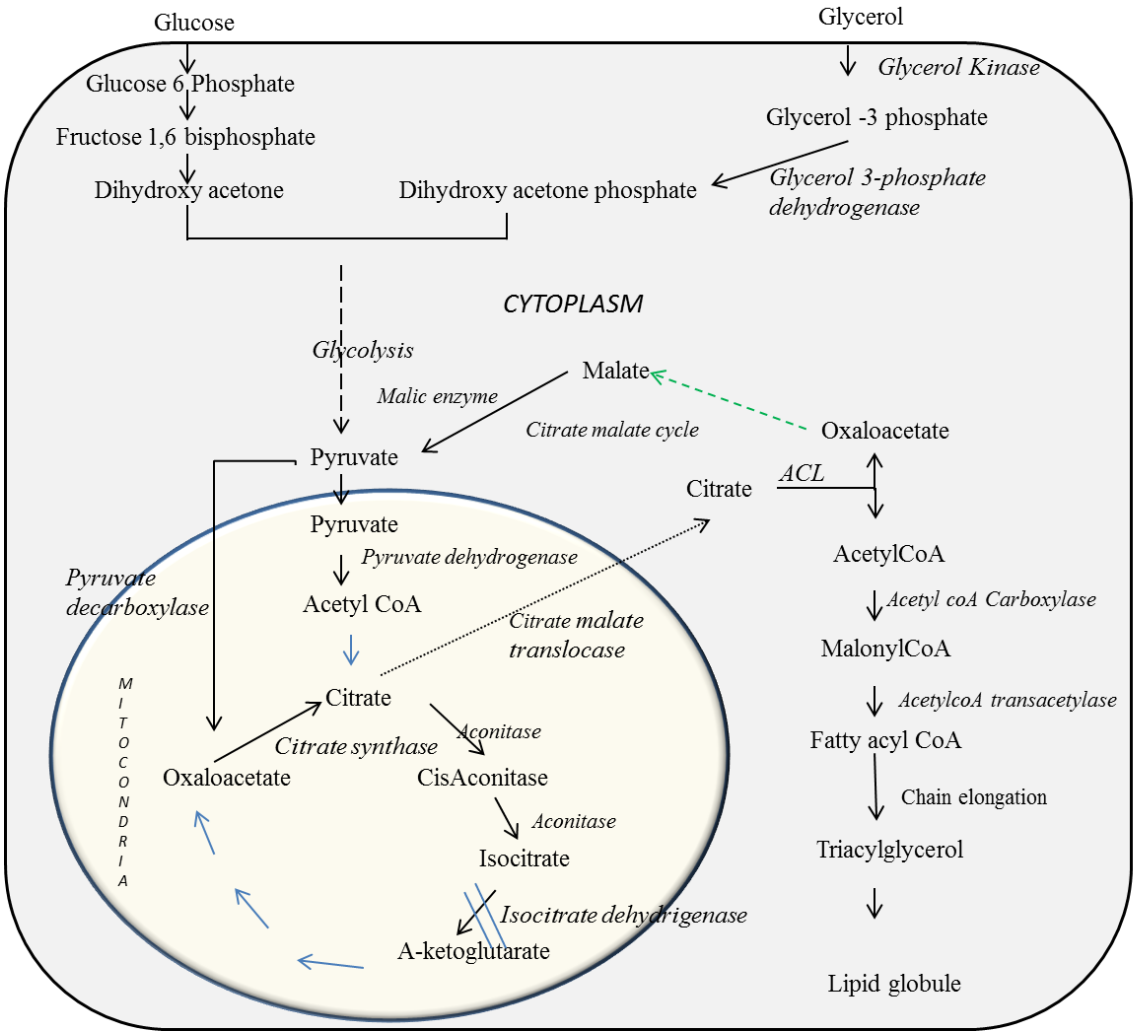


Figure 3 L'assimilation de glucose et de glycérol par les microorganismes pour la production de lipides: une voie

4. Les sources de carbone pour la production de lipides

4.1. Glucose

Le glucose est le glucide le plus abondant sur terre. Il joue un rôle structurel comme composant principal de la cellulose présente dans les matières végétales. Le besoin global de glucose a augmenté régulièrement à 165 millions $ en 2010 et il est prévu d'atteindre 356,5 millions $, avec un taux de 14,2% d'inflexion pour la période de 2011-2016 (BCCresearch, 2012) .

Le glucose est un monosaccharide simple et une source de carbone bien étudiée en tout type de croissance microbienne. En général, quand le glucose est utilisé comme source de carbone, la

levure commerciale (*S. cerevisiae*) et *E. coli* peuvent atteindre un des taux de croissance spécifiques maximales ou μmax de 0,4 h^{-1} (Chaudhary. et al., 2012; Walker & Chichester, 1997). La levure possède une grande affinité pour le glucose : la principale raison est l'entrée de fortess concentrations de glucose dans la voie de la glycolyse pour former divers métabolites ainsi que l'éthanol. Bien que cette souche ait une tolérance à l'éthanol et reconnue par une productivité élevée, elle est incapable d'assimiler des sources de carbone non usuel telles que le glycérol. Le μmax reporté chez ces espèces était d'environ 0,02 h^{-1} en présence du glycérol comme source de carbone, (Liu et al., 2013).

La production des lipides à partir du glucose a été utilisée depuis des décennies, il a été certifié en tant qu'une source de carbone idéale pour la production de lipides. Cependant, le coût élevé de vente du glucose utilisé comme source de carbone peut affecter le coût total de production des lipides. Des études récentes de Zhu et ces collaborateurs démontraient une biomasse élevée avec une accumulation considérable de lipides pour la fermentation microbienne à base de glucose. La biomasse et les lipides ont atteint jusqu'à 28,1 g/L avec 62,4 % (poids/poids) de lipides accumulés (Zhu et al., 2008). Toutefois, lorsque la fermentation était entretenue en mode discontinue (fed-batch), la biomasse et les rendements de production de lipides ont été notamment augmentées à 151,5 g/L et 72,72 g/L dans le cas de *Rhodosporidium toruloides*Y4 (Li et al., 2007). Cela prouve que la croissance cellulaire dépend fortement de la concentration initiale du substrat et qu'entre autres, lorsque la concentration du glucose est élevée (plus de 100 g/L), la croissance est inhibée (Chang et al., 2013). Un grand nombre des microorganismes sont vulnérables à un milieu de culture à concentrations excessives en carbone. En raison du déséquilibre osmotique, la croissance microbienne est significativement inhibée et affecte alors la productivité. Néanmoins, le procédé d'utilisation du glucose varie en fonction du microorganisme, l'état de la culture et d'autres paramètres physico-chimiques. Dans ce même contexte, une autre souche de *R.turoloides* fermentée en mode discontinue dans un milieu à base de glucose, révèle des rendements de 37 g/L de biomasse et seulement 49% (p/p) de lipides accumulés. Lorsque la fermentation est appréhendée en mode fed-batch les concentrations de biomasse et de lipides ont été améliorées respectivement pour 35 à 47 g/L et de 50 à 75% (Marilyn G Wiebe et al., 2013). D'un autre côté, la méthode de culture à haute densité supplémentée de glucose comme source de carbone en deux étapes de fermentation a été étudiée. (Lin et al., 2011). Les travaux de Lin et al. ont révélé une haute concentration de biomasse par

effet d'enrichissement initial en nutriments. Dans la deuxième étape la teneur en biomasse et en lipides a atteint respectivemnet 104,6 g/L et 67,9 g/L (Lin et al., 2011). La stratégie proposée à ce constat propose de décortiquer la fermentation en deux étapes concomitantes tout en réduisant le procédé à 40 heures. En outre, d'autres études ont confirmé également que l'addition d'une faible concentration d'azote au cours de la phase tardive de la fermentation améliore significativement la production de lipides (Shuib et al., 2014). En effet, l'activité des enzymes lipogéniques ACL nécessite les ions NH_4^+, par conséquent, elle améliore l'approvisonnement de l'AcetylCoA vers la synthèse des lipides.

Récemment, le métabolisme cellulaire d'*Y. lipolytica* a été étudié en détail, où 20 g/L de glucose ont été utilisés comme source de carbone. Une croissance équilibrée a été remarqué jusqu'à 16 h de fermentation et aucun métabolite secondaire n'a été reporté. (Mhairi Workman et al., 2013). D'un autre côté, *R. toruloides* CBS14 a été rapporté produire 76,1% (p/p) de lipides lorsque le glucose est additionné (Zhao et al., 2008). Quand *lipolytica* a été utilisée dans un milieu riche en glucose (30 g/L) et en déficit d'azote; l'accumulation de l'acide citrique est visiblement remarquable avec des rendements en lipides de 0,2 à 0,33 g par g de substrat consommé (Papanikolaou et al., 2009). Ceci suppose que la préférence de substrat à assimiler ainsi que sa concentration relative jouent également un rôle essentiel dans l'accumulation des lipides. Entre temps, les souches de levures oléagineuses sont capables d'assimiler des sources variées de carbone y compris le glucose, le xylose, l'arabinose et du mannose. Indépendamment du glucose, l'xylose est la source potentielle et le deuxième élément carbone à base de plantes disponible sur la planète. Une forte concentration de xylose (100 g/L) a produit 28,8 g/L de biomasse et de 18,5 g/L d'accumulation de lipides dans *M isabellina* (Gao et al., 2013; Mhairi Workman et al., 2013).

4.2. Les sucres dérivés des résidus agricoles

La complexité de la disponibilité des ressources en énergie conduit à l'utilisation de polymères complexes comme une source de carbone fermentescible. La principale source de polymères complexes est la biomasse ligno-cellulosique. En effet, la biomasse ligno-cellulosique est composée de polymères cellulosiques incorporés entre la lignine et l'hémicellulose (Ghosh & Ghose, 2003; Yousuf, 2012). Cependant, l'économie du procédé dépend de l'efficacité des paramètres opérationnels tels que le prétraitement, la saccharification et les conditions de fermentation. Néanmoins, l'utilisation de cette matrice implique aussi la libération de matières

inhibitrices au cours de la fermentation, ce qui réduit le rendement de fermentation. Récemment, des hydrolysats de différents agro-matériaux résiduels ont été testés pour la production de lipides. On y trouve par exemple l'hydrolysat de son de riz, de la paille de blé, les feuilles d'arbres, de déchets de tomate et d'autres ont été utilisés comme sources de carbone pour la production de lipides. Le tableau 3 représente des exemples de sources de carbone alternatives utilisées pour la production de lipides par différents organismes oléagineux.

Une étude menée avec la paille de riz comme matière première pour la production de lipides par *T fermentans* a signalé 26,6 g/L de biomasse et de 11,5 g/L de lipides après 8 jours de fermentation. La paille de riz a été initialement prétraitée avec de l'acide sulfurique suivie par une saccharification enzymatique. Un faible rendement de la biomasse et des lipides a été observé lorsque la culture a été réalisée sans désintoxication de l'hydrolysat (Huang et al., 2009). En outre, la bagasse de canne à sucre a été utilisée par Tsigie et al. (Tsigie et al., 2012). La bagasse a été prétraitée avec 2,5% d'acide chlorhydrique HCl. L'hydrolysat a été détoxifié puis fermenté avec *Y.lipolytica*. Les concentrations finales de biomasse et de lipides étaient respectivement de 11,42 (g/L) et de 6,68 g/L (Tsigie et al., 2011). Sur la base des études mentionnées ci-dessus, il est clair que la détoxication de l'hydrolysat peut conduire à augmenter la biomasse et la concentration de lipides accumulés en réduisant la concentration de l'agent inhibiteur. En outre, il a été rapporté que 2,5 g/L de furfural inhibent complètement la croissance des microorganismes. L'acide acétique généré pendant le prétraitement de la biomasse ligno-cellulosique (BLC) pourrait inhiber la croissance à une concentration de 3 g/L selon les travaux de (Galafassi et al., 2012). Les autres inhibiteurs associés à un prétraitement de la biomasse ligno-cellulosique (BLC) sont l'hydroxyméthylfurfural (5-HMF), l'acide formique, l'acide succinique, des composés phénoliques de faible poids moléculaire ainsi qu'autres produits de dégradation de la lignine.

Autres que les hydrolysats ligno-cellulosiques, divers effluents industriels contenant la composition de sucres différente ont également été utilisés pour la production de lipides en utilisant divers organismes lipogéniques. Le tableau 4 représente différentes sources de carbone utilisées pour la production des lipides. L'acétate est l'une des molécules de carbone simples qui pourraient être assimilés en faibles concentrations par les oléagineux. Une fermentation en mode fed-batch, a signalé 80 g/L de biomasse et 60% (p/p) en lipides obtenus lorsque le rapport C/N a été maintenu à 300.

Table 3 LMI à partir de la biomasse lignocellulosique par des microorganismes oléagineux.

Organismes	**Substrat**	**La biomasse (g/L)**	**Lipide% (p/p)**	**Référence**
T fermentans	La paille de riz	28,6	40,1	(Huang et al., 2009)
R glutinis	déchets d'amidon	40	35	(Xue et al., 2010)
A oryzae	La paille de blé			(Amin Inhm et al., 2010)
C lipolytica	mélasse		59,9	(Karatay & Donmez, 2010)
Y.lipolytica	Les bagasses de canne à sucre	11,42	58,5	(Tsigie et al., 2011)
T cutaneum	La canne de maïs	15,44	23,5	(Huang et al., 2011)
M isabellina	lignocellulosique	22,9	44,5	(Ruan et al., 2012)
garminis R	La canne de maïs		34	(Galafassi et al., 2012)
T cutaneum	La canne de maïs			(Huang et al., 2012)
R kratochvilovae	L'extrait de graines de chanvre	15,1	55,56	(Patel et al., 2014)
R glutinis	hydrolysat de rafles de mais	70,8	47,2	(Liu et al., 2015)

Par ailleurs, le contrôle de la fermentation (pH, DO) tout en utilisant des AGVs ou acides organiques reste très fastidieux par rapport à d'autres sucres. Il a été rapporté que la concentration d'AGVs à plus de 5 g/L peut fortement inhiber la croissance des levures. L'effet inhibiteur de l'acide faible dérive de la modification de la physiologie cellulaire en termes de protéines membranaires responsables des transports transmembranaires. En d'autres termes, la forme non dissociée d'acides organiques faibles pénètre facilement dans la membrane cellulaire et se dissocie au cytoplasme affectant le pH intracellulaire. Par conséquent, on pourrait conclure que le pH du milieu joue un rôle crucial dans l'utilisation de ces substrats (Beligon et al., 2015).

Table 4 Les sources de carbone alternatives pour la production lipidique

Organisme	**Substrat**	**biomasse (g/L)**	**Lipide% (p / p)**	**Référence**
C curvatus	Acétate	80	60	(Beligon et al., 2015)
Y.lipolytica	Acide acétique	31	40	(Fontanille et al., 2012)
toruloides R et C pyrenoidosa	La distillerie et les eaux usées domestiques mixtes	7,25	63,4	(Ling et al., 2014)
Cryptococcus albidus	Les acides gras volatils	1,1	28,3	(Vajpeyi & Chandran, 2015)

4.3. Le glycérol comme source de carbone

Pendant la présente décennie, le développement de l'industrie de biodiesel a favorisé la disponibilité du glycérol, une molécule essentielle pour différentes applications pharmaceutiques alimentaires et industrielles. Jusqu'à ce jour, les principales sources de matières premières pour la transformation du biodiesel sont les huiles végétales et les graisses animales, etc. Chaque 100kg de biodiesel génèrent 10kg de glycérol comme sous-produit majeur du procédé (Ayoub & Abdullah, 2012). La faible pureté de cet élément et l'hétérogénéité des réactions chimiques de transestérification rendent la purification du glycérol brut très laborieuse et affectent également les applications de l'industrie alimentaire et pharmaceutique. La production de biodiesel est estimé à environ 2,73 milliards de kg à l'année 2020 (ABGInc, 2007). De plus, chaque année, l'industrie du biodiesel se développe considérablement à un taux de 17% (2007-2012). Autrement, il est produit à partir de l'industrie de l'éthanol et du savon (Amin Inhm et al., 2010). De ce fait, le glycérol pourrait être considéré comme une matière première potentielle pour la production de biodiesel. Le prix du glycérol était stable au cours des dernières décennies par contre le prix du glycérol purifié a vu une augmentation forfaitaire de 0,27 $ US à 0,41 $ par kg.

Deux milliards de livres ou 0,906 milliards de kg (de glycérol d'une valeur d'un milliard de dollars est produit chaque année dans le monde entier (McCoy, 2005)) et il est utilisé pour la production de divers produits (tableau 5). Les principaux pays producteurs de glycérol brut dans le monde sont l'Europe, les États-Unis et le Sud-Est les pays asiatiques. Le scénario actuel des politiques environnementales aux États-Unis, à l'Union européenne, en Asie du Sud-Est, au

Canada et en Amérique du Sud a conduit à la mise en œuvre de la production d'énergie renouvelable et durable. Il a été rapporté que l'offre de glycérol à base de l'industrie des acides gras et du savon a dominé le marché jusqu'en 2003 pour être remplacé par le glycérol issu du biodiesel en 2008 (Tsobanakis, 2007).

Table 5 Les différentes applications de glycérol offertes dans le monde entier (Stelmachowski, 2011)

Les Produits	Valeur (%)
Aliments	23-25
Produits cosmétiques	37-40
Les produits du tabac	9-10
polyuréthane	7-10
Médicaments	6-8
résine Alkyl	3-9
Autres produits	1-5

Table 6 L'industrie de production du glycérol(Tsobanakis, 2007)

Source / Année	La production mondiale en tonnes métriques / an		
	2003	**2008**	**2010**
Savons	188	146	83
Les acides gras	333	438	521
Biodiesel	167	521	1583
Chimique	63	0	0
Les alcools gras	104	167	250
Autres	63	0	21

4.4. Les problèmes liés à la valorisation du glycérol

Comme mentionné précédemment, il s'agit d'un principal sous-produit de la filière de biodiesel, de produits saponifiables, de pétrochimie, de l'éthanol, etc. La pureté du glycérol obtenu est toujours discutable. La faible pureté du glycérol issu de l'industrie pétrochimique rend cette fraction non utilisable dans différentes applications alimentaires et pharmaceutiques. En outre, le glycérol généré de l'industrie du biodiesel peut être contaminé par des impuretés et des matières dangereuses dictées par l'usage de catalyseurs, la présence d'alcools (principalement le méthanol et / ou éthanol), des métaux lourds et des acides gras libres à concentrations élevés, pouvant ainsi rendre la fermentation microbienne complexe (Gonzalez-Pajuelo et al., 2005; Thompson & He, 2006). Compte tenu des impacts de génération des sous-produits de la filière pour les petites et

moyennes entreprises à faible capital, les procédés de purification du glycérol brut ne sont pas profitables.

La composition et propriétés typique du glycérol provenant de l'industrie du biodiesel est présentée dans le tableau 6&7. La concentration de glycérol dans le produit brut varie de 35 à 95% et il ne dépend que de la méthode de production. Thompson et al. ont déterminé la composition de glycérol obtenu à partir de la transformation de différentes matières premières telles que l'huile de tournesol 30% (p/p), de graines de moutarde 62% (p/p), l'huile de soya 67,85% (p/p) et les huiles végétales 76% (p / p) (Thompson & He, 2006). D'autres éléments ont été mesurés incluant des composés comme le phosphore 1,05 à 2,36% (p/p), le potassium 2,2 à 2,33% (p/p), le sodium 0,9 à 0,11% (p/p). Le calcium, le mercure et l'arsenic sont présents dans des quantités très faibles (Tan et al., 2013).

Table 7 Les caractéristiques physiques et chimiques du glycérol brut (basé sur les données de notre laboratoire)

Caractéristiques	Paramètres	Poids %
Physique	Relative kg / m3 Densité	1102-1280
	Viscosité à RT * (cP)	7-8,5
	teneur en glycérol	12-83
	Couleur (APHA)	Foncé
	Cendre	1,5-4
	Savon	3,0 à 27,3
	Le méthanol	1,5 à 35
	pH	4-11 (dépend du catalyseur)

5 Le glycérol

Le glycérol est transporté à l'intérieur des cellules par l'intermédiaire de deux voies métaboliques : un système de transport par affinité (diffusion facilitée) ou un système symport à affinité élevée (transport actif). En présence du glucose, le glycérol est transporté par une diffusion facilitée. Alors qu'en présence de sucres non-fermentés tels que l'acétate et l'éthanol, l'assimilation du glycérol est effectuée par un transport actif. Une fois la molécule transportée à l'intérieur de la cellule, elle peut être assimilée par phosphorylation ou par oxydation.

La croissance des microorganismes oléagineux en milieu de culture riche en glucose ou en glycérol a montré un taux de croissance spécifique supérieur dans le cas du glycérol ($0,3h^{-1}$) comparativement au glucose ($0,24h^{-1}$). Par ailleurs, la combinaison des deux substrats (glucose et glycérol) a représenté un taux de croissance spécifique de ($0,38h^{-1}$). De plus, l'étude a montré

une demande élevée en O_2 utilisant le glycérol. Ceci indique que l'organisme consomme le glycérol par phosphorylation pendant la phase initiale du métabolisme (Mhairi Workman et al., 2013).

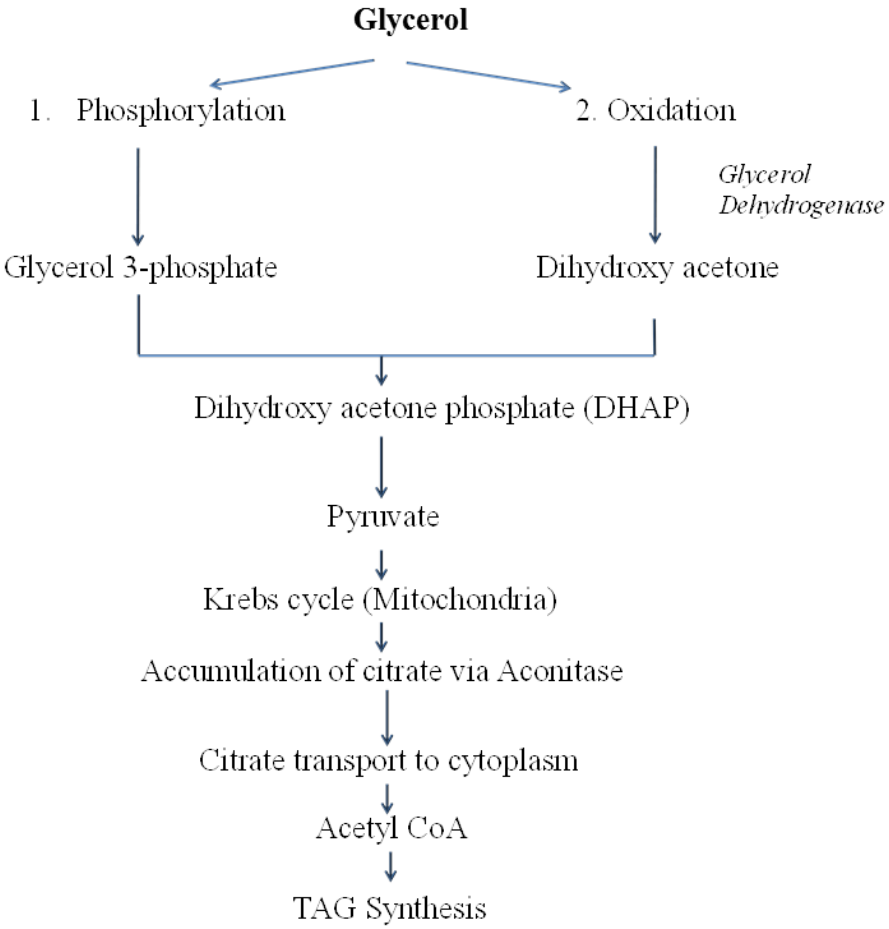


Figure 4 Schéma de la voie métabolique de l'assimilation du glycérol

6. Le glycérol brut et l'accumulation lipidique

La nature simple de la molécule de glycérol en fait une excellente source de carbone pour la synthèse de produits à valeurs ajoutées. Actuellement, la disponibilité du glycérol, pour des applications industrielles diverses, augmente considérablement grâce à l'effort réalisé par l'industrie du biodiesel. L'assimilation du glycérol par les levures, les bactéries et les champignons est bien fondée dans les recherches scientifiques. (Tableau 1&2). L'acide

propionique, le butanol, l'acide succinique et le dihydroacétone sont d'autres composés qui peuvent être produits à partir du glycérol brut (Asad-ur-Rehman. et al., 2008).

Dans ce contexte, le glycérol brut provenant de différentes industries détient la clé de la production énergétique durable. À cause du taux de variation en indicateurs de consommation du glycérol, les microorganismes ont besoin de s'adapter aux nouveaux inhibiteurs aussi qu'aux différentes concentrations de glycérol présents dans le substrat brut. Tenant compte de la forte concentration en carbone requise pour la production de lipides, *Y. lipolytica* appartenant au genre *Candida* a été étudié par différents chercheurs pour son importante production d'acide citrique à partir du glycérol comme source de carbone (Papanikolaou et al., 2002).Néanmoins, *cette souche* possède un potentiel élevé dans l'assimilation du glycérol et l'accumulation de lipides intracellulaires. La substitution des substrats simples par autres non usuels est différemment tolérée selon l'organisme. Par exemple, *Y. lipolytica* peut tolérer une concentration en glycérol allant jusqu'à 120g/L. alors que la croissance de *C. curvatus* est inhibée à une concentration excédant 64g/L (Papanikolaou & Aggelis, 2002). La production lipidique à partir du glycérol utilisé par différents microorganismes a également été illustrée par diverses recherches. Ainsi, *Rhodotorula glutinis*, *Cryptococcus albidus*, *Lipomyces starkeyi*, *Candida curvata*, et *Y. lipolytica,* sont, selon la littérature, les microorganismes présentant les contenus lipidique les plus remarquables à partir d'un substrat de glycérol brut en fermentation lipogène (Gwendoline Christophe et al., 2012; Meng et al., 2009).

Plusieurs études réalisées en 1976 ont porté sur des souches de champignons capables de produire des lipides à valeurs ajoutées (Wynn & Ratledge, 2005). Même si le but recherché n'a pas été atteint, plusieurs paramètres influant la lipogenèse ont été évalués et caractérisés comme par exemple l'effet de sécrétion d'acide citrique. Comparativement aux bactéries et levures, les champignons présentent des avantages et des inconvénients innombrables en considérant le glycérol ou d'autres substrats carbonés non usuels. La plupart des champignons sont capables d'assimiler des concentrations élevées de glycérol. En outre, au cours de la fermentation à grande échelle, le taux de cisaillement élevé, le colmatage et la prolifération cellulaire en filaments rendent la production lipidique à partir de champignon plus compliqué (Azocar et al., 2010). Les champignons ont un taux de croissance plus lent mais sont bien reconnus comme des oléagineux

capables de produire en particulier les acides gras polyinsaturés. D'après la littérature, les espèces de champignons les plus répertoriées en lipogenèse sont *Mucor sp.*et *Mortierella sp.*

Si on compare à partir de la littérature, par exemple, 3 types d'organismes, l'accumulation de lipides varie considérablement entre : *Rhodococcus 87% p/p, cryptococcus et Lipomyces supérieur à 60% p/p et Mortierella sp. 65% p/p* (Alvarez et al., 1996; Beligon et al., 2015; Gao et al., 2013; Saenge et al., 2011; Yang et al., 2014).Dans le cas de *Mortierella*, les lipides spécifiques sont riches en acide Gamma-linoléique. (AGL). Même si le taux de croissance des champignons est faible, l'accumulation de la biomasse totale excède celle d'espèces bactériennes dans bien des cas. Une accumulation de biomasse jusqu'à 28,1g/L avec un contenu lipidique de 62,4% a été démontrée chez *T. fermentans* (Zhu et al., 2008).

7. Inhibition de la croissance cellulaire et l'accumulation des lipides par des composés présents dans le glycérol brut**.**

Le glycérol brut est une source de carbone adéquate pour la production lipidique. Toutefois, si les impuretés présentes dans ce substrat sont à de fortes concentrations, elles peuvent être la cause d'une inhibition de la croissance cellulaire. De plus, la molécule de glycérol elle-même est inhibitrice à forte concentration, selon la capacité et tolérance du microorganisme (Liang et al., 2010). De nombreuses recherches ont explorés une panoplie de microorganismes avec des très grandes marges de tolérance aux concentrations élevées en glycérol. À titre d'exemples *R. glutinis* peut tolérer 8,5% p/p de glycérol dans le milieu de fermentation. Lorsque cette concentration est franchie, le taux de croissance de l'organisme est inhibé. Alors qu'*Y. lipolytica*, présente une croissance normale à 10% p/v de glycérol (Beopoulos et al., 2009a; Papanikolaou & Aggelis, 2002). Dans le cas de *Cryptococcus*, la concentration en glycérol varie entre 40 et 60 g/L.

Les composantes majeures associées au glycérol brut sont les catalyseurs de la réaction de transestérification (acide ou base), le savon, le méthanol, les métaux traces et les acides gras libres. L'inhibiteur le plus prévalent étant le méthanol mais celui-ci reste plausible en tant que source de carbone pour la croissance cellulaire chez certaines algues et levures. La concentration de méthanol dans le glycérol brut varie de 1,5 à 35% (v/v) dépendamment des étapes de transformation en aval du procédé de fermentation. Une concentration de 1% v/v en méthanol améliore la production totale de lipides de 1,2%. Dans le cas *Chlorococcum sp.*, Liang *et al.* (2010) ont indiqué que l'addition de 23,6 g/L de méthanol utilisant le mode fed-batch, la souche

Cryptococcus a indiqué une certaine tolérance physiologique au cours de la fermentation. Bien qu'une perte de concentration du méthanol par l'effet de son évaporation n'a pas été justifiée par les études précédentes , l'organisme est capable à un seuil assez haut de tolérer le méthanol et de l'assimiler pour une prolifération cellulaire non négligeable (Woon-Yong et al., 2011).

Comme évoqué plus haut, la formation du savon durant la réaction de tranestérification dépend du catalyseur utilisé dans la réaction de trans-esterfication. Lorsque le catalyseur utilisé est une base telle que le NaOH ou le KOH, l'acide oléique présent dans la fraction lipidique est converti en oléate de sodium ou de potassium. Bien qu'il soit un excellent surfactant, lorsque sa concentration excède 2g/L dans le milieu, le catalyseur basique inhibe la prolifération de *Schizochytrium limacinum,* pour la production de ADH (acide docosahexaenoique) (Denver j. Pyle et al., 2008). Un effet positif de l'oléate de sodium sur la production de la biomasse et de lipides à une concentration de 4g/L a été montré par les études de Xu et al (Xu et al., 2012).
En effet, l'inhibition de la croissance cellulaire par l'effet du savon dépend de l'interaction de celui-ci avec la membrane cellulaire en raison de l'interaction complexe entre la couche phospholipidique de la membrane et la propriété du savon comme surfactant. Néanmoins, l'effet positif du savon peut être résumé par un afflux de nutriments vers la cellule microbienne et une augmentation de la perméabilité cellulaire (Wei et al., 2008). Il est fondé que le pH constitue un facteur d'influence en termes de décantation du glycérol brut après séparation de phases. Le procédé de décantation favorise l'accumulation des glycérides et d'une certaine quantité d'esters méthyliques d'acide gras ou EMAG dans la fraction du glycérol. L'oléate méthylique est la fraction la plus communément retrouvée dans les échantillons de glycérol dérivés du biodiesel. L'oléate méthylique est reconnu pour améliorer l'accumulation lipidique chez *R. toruloides* (Xu et al., 2012). On présume généralement que les organismes oléagineux sont capables d'assimiler naturellement certains substrats hydrophobes tels que l'alkane, les acides gras, les huiles (Fickers et al., 2005). Chez *Y. lipolytica,* les voies métaboliques dites « de novo » et « ex novo » (substrats hydrophobiques) sont reconnues et bien illustrées dans la littérature. Ce sont des outils moléculaires pour la modification des levures (Beopoulos et al., 2009b). Les sels représentent une autre classe de composés retrouvés dans le glycérol brut (NaOH). Certains articles scientifiques ont mentionné que la présence de ces composés améliore plutôt la production lipidique. Fréquemment, la concentration des sels dans le glycérol brut est d' environ de 5 à 7% p/p et favorise la croissance cellulaire (Xu et al., 2012). Ainsi, l'ajout de chlorure de sodium

entre 4 à 16 g/L améliore le rendement lipidique respectivement de 12 à 40% (Xu et al., 2012). L'analyse des microéléments présents dans le glycérol brut et d'autres matières premières est présentée dans le tableau 8.

Table 8 Analyse des microéléments présents dans le glycérol brut et différentes matières premières (concentration en ppm) (Thompson & He, 2006)

microéléments en ppm	colza	Canola	Soja	Crambe
Calcium	24	19.7	11	163,3
Potassium	BDL	BDL	BDL	216,7
Magnesium	4	5,4	6,8	126,7
Phosphore	65	58,7	53	136,7
Sulfure	21	14	BDL	128
Sodium	1,06	1,07	1,20	1,1
Azote	0,05	0,05	0,04	0,06

8. Les facteurs affectant l'accumulation des lipides

La faisabilité économique de la production de lipides microbiens dépend majoritairement de la source de carbone utilisée. Il a été estimé à 75% du cout de production totale. La disponibilité du glycérol brut issue du biodiesel industriel ouvre une nouvelle opportunité et implique une amélioration considérable dans la production des énergies renouvelables. Bien que la source de carbone soit disponible en quantité importante, le procédé dépend également d'autres facteurs, qui déterminent l'efficacité de l'accumulation lipidique chez les organismes oléagineux (Wynn & Ratledge, 2005). Les principaux facteurs sont le rapport C/N, la concentration d'inoculum, l'âge de l'inoculum, la présence de micro et macro nutriments, le pH et la température (Li Y-h, 2005). En outre, l'efficacité du procédé dépend de la capacité de l'organisme à assimiler une source de carbone donnée avec un niveau nutritif faible et de l'accumuler sous forme de lipides.

8.1. Le rapport C/N

La nature de la source d'azote (inorganique ou organique) est cruciale pour la qualité et la quantité de biomasse et des lipides accumulés. L'ajout de source inorganique améliore la croissance microbienne alors que l'apport d'une source d'azote organique réduit la biomasse, mais permet l'accumulation lipidique jusqu'à un certain point.

Le rapport C/N est un paramètre bien étudié à ce sujet. Des études de Beopoulos et ses collaborateurs ont montré que l'augmentation du carbone avec un rapport de C/N élevé entraîne une meilleure accumulation des lipides (Beopoulos et al., 2009a). 68 % (p/p), les lipides ont été accumulés par *L. starkeyi* en utilisant un C/N 150 alors que, le contenu lipidique a diminué à 40% lorsque le rapport C/N utilisé était de 60. Il a été démontré qu'avec un rapport C/N entre 80 et 120, l'accumulation de l'acide citrique est remarquable au début de la fermentation contre une faible teneur en lipides. Des résultats similaires ont été significativement constatés et démontrés chez *Y. lipolytica*. Ce phénomène est relié à la concentration en carbone dans le milieu. Par exemple une forte concentration en carbone inhibe l'ACL, ce qui provoque l'accumulation de l'acide citrique dans le milieu au lieu d'être acheminé vers la synthèse des TAGs (Beopoulos et al., 2009a; Wynn & Ratledge, 2005). La concentration critique en azote requise pour la production de l'acide citrique par *Y. lipolytica* est à 10^{-3} mol l^{-1} mais il est important de ne pas atteindre la valeur limite. Alors que dans le cas de *R. glutinis*, l'augmentation du rapport C/N entre 150 et 350 permet d'améliorer le rendement global de lipides de 0,25 à 0,40 g/g de glucose consommé dans une fermenatation en batch. Cependant, l'augmentation du rapport C/N au-delà de 350 affecte de manière significative la synthèse des lipides par l'altération de l'activité enzymatique due à un déficit en azote.(Beopoulos et al., 2009b; Cescut, 2009) . En effet, l'ACL nécessite l'ion ammonium pour son activation et un rapport C/N élevé durant la phase lipogénique améliore le rendement en lipides particulièrement durant la phase lipogénique, qui ne peut être initiée qu'en présence d'une source d'azote minime.

Les composés inorganiques (micronutriments) ont également une forte influence sur l'accumulation de lipides. Dans la plupart des cas, ces éléments agissent comme des cofacteurs de l'activité enzymatique. Le magnésium, l'ammonium, le zinc, le fer et le phosphate sont des composés inorganiques bien étudiés à ce sujet. Naganuma et ses collègues ont révélé qu'une carence en NH_4^+, K^+, Mg, PO_4^-, SO_4^{2-}, Zn^{2+}, Fe^{3+}, ou Mn^{2+} entraînent un effet important sur la production de la biomasse, alors que les éléments tels que NH_4^+, K^+, Ca^+, Zn^{2+}, Fe^{3+}, and Mn^{2+} influencent l'accumulation de lipides (Naganuma et al., 1985).

8.2. Impact du pH et de l' oxygène dissous (OD)

L'effet du pH et de l'oxygène dissous (OD) ont été identifiés comme étant les principaux indicateurs de la croissance cellulaire et de la production lipidique (Yen & Zhang, 2011).

Lorsque l'organisme est en phase de croissance exponentielle, le pH varie peu chez les oléagineux. Durant la phase de transition, *Y. lipolytica* commence à produire une grande quantité d'acide citrique et d'autres acides organiques aboutissant à la baisse du pH dans le bouillon de fermentation (Beopoulos et al., 2009a). Il est bien connu que le mécanisme de transport de toute source nutritive dépend de la perméabilité de la paroi cellulaire et des conditions environnantes affectant le transport membranaire, dont le pH. Ce dernier interfère pour influence le flux de transport via les canaux ioniques H^+ K^+ de part et d'autre de la membrane cellulaire.

La surproduction d'acides organiques peut également inhiber les enzymes intracellulaires nécessaires à la synthèse lipidique. Afin de connaître l'effet du pH sur la croissance cellulaire et l'accumulation de lipides par *R. glutinis*, des expériences à pH contrôlé et non contrôlé ont été réalisées. Dans le réacteur à pH non contrôlé, sa valeur a diminué de manière significative de 6,5 à 4,3 au cours des premières 48 heures de la fermentation. La teneur en lipides où le pH est contrôlé était supérieure de 6% à celle avec un pH non contrôlé. Aussi, l''utilisation du substrat s'est avérée plus remarquable en conditions de pH contrôlé (Saenge et al., 2011).

L'OD est un autre facteur critique de la lipogenèse microbienne. La phase de croissance nécessite un taux élevé d'OD alors que la phase de la lipogenèse n'en exige que peu. Il a été rapporté que pendant la phase de lipogenèse chez *Y. lipolytica*, il y a une forte diminution de l'utilisation d'O_2. Ainsi, l'O_2 fourni continuellement entraine une augmentation du taux d'OD au cours de la fermentation. À un niveau plus faible (15%) on peut améliorer l'accumulation totale de lipides chez *Mucor sp.* RRl001 et *Candida lipolytica* (Ahmed et al., 2009), alors qu' un OD (80%) plus élevé favorise la production de l'acide citrique (Bati et al., 1984). L'effet d'OD sur l'accumulation des lipides et la croissance cellulaire a été élucidé à différents degrés par par Yen et ses collaborateurs (Yen & Zhang, 2011). Il a été démontré que l'OD fourni à faible taux augmente l'accumulation des lipides, mais inhibe la croissance des cellules tandis qu'un taux plus élevé augmente l'accumulation de la biomasse plutôt que la concentration totale en lipides. En outre, Des études ont été faites avec un OD contrôlé en deux étapes: un OD initial a été maintenu à 60% ± 10 pendant environ 70h ensuite réduit à 25% ± 10 jusqu'à la fin de la fermentation (180h). Ceci a pu améliorer la concentration totale en biomasse de 36,2 g/L à 45,4 g/L, mais la teneur en lipides a baissé de 62 ± 7% à 42 ± 3% en p/p de biomasse cellulaire sèche (Yen & Zhang, 2011) .

9. Les Modes de fermentation

Les modes de fermentation en batch, fed-batch et en continu ont été étudiés pour la culture des microorganismes oléagineux en utilisant différentes sources de carbone. Des études basées sur le glycérol et le glucose sont bien décrits pour la culture microbienne à haute densité.

9.1. La fermentation en mode batch

Il s'agit d'un mode de fermentation où le carbone et d'autres nutriments essentiels ont été additionnés initialement dans le milieu de culture. Le principal paramètre étant le rapport C/N a été ajusté afin de déclencher ou stimuler la lipogenèse. La source de carbone est ajoutée en excès afin de répondre à la condition d'excès de carbone durant la phase lipogénique. Dans un mode de culture en batch, l'utilisation des nutriments est limitée en raison de la production de la biomasse, qui reste également limitée par la disponibilité du substrat. De plus, le mécanisme de lipogenèse exige l'augmentation du rapport C/N qui est une condition difficilement contrôlée en mode batch (le carbone diminue et le rapport C/N diminue également). Pendant la phase de croissance, le carbone assimilé est principalement utilisé pour la prolifération cellulaire ; donc la biomasse (free lipid biomass ou biomasse absolue) est accumulée dans le milieu. Pour un rapport C/N élevé, la croissance est relativement faible et le carbone assimilé est acheminé vers la synthèse des acides gras libres par l'activation d'un pool d'enzymes lipogéniques présentes dans l'organisme. En mode batch, la limitation des nutriments est cruciale (le rapport C/N) pour la production de lipides. Par conséquent, l'optimisation du rapport initial C/N dans le cas de la fermentation en mode discontinu exige une plus grande attention et, en général, un rapport molaire C/N de 80 à 350 est considéré comme approprié pour la synthèse des lipides (Beopoulos et al., 2009a). Cependant, cela peut varier en fonction de l'organisme et de la concentration initiale en carbone. Néanmoins, la forte concentration de glycérol dans les fermentations en mode batch a un impact négatif sur la croissance de la biomasse. Pour cette raison, dans de nombreux cas, la concentration de glycérol initiale était limitée à environ 20 à 60 g/L. Cependant, la faible concentration du substrat initial peut affecter la production de lipides. Cette situation est due à l'épuisement rapide de la source de carbone, ce qui se traduit par une faible disponibilité de carbone pendant la synthèse des lipides en phase métabolique de la fermentation.

9.2. La fermentation en mode Fed batch

A la différence du procédé par mode batch, la fermentation en mode fed-batch fournit du carbone et des substances nutritives de façon contrôlée. Le taux de croissance, le flux de carbone et

d'azote sont contrôlés et fournis en conséquence. Afin de maintenir les organismes oléagineux dans des conditions métaboliques actives, un rapport optimal C/N doit être retenu durant les différentes périodes de culture (la phase de croissance, de transition et de la lipogenèse) (Ramalingam et al., 2010). Pour la synthèse des lipides, la phase lipogénique est considérée comme la plus cruciale. Par conséquent, si le rapport molaire du carbone et de l'azote dans le milieu de fermentation n'est pas optimal, le métabolisme peut se traduire par une synthèse d'acides organiques. Sur la base d'études précédentes, le rapport C/N de 20 C mol / N mol empêche la production d'acide citrique chez *Y. lipolytica*. Un contrôle précis du rapport C/N est conseillé pour une meilleure production de lipides en mode fed-batch (André et al.; Beopoulos et al., 2009b). Cette stratégie de fermentation pourrait entraîner une augmentation de la productivité en biomasse et en teneur de lipides. Une étude utilisant le mode fed-batch a été réalisée par Li et al qui ont pu montrer que que la concentration en biomasse de 151,5g/L avec une teneur en lipides de 48,0% p/p ont été obtenues pendant 25 jours de fermentation en erlenmeyer (Li et al., 2007). Les expériences ont été répétées à grande échelle avec des conditions contrôlées et la biomasse obtenue était de 106g/L avec un contenu lipidique 67,55% p/p à 134h (> 6 jours) de fermentation. Dans les deux cas, le glucose a été additionné à un débit de 20 g/L/jour. En accord avec les études de Saenge et ses collaborateurs , la fermentation en mode fed-batch a été réalisée avec la levure *Rhodotorula glutinis* pour produire des lipides en utilisant le glycérol comme source de carbone (Saenge et al., 2011). Le but du processus fed-batch était d'éviter l'inhibition due au substrat et d'améliorer la production de lipides en ajoutant une source supplémentaire de carbone. Une fermentation en mode batch avec un volume de 800 ml a été opéré. Une concentration initiale en glycérol (9,5% (p/v)) avec un rapport C/N de 85 ont été utilisés.

50 ml de glycérol brut ont été ajoutés toutes les 12 h jusqu'à 48 h de fermentation pour maintenir la concentration du substrat carbone à un niveau optimal de 9,5% (p/v). L'alimentation du substrat en mode fed-batch a augmenté la biomasse et la teneur en lipides jusqu'à 10,05 g/L et 60,70%, respectivement. La consommation de glycérol par les microorganismes était plus importante en fermentation fed-batch. La concentration finale en lipides atteint 8,36 g/L, soit pratiquement deux fois plus que la teneur en lipides obtenue en mode batch (4,33 g/L) (Saenge et al., 2011).

9.3 La fermentation en mode continu

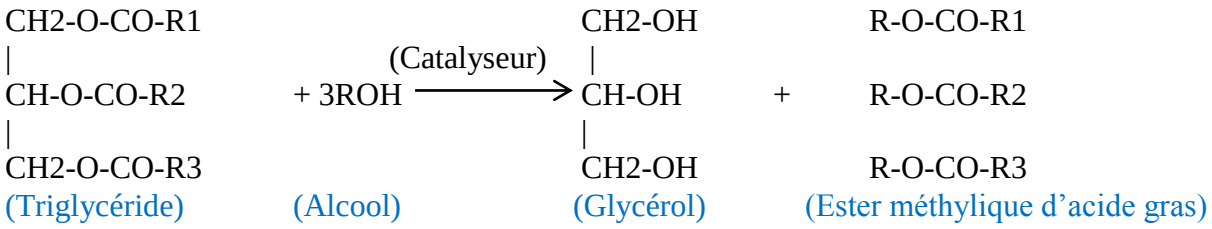
La fermentation en mode continu se réfère à l'assimilation du carbone et d'autres substances nutritives à une vitesse constante en fonction du taux de croissance microbienne et de la dilution du milieu de culture. La concentration des substrats dans le bioréacteur est maintenue également de façon constante et en fonction du taux de dilution; le rapport C/N reste constant au cours de la fermentation contrairement au procédé en mode batch. comparativement à cette dernière, le rapport C/N ajusté dans le milieu continuellement doit être supérieure à celui du ratio initial afin d'obtenir une forte accumulation de lipides. Toutefois, le taux de dilution élevé peut affecter l'équilibre métabolique pour la synthèse des lipides quand il s'agit d'une fermentation en mode continu. Par conséquent, le taux de dilution représente un facteur critique déterminant dans le contrôle et la réussite de ce mode de culture (Huijberts & Eggink, 1996). Papanikolaou et Aggelis ont rapporté que *Y. lipolytica* était capable de produire une grande quantité de lipides lorsqu'elle est cultivée dans du glycérol brut sous un procédé en mode continu, où l'azote est limité (Papanikolaou & Aggelis, 2002). Dans un procédé continu avec une forte aération, la production de lipides est favorisée à des taux de dilutions faibles, et la productivité la plus élevée de lipides obtenue était de 0,12 $g.l^{-1}$ h^{-1} au plus bas taux de dilution à environ 0,03 h^{-1}. L'augmentation des taux de dilutions conduit à une augmentation du rendement de la masse cellulaire, mais résulte d'une fraction lipidique faible. Par ailleurs, la composition en acides gras des lipideses extraits n'a pas été affectée par le taux de dilution.

10 La tranestérification

10.1 La transestérification chimique

Les huiles provenant de sources végétales, animales ou microbiennes sont principalement composées de TAGs qui sont également connus comme des composés d'acides monocarboxyliques saturés et insaturés. Ils sont principalement constitués d'acides palmitique, stéarique, oléique, linoléique et linolénique. La transestérification des TAGs implique une réaction catalysée principalement par un acide (H_2SO_4) ou une base (KOH, NaOH) en présence de mono-alcools, le méthanol plus communément, ou l'éthanol en moindre usage. La réaction catalytique simplifiée de la réaction de transestérification est représentée par l'Équation 6 ci-dessous. La transestérification est un procédé chimique où les TAGs sont convertis en esters méthyliques d'acides gras en 3 étapes consécutives aboutissant à la formation d'une mole de glycérol.

Équation 6: Réaction chimique de Transesterification des TAGs en EMAGs



La réaction de tranestérification catalysée par une solution basique libère aussi de l'eau comme indiqué dans l'**Équation 7**:

Équation 7: Réaction de transesterification par catalyse alcaline

$$R_1 - COOH + NaOH \rightarrow R_1 - COONa + H_2O$$

AGLs catalyseur savon eau

Il existe divers problèmes associés à la transestérification des lipides utilisant un catalyseur alcalin. La formation de savon dans la réaction catalysée par une base est indésirable et elle réduit considérablement le rendement de transformation des esters. Elle entrave également la séparation de phases. Le savon formé a tendance à se lier avec le catalyseur, ce qui se traduit par sa consommation accrue qui induit finalement des coûts supplémentaires au procédé global.

Un autre problème majeur est l'hydrolyse des triglycérides en diacylglycérides en raison de la présence de molécules d'eau qui entraînent la formation d'acides gras libres (AGL). En présence d'eau et d'AGL, la transestérification en milieu alcalin tend à se retarder et réduire le rendement du procédé.

Cependant, dans le cas de réactions catalysées par une solution acide, les AGLs sont convertis par la suite en esters, et par conséquent, les huiles contenant une teneur élevée en AGL sont plus appropriées pour une transestérification utilisant un catalyseur acide. Dans la plupart des cas, l'acide sulfurique est le catalyseur et le méthanol est l'alcool le plus souvent exploité. Toutefois, en raison de la réaction hyperthermique et de la lenteur de la réaction, cette approche n'est pas très sollicitée par les industriels du domaine.

En pratique courante, la transestérification à l'aide d'un catalyseur alcalin est plus appréhendée que la tranestérification utilisant un acide. Un organigramme simplifié est présenté ci-dessous pour expliquer le processus (**Error! Reference source not found.**). Les huiles contenant des AGLs inférieure à 2,5% en p/p sont utilisées directement pour la tranestérification, mais si la

teneur est supérieure à 2,5% en p/p, l'huile est soumis à un prétraitement précédant la tranestérification. En général, le prétraitement à base d'acide est recommandé dans plusieurs études pour réduire la teneur en AGLs. Quand cette teneur est réduite, la transestérification est effectuée avec un catalyseur basique. Une des alternatives à cette méthode était l'utilisation d'un ratio élevé alcool: huile (40:1) notamment pour des solutions à forte teneur en AGLs en contact avec l'huile. Cependant, la récupération de l'alcool qui n'a pas réagi exige un investissement à capital élevé. Pour améliorer le rendement du produit, l'huile disponible doit être évaluée avant la transestérification. Etant donné que les huiles proviennent de sources végétales et animales, les AGLs, la teneur en phospholipides et l'eau varient. Il existe deux grandes techniques proposées pour éliminer ces composants perturbateurs. Un procédé comprend une filtration sur membrane suivie d'un traitement à l'aide d'un solvant organique. La qualité de l'huile récupérée par ce processus dépend principalement de la taille des pores de la membrane filtrante et le type de phospholipides présents dans l'huile d'alimentation. La seconde méthode est la déshydratation de l'huile à une température de 200°C. Grâce à la simplicité du procédé et une élimination efficace des AGLs et des phospholipides, l'hydratation est la plus couramment utilisée comme étape de prétraitement dans le secteur industriel. L'inconvénient du prétraitement par hydratation est, néanmoins, qu'il ne peut pas éliminer les phospholipides non hydratables. D'autres méthodes utilisées pour éliminer les phospholipides comprennent l'addition d'acide phosphorique ou de l'acide citrique à l'huile afin de réduire le pH et une séparation ultérieure des phospholipides se fait par formation de phases.

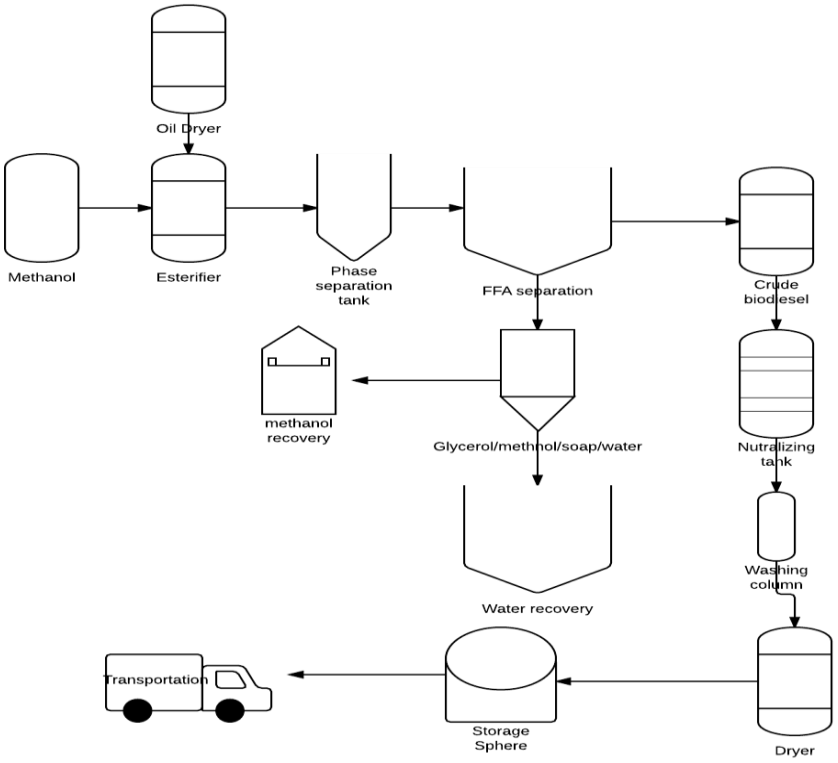


Figure 5 Étapes schématiques du processus de la transésterification

À la fin de la transésterification, le mélange est constitué d'esters méthyliques d'acides gras (TAGs), de catalyseur non réactif, d'alcool, de savon et de l'huile. Bien que la fraction de glycérol se décante, la fraction de biodiesel resterait contaminée par les constituants mentionnés ci-dessus. Consécutivement, l'élimination des impuretés nécessite une étape supplémentaire après transestérification acide ou alcaline. La purification du biodiesel brut peut être réalisée par plusieurs procédés : le lavage à l'eau, le lavage à sec et l'extraction membranaire sont les méthodes les plus couramment utilisées.

Les principaux facteurs qui affectent la transestérification dépendamment de la composition d'huile sont : la concentration d'alcool, le temps de réaction, la température de réaction et la concentration du catalyseur. L'équation simple pour l'alcool et le rapport molaire de l'huile est de 3:1. Mais quand la concentration d'alcool augmente au-delà de 3, le rendement s'améliore également. En outre le rapport molaire d'alcool, le type de catalyseur associé à la réaction joue un rôle clé dans la transestérification. Ce dernier facteur peut être déterminé par la quantité des

AGLs présents dans la quantité d'huile. L'augmentation du temps de réaction peut conduire à un taux de conversion important. Dans la littérature, on retrouve des rapports suggérant que le temps de réaction de plus de 90 minutes conduit à des rendements relativement élevés en TAGs. Mais la prolongation du temps de transestérification peut entraîner une réaction inverse et par conséquent résulter d'une formation accrue de savon. La température de réaction est un facteur critique dans la transestérification chimique. La viscosité de l'huile diminue à mesure que la température de réaction augmente, mais ce facteur ne peut pas dépasser la température d'ébullition de l'alcool résultant d'une évaporation du produit et de la réduction de la performance de la réaction. La température la plus couramment utilisée est de 50 à 60^{o}C. La concentration du catalyseur affecte aussi le taux de conversion tandis qu'il influence positivement la réaction quand il est utilisé à fortes concentrations. En outre, la concentration insuffisante du catalyseur peut entrainer une transestérification incomplète (Avila-Neto et al., 2014). Freedman et al ont constaté que lorsque le catalyseur est méthylé avant son addition dans la réaction, le rendement de production est amélioré (Freedman et al., 1986).

10.2 La transesterification enzymatique

Les avancées scientifiques en technologie verte ont touché les problématiques liées à la transestérification. La lipase est l'enzyme qui hydrolyse les lipides et son application dans la transestérification a été bien étudiée au cours des dernières années. Les lipases sont produites sous deux formes différentes: intra et extracellulaires (Adriana Gog et al., 2012). Les lipases extracellulaires de novozymes (Li et al., 2006) lipozyme, Lipozyme RM IM (Oliveira & Rosa, 2006) et la lipase PS-C (Li Deng et al., 2005) sont les plus largement rapportés dans des processus de transestérification. En outre, les lipases sont classées en fonction de la spécificité de position et celle des acides gras.

Les lipases extracellulaires sont produites principalement *par Mucor miehei, Rhizopus oryzae, Candida antartica et Pseudomonas cepacia* (Ban. K et al., 2012). Basé sur le type d'organisme, la spécificité de la lipase produite varie également. Par exemple, *C. viscosum* a été caractérisée d'avoir des sites différentiels actifs sn^{-3} à sn^{-1} (Fox, 2003). La présence de phospholipides, de fortes teneurs en AGLs et une faible teneur en eau ne peut pas empêcher la réaction enzymatique. Selon la littérature, certains microorganismes peuvent produire une enzyme avec une affinité élevée à une variété d'huiles. L'huile de tournesol peut être transestérifiée avec l'enzyme produite par *C. antartica, C. rugosa, A. Niger* (Valerie Dossat et al., 2002). Aussi,

l'huile de soja peut être transestérifié efficacement par les lipases produites chez *R. oryzae* et *P. fluorescens* (Wei Du et al., 2003). Bien que les enzymes présentent plusieurs avantages par rapport aux méthodes chimiques, ils sont défavorisés par le fait d'un souci de leur inhibition probable au contact de l'alcool utilisé dans la réaction ainsi que la formation du glycérol. Les alcools à chaîne courte, comme le méthanol et l'éthanol, peuvent inactiver l'enzyme. Mais, selon les études de Belafi-Bako et al, les étapes d'addition d'alcool peuvent améliorer l'activité enzymatique et permettre une meilleure conversion, soit un rendement de l'ordre de 97% (K. Bélafi-Bakó et al., 2002). La réduction de l'efficacité de transestérification due à la liaison de l'enzyme libre au glycérol peut être plus maîtrisée en utilisant du 2-méthylpropan-2-ol communément connu par le tert-butanol. La polarité modérée de ce solvant peut solubiliser le glycérol ainsi que le méthanol et éliminer leur impact négatif (Wang et al., 2006). Une comparaison du procédé chimique et enzymatique de la transestérification est présentée dans le Error! Reference source not found..

Table 9 La comparaison de transestérification chimique et enzymatique(Adriana Gog et al., 2012)

Paramètre	Base	Acide	Enzyme
température	60 à 80	>90	30 à 50
Teneur en AGL	Formation élevée de savon	Convertie en FAME	Convertis en FAME
Récupération du glycérol	Fastidieux, glycérol bas de gamme	Fastidieux, bas de gamme glycérol	facile, glycérol de haute qualité
Contenu en eau dans le substrat	Formation de savon et hydrolyse d'huile	Désactivation du catalyste	favorise la réaction jusqu'à 5%
Purification des FAME	Par lavage	Par lavage	Nul
Coût de Production	Élevé	Élevé	Élevé
Traitement et disposition de déchets	Élevé, traitement nécessaire	élevé, traitement nécessaire	Peu couteux, déchets minimes non dangereux

11. Conclusion

La croissance mondiale ou globale dans l'industrie du biodiesel a conduit à la disponibilité de l'excédent de glycérol brut sur le marché mondial du coproduit ayant un impact sur son coût d'approvisionnement. Bien que les données soient disponibles sur la combustion du glycérol pour produire de l'énergie d'une manière facile, le rendement énergétique est faible par rapport aux carburants classiques et la combustion est difficile à des températures très basses ou contrairement très élevées. Sa viscosité élevée le rend également difficile à s'évaporer. Pour ces raisons, la conversion microbienne du glycérol en différents produits à valeur ajoutée semble être une approche réaliste et respectueuse de l'environnement. Bien que plusieurs microorganismes soient tolérants à l'assimilation du glycérol comme substrat, un procédé économiquement viable est actuellement loin de la réalité. Plusieurs souches microbiennes ont été rapportées comme oléagineuses avec des rendements de 20-85% de lipides par unité de biomasse sèche, cependant ces constats ne sont pas applicables dans un contexte de valorisation de résidus et de déchets industriels comme le glycérol brut. Afin de développer un processus stable, les facteurs opérationnels et les conditions de culture et les paramètres relatifs à la nature et la performance de la souche doivent être abordés et une recherche approfondie dans en disciplines diverses comme physiques et biochimiques, et génie génétique et qui doivent être étudiés en détail afin de comprendre les mécanismes cellulaires des oléagineux. Les levures ont été plus étudiées comparativement aux champignons filamenteux en raison de leur taux de croissance plus rapide, la facilité de manipulation, l'accumulation efficace de lipides, et la familiarité de la base de biochimie de la synthèse des lipides, etc. En général, l'avenir des huiles microbiennes pour l'application en bioénergies offre un grand potentiel. De plus, plusieurs microorganismes éventuels ont été identifiés quoique le développement du processus soit toujours limité à l'échelle du laboratoire. Pour surmonter les problèmes liés à l'optimisation des processus par les paramètres de culture et l'amélioration des souches en termes de biomasse élevée, la production de lipides devrait faire la priorité de la recherche dans le domaine de bioénergies et valorisation de produits à valeur ajoutée.

Problématiques

En se basant sur l'étude de la littérature présentée dans ce rapport, les problématiques de recherche suivantes ont été identifiées.

Problématique-1

Il existe près de 33 souches microbiennes potentielles isolées et étudiées pour la production de lipides à partir de différentes sources nutritives. Même si un organisme pourrait être capable d'utiliser une large panoplie de sources de carbone, seuls quelques-uns seraient en mesure de se développer et activer leur métabolisme de réserves lipidiques dans des conditions contraignantes et avec des sources nutritives non usuelles. La plupart des isolats ont été criblés pour la production de lipides en utilisant des sucres simples comme le glucose, le glycérol, le xylose, mais les sources de carbone complexes issues des boues d'épuration, lignocellulose et les effluents industriels ont été moins explorés. La plupart des microorganismes sont vulnérables face à des composés inhibiteurs contenus dans les coproduits résultant du processus de transestérification, ainsi que du prétraitement lignocellulosique ou autres composés inhibiteurs des boues industrielles ou des eaux usées.

- Trouver un organisme capable de tolérer la plupart de ces inhibiteurs et d'utiliser un large éventail de sources de carbone contribuerait à développer un procédé de production de lipides nécessitant une source diversifiée de carbone

Problématique-2

Les paramètres de croissance tels que la concentration du carbone et le rapport carbone/azote sont les principaux éléments à résoudre. La plupart des études sur le rapport carbone/azote ont montré que le ratio idéal de ce dernier varie d'un organisme à un autre. Par conséquent, les paramètres de culture doivent être évalués pour le nouvel isolat. Le temps de fermentation rapporté dans la littérature pour la production de lipides est assez élevé. La durée de fermentation, la concentration de biomasse et sa teneur en lipides sont les facteurs clés pour le succès du procédé en application industrielle.

Problématique -3

L'économie de la production de lipides microbiens à partir d'organismes hétérotrophes a été étudiée pendant des décennies. Cependant, un plan économiquement faisable n'a pas été encore développé. Le principal obstacle identifié dans le processus étant le coût de la matière première, suivi par le procédé de fermentation et enfin la nécessité de micro et macro nutriments. Récemment, plusieurs matières premières à bas prix ont été étudiées et une production élevée de biomasse et de lipides ont été répertoriées. Cependant, le processus n'a pas été concrétisé en raison d'autres facteurs tels que l'exigence des micros et macros éléments.

Problématique-4

Il y a très peu de recherches disponibles sur la fermentation non stérile sans addition d'antibiotiques (coûteux). Très peu de travaux aussi ont décrit l'utilisation du méthanol comme source de carbone pour la culture de certaines micro-algues et levures. La stérilisation des milieux de culture à l'échelle industrielle est un processus très exigeant en énergie. Éliminer la stérilisation peut balancer favorablement toute l'économie du processus. Dans une telle situation, une fermentation non stérile à base de méthanol permettra de rendre le processus économiquement viable. Afin de suivre cette stratégie, il est nécessaire de dépister des isolats microbiens avec une grande tolérance à une concentration élevée de méthanol.

Problématique-5

La revue de littérature fait mention de nombreuses tentatives d'utilisation d'organismes génétiquement modifiés en vue d'augmenter la biomasse et d'accroitre la quantité de lipides. Toutefois, la réglementation sur l'utilisation des organismes génétiquement modifiés (OGM) pour des opérations à échelle industrielle est encore discutable et controversée. En outre, l'intégrité des organismes génétiquement modifiés peut diminuer au cours des repiquages de sous-cultures successives. D'autres procédés ont été développés pour améliorer le processus de fermentation en général dont par exemple l'ingénierie biochimique en fermentation microbienne. Cependant, très peu d'études ont été consacrées à son évaluation. La biotine est un des éléments les plus importants dans la synthèse des lipides. Elle agit en tant que groupe prosthétique d'acétyl-CoA carboxylase. La conversion de l'acétyl-CoA en malonyl-CoA ait lieu en une réaction à deux étapes. Initialement, le composant carboxylase de la biotine catalyse la phosphorylation du bicarbonate par l'ATP pour former le carboxyphosphate intermédiaire.

Ce dernier transfère, ensuite, le groupe carboxyle à la biotine pour former la carboxybiotine. En deuxième étape, le carboxyltransférase transfère le groupe carboxyle à l'acétyl-CoA pour former la malonyl-CoA qui suit ultimement la synthèse des TAGs. Par conséquent, cette étape est connue comme la première phase incontournable pour la synthèse des TAGs. Cependant, les études sur l'importance de la biotine demeurent très limitées jusqu'à présent. Pour cela, son importance doit être évaluée mutuellement avec la synthèse des lipides.

Hypothèses, objectifs et originalité

Hypothèses

- Tous les microorganismes synthétisent les lipides sous forme de phospholipides comme revêtement cellulaire durant le processus de croissance et de reproduction. Seulement quelques-uns sont reconnus comme oléagineux en raison de leur capacité à accumuler des lipides intracellulaires (autres que les phospholipides) sous forme de réserves, en présence d'un excès de carbone dans le milieu environnant, combiné avec une limitation d'autres nutriments. Les organismes qui se développent dans un environnement naturel comme les forêts, les zones contaminées par des hydrocarbures et d'autres endroits avec une grande disponibilité de carbone pourraient avoir un potentiel naturel à accumuler des éléments tels que les lipides, les Poly-Hydroxy-Alcanoates (PHA), les polyols et d'autres matériaux de réserve énergétique. La sélection de ces microorganismes au profit d'une production lipidique est une technologie sollicitée actuellement et pourrait éliminer la nécessité d'une modification génétique non garantie à long terme.
- Le méthanol est un inhibiteur toxique naturel pour les cellules microbiennes en croissance, pourtant, à des concentrations très faibles, il pourrait soutenir la prolifération cellulaire (comme source de carbone). Certains microorganismes sont capables d'utiliser le méthanol comme source de carbone à des concentrations à la fois faibles et élevées. Le recours à une souche microbienne ayant une tolérance élevée au méthanol permettra d'éviter l'étape de stérilisation pour la fermentation des lipides. Ceci aidera à rendre le processus économiquement faisable.
- Les déchets organiques sont disponibles en abondance. L'utilisation de matériaux organiques de l'agro-industrie comme source de nutriments pour la production des lipides est une approche très intéressante pour la recherche actuelle dans le domaine des ressources renouvelables. Dans notre cas, les sels minéraux inorganiques seront remplacés par des déchets organiques tels que les lixiviats (lixiviats de silos de grains). Cela permettra de réduire le coût des éléments nutritifs nécessaires et pourrait également être considéré comme un processus de décontamination de déchets indésirables en réduisant notamment la DBO / DCO. Ceci contribuera à l'élimination des nutriments dans le flux des déchets chargés nécessitant un prétraitement en amant de leur disposition dans l'environnement.

- Amorcer la synthèse de lipides par un ajout externe de vitamines (Biotine) est un autre facteur remarquable dans métabolisme de synthèse lipidique, sachant que la phase synthèse est de très courte durée chez la plupart des organismes. L'addition de la biotine et d'une quantité d'ions ammonium à l'état de trace pendant la lipogenèse soutiendra le processus en fournissant suffisamment de cofacteurs pour les enzymes clés impliquées dans la lipogenèse. Cela permettra de prolonger la phase lipogénique et finira éventuellement par améliorer la productivité globale des lipides.

Objectifs

En se basant sur les problématiques de recherche identifiées, les objectifs suivants ont été formulés :

- Le criblage de souches de champignons et de levures ayant la capacité d'assimiler le glycérol brut pour la production de lipides. L'identification des isolats en utilisant le système Biolog et les méthodes de séquençage d'ARNr 18S et 26S.
- La sélection de souche performante pour la production de lipides ayant une concentration élevée de biomasse associée à une grande teneur en lipides pendant une durée minimale de fermentation.
- Des études visant à identifier la concentration idéale en glycérol, le rapport Carbone/Azote (C/N), le pH, les éléments traces, l'âge de l'inoculum ainsi que sa taille afin d'améliorer la concentration de biomasse et l'accumulation de lipides A la base de se substrat non usuel.
- Le remplacement des sources synthétiques d'azote (extrait de levure, l'urée, le dihydrogénophosphate d'ammonium) avec les boues de l'agro-industrie.
- Étudier l'influence de la concentration du méthanol sur celle de la biomasse et sur l'accumulation de lipides ainsi que le développement d'un procédé de fermentation non stérile pour la production de lipides.
- Une approche biochimique d'amélioration de la synthèse des lipides par l'usage de la biotine en promouvant l'activité des enzymes de la lipogenèse.

Originalités

- Les organismes utilisés dans cette étude ont été isolés de l'environnement naturel et criblés pour la production de lipides dans les médias standards puis confirmés comme accumulateurs de lipides dans les milieux à base de glycérol (ou microorganismes oléagineux). Les organismes énumérés (SK, SKY 2, SKY7, SKY 8, SKY 10, SKY 11, SKY 28, A 1, N1) ont été isolés à partir du sol et des échantillons du déversement de glycérol prélevés de la région du Québec et des industries de biodiesel de la même province (Québec). Les souches fongiques et les levures et ont été criblées sur un média de sélection gélosé à base de glycérol supplémenté avec des antibiotiques.
- Le développement d'un nouveau procédé à base de méthanol sans recours à une stérilisation du milieu exigée en fermentation lipogénique constitue une nouvelle approche économique du projet. Bien que peu d'études aient utilisé le méthanol comme source de carbone pour les micro-algues et les levures, le développement d'un procédé non stérile et l'impact d'une concentration élevée du méthanol sur la synthèse de lipides par *Y. lipolytica* (SKY7) n'a pas encore été appréhendée.
- L'évaluation du rapport molaire C/N approprié à la phase lipogénique ainsi que les études d'ajout d'azote peuvent révéler l'étendue exacte de la concentration d'azote (rapport de C/N) nécessaire pour les enzymes de la lipogenèse pendant cette phase métabolique critique.
- Une étude biochimique a pour but de prolonger la phase lipogénique par addition de biotine n'a pas été bien évaluée jusqu'à présent. Une alimentation continue de NADPH est nécessaire pour l'activité de l'ACC, et ceci est dérivé de l'activité du ME. ACC est connu pour être une enzyme dépendante de la biotine, par conséquent, un apport extérieur de biotine peut améliorer l'activité de l'ACC, ce qui peut prolonger la phase lipogenèse.
- Le remplacement de l'azote organique par des matériaux de déchets agro-industriels peut réduire le coût de la production de lipides à partir du glycérol. Cela pourrait aider à protéger l'environnement local en réduisant les nutriments dans les flux de déchets à traiter avant disposition. Pour l'étude de lixiviat de silo comme substitut de l'azote commercial, une approche comparative sera réalisée afin d'en retirer les conclusions.

Résultats et discussion

Les résultats obtenus lors de cette étude sont dressés selon 3 sections. La première section invoque l'isolement de microorganismes potentiellement oléagineux (levures et champignons supérieurs) à partir de sols dépourvus d'azote. L'isolement a été suivi par un criblage de ces isolats dans un milieu limitant en azote, soutenu par l'ajout de glycérol brut à différentes concentrations et avec différents rapports C/N. Les souches bien adaptées au glycérol brut et ceux produisant des biomasses plus élevées et accumulant plus de lipides ont été choisies pour des études ultérieures. Basé sur l'isolement et la sélection d'une souche robuste assimilant absolument le glycérol brut, *Y.lipolytica* SKY7 a été sélectionnée comme souche potentielle pour la fermentation lipidique dans milieu riche en glycérol. La concentration de glycérol, le rapport C/N, le volume d'inoculum, et les micro et macronutriments ont été évalués dans la section 3. En se basant sur des analyses univariées des paramètres indépendants, une optimisation statistique à part entière a été planifiée dans la finalité d'obtenir les conditions optimales pour maximiser la production en biomasse et en lipides accumulés. La méthodologie appréhendée repose sur le logiciel Design expert version 8. Consécutivement, les effets du pH sur la biomasse et la production en lipides ont été évalués et expliqués.

Dans le cas d'une production de «lipides unicellaires», la richesse des matières premières et le coût opérationnel sont les deux principaux facteurs affectant l'économie globale du processus. Cependant, les substrats à faible coût tels que les déchets lignocellulosiques, les eaux usées municipales, les eaux usées industrielles et le glycérol provenant de l'industrie du biodiesel auraient récemment remplacés les matières premières à coût élevé. Toutefois, d'autres composants du milieu de fermentation lipogène n'ont pas encore fait l'objet de substitution par des déchets à valeur ajoutée dans une projection de faisabilité économique. Cette recherche mise aussi sur la substitution des sources d'azote commerciales par des effluents industriels ce qui constitue un verrou pour éliminer complètement la dépendance en matière première commerciale de la filière de biodiesel. Le lixiviat de silos de grains est utilisé dans la présente étude pour remplacer les sources d'azote commercial tel que l'extrait de levure et le sulfate d'ammonium. Les résultats élaborés sont présentés dans la section 4.

En outre, un processus non stérile de fermentation microbienne moyennant le méthanol comme agent de prévention contre la contamination a été élaboré et les résultats correspondant font objet

de discussion dans la section 5. Enfin, suite à l'étude d'optimisation, une approche biochimique basée sur l'usage de biotine comme stimulant de production, a été mise au point pour une production poussée de lipides chez *Y.lipolytica* SKY7. Les résultats obtenus ont été détaillés dans la section 6.

Les tendances récentes en matière de valorisation et intégration de carbone à partir de sources classiques et des sous-produits pour la production des lipides
Revue de littérature (Chapitre II) :

Ce chapitre survole en aperçu général la production de lipides à partir de différentes classes de microorganismes. La partie I détaille les cheminements de réactions biochimiques impliquées dans la synthèse des TAGs (triacylglycérols). L'importance des enzymes intracellulaires tels que ACL (ATP de citrate lyase, ME (enzyme malique et ACC (acétyl Co-A carboxylase) a été également élucidée. Bien que les événements biochimiques impliqués dans l'accumulation des lipides soient bien expliqués, la production de lipides microbiens à partir de microorganismes hétérotrophes n'est pas facilement applicable à l'échelle industrielle, ceci est dû à plusieurs facteurs. En premier lieu vient le coût de la matière première, car elle contribue à environ 60 à 75% du coût total de la production de lipides à partir des microbes. De nombreuses études ont été réalisées en utilisant des substrats tels que le glucose, la xylose, la biomasse lignocellulosique, les eaux usées municipales, la graisse animale et le glycérol. Cependant, le glycérol provenant de l'industrie du biodiesel a attiré plus d'attention de la part des chercheurs en raison de sa disponibilité abondante à faible coût. En outre, le glycérol est considéré comme la source de carbone la plus utilisée par les microbes après le glucose. L'étude suivante, explore l'utilité de ce coproduit (glycérol brut) de la filière de biodiesel pour la production intensive de lipides microbiens via la fermentation lipogénique. Autres sources de carbone alternatives ont été également évoquées dans la revue. Il est important de savoir que, le mode de fermentation joue un rôle crucial dans la production de la biomasse et des lipides. En effet, différents modes de fermentations ont été comparés et présentés dans la revue.

Une approche sélective pour l'isolement de souches oléagineuses en milieu déficient d' azote : approche par substrat sur des échantillons de sol.
(Chapitre III)

Le microorganisme utilisé dans cette étude a été isolé à partir d'échantillons de sol prélevés de déchets de bois de la sylviculture et de sol de humides à. Les isolats ont été initialement soumis à une analyse phénotypique BIOLOG. Le recours à cette méthode a prouvé que la plupart des microorganismes appartenaient au genre *Candida*. Deux souches (SKY7 et SKY 28) ont été identifiées comme *Y.lipolytica* et N1 a été identifiée comme *Rhodococcus sp*.

Le profil d'utilisation de substrat de l'ensemble des 9 isolats a été considéré. À l'exception de N1 (*Rhodococcus*), toutes les souches se sont avérées positives à l'assimilation du glycérol comme seule source de carbone. Après la caractérisation biochimique basée sur BIOLOG, les organismes ont été identifiés à travers la méthode de séquençage ADNr 16S. C'est ainsi que SKY7 et SKY 28 ont été authentifiés comme *Y.lipolytica* par le séquençage d'ADNr16s. Les autres levures ont été identifiés comme *Candida membranaceous*. Après identification, les isolats ont été examinés sous microscope après coloration au Soudan noir B pour différencier les souches accumulatrices et non accumulatrices de gouttelettes d'huile intracellulaires. Ces souches sélectionnées ont été cultivées à différentes concentrations de glycérol de rapports C/N. La production maximale de biomasse a été obtenue chez SKY 11 (18,47 ± 0,13 g/L). Cependant, la concentration de lipides accumulés s'avérait très faible.. Il a été aussi remarqué que SKY7 représente un bon potentiel pour la production de biomasse (7,53 ± 0,27 g/L) avec 42,04% p / p d'accumulation de lipides. La composition des lipides produits par SKY7 a été déterminée par GC-FID et a révélé que l'huile proles lipides accumuléspar SKY7 sont propices à la production de biodiesel.

Élucider l'effet de la concentration du glycérol et le rapport C/N sur la production de lipides en utilisant *Yarrowia lipolytica* SKY7

(Chapitre IV Partie 1)

D'après les analyses et l'identification moléculaire réalisées, la souche SKY7 (*Yarrowia lipolytica*) a été identifiée comme un microorganisme doté d'un bon potentiel pour la production de biomasse et de lipides à partir du glycérol brut. Toutefois, lorsqu'un microorganisme est nouvellement isolé, les paramètres de culture pour une production optimale doivent être évalués au premier abord. À cet effet, le nouvel isolat a été soumis à croître à des concentrations différentes de glycérol (34.4; 52.3; 112,5 et 168,2g/L) pour produire de la biomasse et des lipides. Les résultats ainsi obtenus ont révélé que la concentration de glycérol de 112,5g/L est la plus favorable pour le rendement en biomasse et lipides. Lorsqu'elle est de l'ordre de 112.5g/L, les résultats en biomasse et lipides ont respectivement atteint 14,84g/L et 6,14g/L. Néanmoins, une faible concentration en biomasse (11,8 g/L) a été obtenue par l'utilisation de 34,4g /L de glycérol. Une amélioration remarquable a été notée en augmentant la concentration de glycérol à 52,3g/L, la concentration de la biomasse a ainsi atteint 16,14g/L. Concernant la production des lipides, des expériences employant 112,5; 168,2; 52,3et 34.4 g/L de glycérol ont été procédées. Les résultats ont montré que 112,5g/L de glycérol est la concentration la plus appropriée pour la production de biomasse et de lipides. Cette concentration a permis l'obtention de (6,14g/L) de lipdes. Tandis qu'une concentration relativement plus faible (5,75g/L) en lipides a été obtenue avec 168,2g/L de glycérol, ans deux autres cas (34,4 et 52,3g/L) les concentrations en lipides étaient encore moindres.

Le rapport C/N a été étudiée selon 4 variables différentes (25, 50, 100, 150) en utilisant la concentration optimale en glycérol.; la concentration en azote dans le milieu étant un facteur très important régissant la production lipidique et microbienne. Une forte concentration d'azote favorise la formation de biomasse plutôt que l'accumulation des lipides. D'après les expériences réalisées, il est clair que lorsque le rapport C/Nétait faible (25 et 50), la concentration de la biomasse atteint son maximum. Cependant, un rapport plus élevé C/N utilisé initialement a aboutità des concentrations inférieures de biomasse. Il est à noter que ce même faible rapport C/N (C/Nratios 25 et 50) n'a pas reproduit des résultats satisfaisants en termes de concentration de lipides extraits. Tandis que le ratio C/N (C/N 100-6,66 g/L) a démontré des taux plus élevées

en lipides. Suite à cette expérimentations par rapport au ration C/N, un choix optimum a été fixé : le rapport C/N 100 est choisi comme condition de fermentation pour les études ultérieures. Simultanément , une concentration de glycérol de 100 g/L avec un rapport C/Nde 100 considéré optimal pour la lipogenèse et la production de biomasse chez les souches sauvages par *Y.lipolytica* (Ageitos et al.) a été prise en charge.

Une fois que la concentration en glycérol (112,5g/L et le rapport C/N100) et le rapport C/N ont été fixés, le volume de l'inoculum a été étudié. Quatre concentrations différentes d'inoculum (2,5, 5, 7,5 et 10% v / v) ont été expérimentées. Lorsque le volume de l'inoculum a été augmentée de 2,5% v/v à 5% v/v, une élévation considérable de la biomasse et de la teneur en lipides a été observée (biomasse de 11.6 g/L à 14,08g/L, lipides 4,32g/L à 6,28g/L) . Cependant, une augmentation supplémentaire du volume d'inoculum n'a pas révélé de meilleurs résultats. On en déduit alos que la concentration d'inoculum de 5% v/v pourrait etre la condition optimale pour la fermentation de *Y.lipolytica* et cette concentration est retenue pour des études plus avancées. En outre, l'effet des oligo-éléments sur la production de la biomasse et de lipides a été élucidé. Il a été intéressant d'en déduire que l'ajout des éléments traces augmente de manière significative la formation de la biomasse. Cependant, cet apport d'oligo-éléments s'avère insignifiant sur la réponse en lipides accumulés (g/L). En général, le glycérol brut provenant de l'industrie du biodiesel est connu pour avoir une quantité suffisante d'ions métalliques (sous forme de catalyseurs ajoutés au cours du processus de transestérification) ainsi que la matrice soumise à la transestérification (l'échantillon d'huile) pouvant contenir notamment quelques traces de macro et micronutriments. À l'issu de ce raisonnement, il semble non nécessaire et significatif l'ajout additionnel d'oligo-éléments à la composition du milieu de fermentation riche (par les micro-éléments du glycérol) pour la maximisation de la production de lipides intracellulaires. Le substrat de base de cette fermentation est un coproduit de la filière de biodiesel revalorisé dans le même procédé par biofermentation.

Étude de l'effet du pH sur la production de lipides dans des cultures en batch de *Yarrowia lipolytica* SKY7
(Chapitre IV, Partie 2)

L'objectif principal de cette étude était d'évaluer l'impact du pH sur la production de la biomasse et des lipides en culture discontinue ou dite en batch. Un pH légèrement acide (5,5 à 6,5) a été rapporté pour favoriser la production de lipides chez les organismes oléagineux. Dans la présente étude, les paramètres de culture préalablement sélectionnés (glycérol de concentration 100 g/L, C/N100, le volume de l'inoculum à 5% (v/v)) ont été utilisés pour produire de la biomasse et des lipides avec et sans contrôle du pH. Deux réacteurs de 15 L ont été opérés simultanément (toutes les expériences sont réalisées en duplicata). Une courte phase de latence a été observée (0-6h) au début des deux expériences. Concernant la concentration de la biomasse, aucun effet significatif n'a été constaté. Dans les deux conditions, une concentration en biomasse de 20,48 ± 0,22 g/L et de 20,58 ± 0,58 g/L ont été obtenues respectivement ce après 120 heures de fermentation. Quant aux lipides, une concentration peu élevée a été observée quand le pH est sous contrôle. Une concentration en lipides de (7,78 ± 0,11 g/L) a été obtenue à 60h de fermentation dans le cas où le pH est contrôlé et résulte ainsi de à 45,4% en poids / poids de teneur en lipides à partir de la masse cellulaire totale. Dans le cas pH non contrôlé, une concentration lipidique de 5,78 ± 0,19 g/L a été obtenue à 60h de la fermentation et qui ne contribue que par 30,9% p / p de la biomasse totale. En outre, le rendement de la biomasse et des lipides obtenus montre que, dans le cas des expériences avec pH contrôlé, la conversion du substrat en lipides était comparativement plus élevée. À 60 h, le rendement en biomasse et en lipides respectivement atteint de 0,377g / g et 0,171g / g en condition de pH contrôlé. Cependant, il est de 0,309g de biomasse par g de substrat avec un rendement lipidique de 0,134 g / g de quand le pH est non contrôlé. D'autre part, la production d'acide citrique a été mesurée dans les deux cas. Les résultats correspondant montrent une forte sécrétion d'acide citrique dans le l'expérience à pH contrôlé (11,1 ± 0,08 g/L). Néanmoins, une très faible concentration d'acide citrique a été obtenue dans le cas des expériences dépourvues du contrôle de pH. La formation de l'acide citrique est un facteur important dans la synthèse des lipides. En se penchant sur la synthèse « de novo », on peut élucider le fait que l'excès de carbone est d'abord converti en acide citrique, puis transformé en acétyl-CoA par l'ATP citrate lyase. L'Acétyl-CoA est ensuite converti en malonyl-CoA par l'acétyl-CoA carboxylase. Par conséquent, l'acide citrique peut être considéré comme le

précurseur de la synthèse de novo des lipides microbiens. L'accumulation continue de l'acide citrique. Le milieu décrit indirectement une activité efficace du glycérol en acide citrique consécutivement converti en lipides si on maintient le pH sous contrôle. Contrairement, en raison de la sécrétion d'acide citrique et d'autres métabolites cellulaires, le milieu de culture, en absence de contrôle de pH, s'acidifie et atteint un pH de 4. Après 96 heures de fermentation sans contrôle de pH, les équilibre de charge ioniques entre les cellules microbienne et le milieu (surnageant) acide initie leur agrégation et décantation en raison du faible pH. En conclusion à ces résultats expérimentaux, nous avons pu constater que le maintien du pH par contrôle d'acidité attendue, favorise une meilleure accumulation des lipides intracellulaires pour la culture en batch de *Y.lipolytica*.

Optimisation des paramètres de culture pour la production de lipides en utilisant la méthodologie des surfaces de réponses et en élucidant la cinétique de la production des lipides dans un fermenteur moyennant la souche *Y.lipolytica* SKY7 isolée au laboratoire (Chapitre IV, Partie 3)

À l'issus de données d'expérimentation antérieures, l'optimisation des paramètres de culture a été procédée en utilisant la méthodologie de surface de réponse. Le plan d'expérience conçu de « Box-Behken » a été choisi pour évaluer l'interaction entre les paramètres les plus signifiants sur les réponses établies à savoir : concentration en biomasse (g/L) et la concentration de lipides extraits à partir de différents essais de fermentation sous diverses conditions. Les paramètres tels que la concentration en glycérol (75 à 100 g/L), C/N (50 à 150) et le volume de l'inoculum (2,5 à 10% v / v) ont été examinés. En considérant les conditions générés par le modèle, les paramètres simples de concentration en glycérol et le volume d'inoculum ainsi que l'effet combiné des 2, se sont avérés importants avec un seuil de probabilité hautement significatif de p-value 0.0001 et 0.01 . La validation a été effectuée par un teste expérimental de validation des résultats attendues selon le modèle sous les conditions optimales. On en conclut que les paramètres définitifs pour l'amélioration du rendement en lipides accumulés est possible dans un milieu contenant 85.5g/L de glycérol avec un rapport C/N de 75 et un volume d'inoculum de 6,25% v / v.

Les paramètres de culture obtenus à partir de l'étude RSM ont aussi été évalués à l'échelle du fermenteur 15L. Lorsque les paramètres de culture optimaux ont été utilisés, la concentration de

la biomasse a atteint 29.46g/L à 72 heures de fermentation. La concentration lipidique était de 15.16g/L à 72h ce contribue à 54,1% de la masse totale des cellules. Le rendement de la biomasse et des lipides a été légèrement amélioré par rapport aux expériences précédentes. La valeur de la biomasse et des lipides obtenus est de 0,332g / g et 0,179g / g, respectivement. Le rendement en lipides augmente nettement en appliquant les conditions d'optimisation. Ceci nous mène à déduire des conditions de culture idéales pour notre isolat d'*Y.lipolytica* dans un milieu de culture à base de glycérol Ils sont comme suit : 85.5g/L de glycérol, un rapport C / N= 75 et avec un volume d'inoculum de 6,25% v / v.

Remplacement des sources de nutriments commerciaux par des lixiviats de silos de grains pour la production de la biomasse et des lipides en utilisant le glycérol brut comme seule source de carbone par *Y.lipolytica* SKY7
(Chapitre V)

Le développement d'une production économiquement viable de « lipides unicellulaires » ne dépend pas seulement de la charge et des coûts d'exploitation. Les sources d'éléments nutritifs tels que l'azote, le phosphate, le magnésium, etc., sont essentiels pour la croissance cellulaire et le métabolisme lipidique. Cependant, les sources nutritives disponibles dans le commerce s'avèrent très coûteuses. A cet effet, une alternative de substitution a été développée. Celle-ci est basée sur l'utilisation de lixiviats de silos (L.S) comme source d'azote. Dans un premier temps, différentes concentrations (100ml/L et 200ml/L) de L.S ont été utilisées dans des milieux de fermentation apportée comme source d'azote. Lorsque la concentration des L.S était 100ml/L, on souligne la concentration plus faible comparativement à celle contenant 200ml /L. De plus, la biomasse obtenue au cours de cette étude a été comparée à celle de la fermentation témoin (avec ajout de l'extrait de levure). Dans l'échantillon témoin (contrôle), une concentration en biomasse de 10.68g/L a été obtenue à 72h de fermentation, avec 4,04 g/L de lipides. Lorsqu'on remplace l'extrait de levure par 200 ml/L de L.S, la concentration en biomasse et lipides atteint 8,72 et 3,6 g/L. Toutefois, avec 100 ml/L de L.S, les concentrations de biomasse et de lipides ne dépassaient pas 5,2 et 1,16 g/L respectivement. Ce faible rendement en biomasse et lipides peut être expliqué par la quantité d'azote ajouté au milieu de fermentation. Les L.S contiennent une concentration en azote de 2,048g/L. Cela indique que la quantité d'azote et autres éléments nutritifs fournis dans les 100 ml/L de L.S pourrait être plus faible que la concentration optimale nécessaire en

azote. D’où le faible gain de biomasse et lipides en doublant la concentration de L.S (condition 200 ml/L). Cette concentration étant meilleur, elle a été retenue pour des étapes ultérieures.

Lorsque les conditions similaires ont été reproduites à l'échelle du réacteur de 15L au laboratoire, les concentrations de la biomasse et des lipides ont atteint 29,16 g/L (72h) et 15.76 g/L (51h). Une augmentation monotone de la concentration lipidique a été remarqué jusqu'à 51h de fermentation. Comparé aux expériences précédentes, les concentrations de la biomasse et des lipides obtenues ont été semblables à celles réalisées dansle milieu avec L.S. Les rendementsen biomasse et lipides donc obtenus sont respectivement de 0,502 g/g et 0,32 g/g. Cela montre que le rendement est relativement plus élevé à un faible temps de fermentation. En outre, on confirme que les eaux usées industrielles pourraient donc être utilisées en tant que source de nutriments pour la production de lipides, car la production de lipides à partir des microorganismes hétérotrophes nécessite peu d'azote et une certaine teneur en éléments fertilisants,ainsi présent dans l’échantillon de L.S. En outre, l’utilisation des L.S pourrait avoir une valeur ajoutée pour la filière de traitement des eaux usées, en diminuant les charges d’effluents tout en les exploitant pour une production valorisante tel qu’ en Biodiesel. Une réduction de DBO est garantie etle risque de contamination de l’environnement local est atténué (DBO initial élevé environemental12-19 g/L).

Étude de l'effet du méthanol sur la production de biomasse et de lipides microbiens et le développement d'un procédé de fermentation non stérile à partir de glycérol coproduit par la filière de biodiesel

(Chapitre VI)

Le biodiesel est un biocarburant liquide produit à partir de l'huile usée de cuisson, des graisses animales, et d'autres huiles usagées considérées comme une alternative potentielle aux combustibles fossiles. Cependant, plusieurs études sont en cours, visant la production durable de biodiesel à partir de ressources renouvelables, les principaux paramètres à prendre en considération étant la charge et le coût d'exploitation. Parmi les activités opérationnelles galbant les charges économiques, on retrouve la stérilisation du réacteur qui est un processus exigeant et à très haute énergie. L'élimination de la stérilisation permettra de réduire potentiellement les couts totaux de la fermentation lipogène en culture pure. Dans la présente étude, un procédé de fermentation non stérile a été étudié.

Le méthanol est un alcool monocarboné, il est toxique pour les microorganismes à des concentrations élevées. Toutefois, certains microorganismes sont capables d'utiliser le méthanol comme source de carbone pour leur croissance, *Y.lipolytica* en est un exemple. Cependant, afin de prévenir la contamination au cours de la fermentation, une forte concentration de méthanol est nécessaire. Conjointement, cette concentration ne doit pas pour autant inhiber la croissance de *Y.lipolytica*. Pour ce faire, *Y.lipolytica* a été initialement mise en culture dans un milieu contenant différentes concentrations en méthanol à différentes concentrations (1, 2, 3, 4, et 5% v / v). Les données de la méthode d'UFC confirme une tolérance élevée de *Y.lipolytica* à 5% v / v.

En ajoutant des concentrations relatives à1 et 2% v / v de méthanol, la contamination a inévitablement apparu dès les 1ères heures de la fermentation. Toutefois, au-delà de 3% v / v de méthanol, nous avons pu retarder le risque de contamination, qui apparait après 96h de fermentation. Avec des concentrations plus élevées en méthanol 4 et 5% v / v de, aucune contamination n'a été perçue. Par conséquent, la concentration de 3% v / v de méthanol a été retenue comme résultat plausible de l'essai de fermentation non stérile à base de méthanol contenu dans le substrat de glycérol. Dans l'objectif d'étudier l'efficacité du méthanol comme élément de prévention et de contrôle de la contamination en fermentation, des essais à échelle bioréacteur ont été réalisées. Le milieu de fermentation a été préparé en utilisant l'eau du robinet

avec l'ajout de glycérol et d'autres nutriments. 3% v/v de méthanol a été ajouté au milieu comme agent de contrôle contre la contamination de culture pure de de *Y.lipolytica*. Les valeurs de l'UFC de *Y.lipolytica* augmentaient lentement et à 18h de la fermentation, des colonies contaminantes ont également été observées. En raison de la contamination, la concentration de la biomasse a diminué à 6 g/L et celle des lipides de 2,16 g/L.

L'étude à l'échelle du fermenteur montre que les risques d'une contamination sont plus élevées quand la fermentation est réalisée à grande échelle. Cela pourrait se produire pour deux raisons principales. 1) L'utilisation du méthanol par l'organisme réduit cette concentration et favorise donc la croissance des contaminants, ou 2) la perte par évaporation du méthanol en raison d'aération élevée est aussi probable. En outre, pour confirmer les raisons possibles de contamination, le glycérol brut a été dilué dans de l'eau stérile et de robinet séparément. Un test microbiologique de repicage sur gélose des deux échantillons de glycérol dilué nous a permis d'expliquer la source de contamination Il s'agit évidemment l'eau de robinet pour laquelle la culture sur gélose illustre la présence de colonies gluantes d'origine bactérienne.

Reliant l'étude antérieure, nous avons proposé à cet égard une nouvelle stratégie de prévention de la contamination. Pour cela, un traitement UV de 10 minutes a été utilisé pour la fermentation en gardant l'eau de robinet comme solution de dilution pour le glycérol. À cet effet, l'apparition de la contamination a été prolongée à 42h à l'échelle du fermenteur. En outre, la concentration de la biomasse a atteint 17,57g/L et celle des lipides indique 8,7 g/L. Comparée à la fermentation réalisée sans traitement aux UV, la concentration en biomasse a augmenté d'un facteur de 2, tandis que pour les lipides, une hausse de 4 fois a été observée. D'autres parts, le glycérol brut utilisé dans le présent projet contient une concentration élevée de savon. Pour cette raison, l'activité de la lipase a également été mesurée. Les résultats ont montré une activité lipase de l'ordre de 13,6U/mL à 36 h de fermentation. Cela pourrait favoriser la croissance et l'établissement du métabolisme lipogénique de *Y.lipolytica* en plus courtes durées.

Une approche biochimique pour l'optimisation de la production en biomasse et en lipides par l'usage de la biotine et l'apport intermittent de sulfate d'ammonium pour la fermentation lipogénique de *Y.lipolytica*

(Chapitre VII)

La croissance cellulaire de la levure et la production de lipides sont influencées par la concentration de carbone, d'azote et d'autres micro et macro-nutriments. Afin d'améliorer la production de lipides, des approches technologiques ont été étudiées telles que la manipulation génétique, modification biochimique des milieux de fermentation, le mode de fermentations etc… Dans le domaine du génie génétique, les possibilités d'obtenir des souches efficaces sont souvent limitées. Cependant, les souches sauvages peuvent être cultivées dans un milieu de fermentation conçu pour stimuler les conditions de lipogenèse microbienne.

Dans l'étude actuelle, une approche biochimique a été employée. Celle-ci consiste à ajouter de la biotine au milieu de fermentation pour améliorer la production de la biomasse et des lipides. L'addition de sels d'ammonium a également été étudiée. La biotine est connue comme l'un des facteurs importants pour la synthèse des lipides. Par conséquent, un supplément de biotine pourrait déclencher ou stimuler la lipogenèse. Afin d'évaluer cette hypothèse, les cellules ont été cultivées avec l'ajout d'un supplément de biotine à différentes concentrations de (10, 50 et 100 µg/L) dans le milieu de fermentation. Ainsi, il a été remarqué que la production de biomasse et des lipides concentrations augmentent avec l'augmentation de la concentration de la biotine. Lorsque 10 µg/L de biotine a été ajoutée dans le milieu de fermentation, les concentrations de la biomasse et des lipides ont été se retrouvent pratiquement identiques à celles de l'expérience témoin. Cependant, Pour 50 µg/L de biotine ajoutée, la concentration en lipides a augmenté. Finalement pour une concentration ajoutée de100 pg/L, la concentration en lipides se multiplie d'environ 2 fois.

L'expérience réalisée avec l'ajout de 100 µg/L de biotine a donc été reproduite dans un fermenteur de 15 L avec un contrôle (sans ajout de biotine). Les résultats de la biomasse obtenue est comparable dans les deux cas. Cela montre qu'il n'y a pas d'influence significative de la biotine sur la croissance du microorganisme. Ce qui pourrait être dû à la capacité de la levure à produire la biotine dans le but de synthétiser les lipides membranaires de la cellule microbienne. Cependant, dans le cas de la synthèse des lipides de réserves généralement retrouvés sous forme

de TAGs, une légère augmentation de la concentration en lipides a été observée dans les expériences réalisées avec ajout de biotine. La concentration en lipides ainsi extraits était de 16,16g/L et, 10,56g/L dans l'expérience contrôle. On peut en déduire alors que l'addition de la biotine pourrait améliorer la production de lipides chez les microorganismes oléagineux. Une addition d'azote intermédiaire a également été testée. Les résultats ont montré qu'il n'y a aucune augmentation significative de la concentration en biomasse comparée à l'expérience témoin et celle avec ajout de biotine. Cependant, elle s'avère légèrement plus élevée que la concentration de lipides retrouvés dans l'expérience contrôle.

La communauté microbienne indigène de différents échantillons de boues papetières brutes a été caractérisée par séquençage d'ADN génomique selon la méthode Next Generation des différentes boues de pâtes et papiers brutes et fermentées des eaux usées industrielles par séquençage d'ADN Méthode « Next Generation » (chapitre VIII). Une évaluation de la communauté microbienne des boues d'épuration et de l'industrie de pâtes et papier a été réalisée. Selon la diversité microbienne de cette matrice le potentiel de valorisation des BPPs (Boues de Pâtes et Papiers) a été évalué pour la production à valeur ajoutée telle que de différents produits à valeur ajoutée tels que les lipides, la cire, les biopolymères, les acides organiques et les enzymes industrielles. Les échantillons ont été prélevés à partir des effluents de trois procédés différents de fabrication de papiers (TMP- Thermo-mécanique, Kraft dépulpage et CTMP-thermochimique). L'ADN des boues a été isolé et soumis à un séquençage ARNr 16S (Miseq). Les séquences ont été traitées avec le logiciel FLASH (en utilisant une longueur de chevauchement minimale et maximale entre les deux lectures de 20 et 250 bp respectivement. Ensuite, la séquence fusionnée a également été analysée en utilisant le pipeline d'UPRASE (DRIVE5 Tiburon Californie).

Consécutivement, l'OUT (Unité taxonomique opérationnelle) a été construit et la classification taxonomique a été assignée par la base de données de références standards. Après analyses, on a constaté que 598 différents OTU étaient présents dans les trois échantillons de boues différentes. La diversité de chaque échantillon a été analysée sur la base de la raréfaction d'alpha. La présente analyse a montré une faible diversité microbienne (environ 100 OTU) dans les boues kraft et une haute diversité dans les boues CTMP (250 OTU). En outre, l'analyse phylogénétique des échantillons a été séparée en 4 nœuds différents. Le dendrogramme montre que les microbes ont

été distribués avec une composition pratiquement identique dans les trois procédés. Pour confirmer cela, le diagramme de Van a été réalisé. Les résultats ont montré que parmi 139 OTUs authentiques seulement 20 étaient communes dans les trois échantillons. Cela témoigne de la diversité microbienne dans les trois processus de dépulpage. En outre, sur la base du fichier taxa, les microbes ont été comparés avec la littérature disponible pour évaluer les applications possibles des microbes mixtes. Dans le cas de la production de lipides, la présence de *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Arthrobacter* a confirmé cette possibilité. En comparant ces quatre espèces, Acinetobacter est l'une des espèces les plus communes trouvées dans tous les échantillons de boues. De plus, les producteurs de biopolymères tels que Curvibacter, *Rubrivivax*, *Pseudomonas*, *Acinetobacter*, *Aquaspirillum*, *Comamonas*, *Zoogloea*, *Bacillus*, *Methylobacter*, *Micropurina*, *Cupriavidus* et *Enterobacter* ont été trouvés dans tous les échantillons de boues. En outre, le microbiome montre le potentiel des boues à produire les EPS (substances exopolymériques). La présence de *Rubrivivax*, *Pseudomonas*, *Acinetobacter*, *Zoogloea*, *Bacillus* et *Prevotella* confirme la possibilité de production d'EPS à partir des BPPs. En outre, les Actinobactéries, *Bacteroidetes*, *Acidobacteria*, *Firmicutes*, *Proteobacteria* et *Thermotogae* ont dévoilé des gènes putatifs responsables de la production de l'endo et exocellulase. Sur la base de l'étude actuelle, on peut conclure que les BPPs pourraient être une des ressources renouvelables appropriées pour générer des produits multiples avec un substrat unique sans préparation d'inoculum et de culture pure. Cependant, la quête de l'ingénierie pour ajuster des paramètres de fermentations selon les produits désirés aurait besoin d'une compréhension plus approfondie de la composition des boues ainsi que de la complexité d'interactions au sein du microbiome. Une analyse détaillée est présentée dans le manuscrit.

Références

ABGInc. 2007. Glycerin market analysis.

Adriana Gog, Marius Roman, Tos, M., Csaba Paizs , Irimie., F.D. 2012. Biodiesel production using enzymatic transesterification e Current state and perspectives. *Renewable Energy*, **39**, 10-16.

Ageitos, J.M., Vallejo, J.A., Veiga-Crespo, P., Villa, T.G. 2011. Oily yeasts as oleaginous cell factories. *Appl Microbiol Biotechnol*, **90**(4), 1219-27.

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**(1), 108.

Alvarez, H.M., Mayer, F., Fabritius, D., Steinb chel, A. 1996. Formation of intracytoplasmic lipid inclusions by Rhodococcus opacus strain PD630. *Arch Microbiol*, **165**, 377-386.

Alvarez, H.M., Steinbuchel, A. 2002. Triacylglycerols in prokaryotic microorganisms. *Appl Microbiol Biotechnol*, **60**(4), 367-76.

Amin Inhm, Mohammad A W, Markom M, Peng L C, N., H. 2010. Analysis of deposition mechanism during ultrafiltration of glycerin-rich solutions. *Desalination* **261**, 313-320.

André, A., Diamantopoulou, P., Philippoussis, A., Sarris, D., Komaitis, M., Papanikolaou, S. 2010. Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid. *Industrial Crops and Products*, **31**(2), 407-416.

Anschau, A., Xavier, M.C., Hernalsteens, S., Franco, T.T. 2014. Effect of feeding strategies on lipid production by Lipomyces starkeyi. *Bioresour Technol*, **157**, 214-22.

Asad-ur-Rehman., Wijesekara, R.G., 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), 1072.

Avila-Neto, P.M., da Silva, G.P., Lima, C.J.B., De Paula, F.C., Contiero, J. 2014. Evaluation and optimization of growth and citric acid production by yarrowia lipolytica nrrl y-1095 using glycerol as carbon source as an alternative to use biodiesel byproduct. *Journal of Experimental Biology and Agricultural Sciences*, **2**(1), 25-31.

Ayoub, M., Abdullah, A.Z. 2012. Critical review on the current scenario and significance of crude glycerol resulting from biodiesel industry towards more sustainable renewable energy industry. *Renewable and Sustainable Energy Reviews*, **16**(5), 2671-2686.

Azocar, L., Ciudad, G., Heipieper, H.J., Navia, R. 2010. Biotechnological processes for biodiesel production using alternative oils. *Appl Microbiol Biotechnol*, **88**(3), 621-36.

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.

Bati, N., Hammond, E.G., Glatz, B.A. 1984. Biomodification of fats and oils: trialswith Candida lipolytica,. *J. Am. Oil Chem. Soc*, **61**, 1743-1746.

BCCresearch. 2012. Continuous Glucose Monitoring (CGM): Technologies and Global Markets.

Beligon, V., Poughon, L., Christophe, G., Lebert, A., Larroche, C., Fontanille, P. 2015. Improvement and modeling of culture parameters to enhance biomass and lipid production by the oleaginous yeast Cryptococcus curvatus grown on acetate. *Bioresour Technol*, **192**, 582-91.

Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.L., Molina-Jouve, C., Nicaud, J.M. 2009a. Yarrowia lipolytica as a model for bio-oil production. *Prog Lipid Res*, **48**(6), 375-87.

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

Berg, J.M., Tymoczko, J., Stryer, L. 2002. Acetyl Coenzyme A Carboxylase Plays a Key Role in Controlling Fatty Acid Metabolism. *Biochemistry,NCBI Bookshelf*, **5**.

Buchan, A., Newell, S.Y., Moreta, J.I.L., Moran, M.A. 2002. Analysis of internaly transcribed spacer (ITS) regions of rRNA genes of fungal communities in southern U S salt marsh. *Microbial ecology*, **43**, 329-340.

Carol, Z.B., Anne, C.-S., John, C.W., Grover, L.W. 1999. The Biotin Domain Peptide from the Biotin Carboxyl Carrier Protein of Escherichia coli Acetyl-CoA Carboxylase Causes a Marked Increase in the Catalytic Efficiency of Biotin Carboxylase and Carboxyltransferase Relative to Free Biotin*. *The journal of biological chemistry*, **247**(45), 31767–31769.

Carraretto, C., Macor, A., Mirandola, A., Stoppato, A., Tonon, S. 2004. Biodiesel as alternative fuel: experimental analysis and energetic evaluations. *Energy*, **29**, 2195-2211.

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

Chang, Y.-H., Chang, K.-S., Hsu, C.-L., Chuang, L.-T., Chen, C.-Y., Huang, F.-Y., Jang, H.-D. 2013. A comparative study on batch and fed-batch cultures of oleaginous yeast Cryptococcus sp. in glucose-based media and corncob hydrolysate for microbial oil production. *Fuel*, **105**, 711-717.

Chaudhary., N., Ngadi, M.O., Simpson, B. 2012. comparison-of-glucose-glycerol-and-crude-glycerol-fermentation-by-escherichia-coli K12. *J Bioproces Biotechniq*(S-1).

Cheirsilp, B., Louhasakul, Y. 2013. Industrial wastes as a promising renewable source for production of microbial lipid and direct transesterification of the lipid into biodiesel. *Bioresour Technol*, **142**, 329-37.

Choi, W.J., M.R, H., Chan , W.H., Yeo, S.S. 2011. Ethanol production from biodiesel-derived crude glycerol by newly isolated Kluyveracryocrescens. *Applied Microbial biotechnology* **89**(4), 1255-1264.

Damen, B. Bioenergy and food security in ASEAN:An FAO perspective.

Demirbas, A. 2009. Progress and recent trends in biodiesel fuels. *Energy Conversion and Management*, **50**(1), 14-34.

Dennis Y.C. Leung , Xuan Wu, Leung, M.K.H. 2010. A review on biodiesel production using catalyzed transesterificatio. *Applied Energy* **87**, 1083–1095.

Denver j. Pyle, Rafael a. Garcia, wen, Z. 2008. Producing docosahexanoic acid-rich algae from biodiesel derived-crude glycerol. *j.Agric. Food Chem*, **56**, 3933–3939

Fickers, P., Benetti, P.H., Wache, Y., Marty, A., Mauersberger, S., Smit, M.S., Nicaud, J.M. 2005. Hydrophobic substrate utilisation by the yeast Yarrowia lipolytica, and its potential applications. *FEMS Yeast Res*, **5**, 527-543.

Fisher, D.J., Fernandez, R.E., Adams, N.E., Maurelli, A.T. 2012. Uptake of biotin by Chlamydia Spp. through the use of a bacterial transporter (BioY) and a host-cell transporter (SMVT). *PLoS One*, **7**(9), e46052.

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.

Fox, P.F. 2003. Significance of Indigenous Enzymes in Milk and Dairy ProductsSignificance of Indigenous Enzymes in Milk and Dairy Products. *Hand book of food enzymology*, 2-47.

Freedman, B., Butterfield, R., Pryde, E. 1986. Transesterification kinetics of soybean oil. *J Am Oil Chem Soc*, **63**.

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

Gao, D., Zeng, J., Zheng, Y., Yu, X., Chen, S. 2013. Microbial lipid production from xylose by Mortierella isabellina. *Bioresour Technol*, **133**, 315-21.

Ghosh, P., Ghose, T.K. 2003. Bioethanol in India: recent past and emerging future. *Adv. Biochem. Eng. Biotechnol*, **85**(1-27), 1.

Gong, Z., Wang, Q., Shen, H., Hu, C., Jin, G., Zhao, Z.K. 2012. Co-fermentation of cellobiose and xylose by Lipomyces starkeyi for lipid production. *Bioresour Technol*, **117**, 20-4.

Gonzalez-Pajuelo, M, M.-S., I, Mendes, F., Andrade, J.C., Vasconcelos, I., Soucaille, P. 2005. Metabolic engineering of Clostridium acetobutylicum for the industrial production of 1,3-propanediol from glycerol. *Metabolic Engineering*, **7**(5), 329-336.

Gwendoline Christophe, Vinod Kumar, Régis Nouaille, Geneviève Gaudet, PierreFontanille, Ashok Pandey, Carlos Ricardo Soccol, Larroche., C. 2012. Recent Developments in Microbial Oils Production: a Possible Alternative to Vegetable Oils for Biodiesel Without Competition with Human Food? *Brazilian archives of biology and technology*, **55**(1), 29-46.

Huang, C., Wu, H., Liu, Z.J., Cai, J., Lou, W.Y., Zong, M.H. 2012. Effect of organic acids on the growth and lipid accumulation of oleaginous yeast Trichosporon fermentans. *Biotechnol Biofuels*, **5**, 4.

Huang, C., Zong, M.H., Wu, H., Liu, Q.P. 2009. Microbial oil production from rice straw hydrolysate by Trichosporon fermentans. *Bioresour Technol*, **100**(19), 4535-8.

Huang, X., Wang, Y., Liu, W., Bao, J. 2011. Biological removal of inhibitors leads to the improved lipid production in the lipid fermentation of corn stover hydrolysate by Trichosporon cutaneum. *Bioresour Technol*, **102**(20), 9705-9.

Huijberts, P.A.E.P.M.á.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.

Jin, G., Zhang, Y., Shen, H., Yang, X., Xie, H., Zhao, Z.K. 2013. Fatty acid ethyl esters production in aqueous phase by the oleaginous yeast Rhodosporidium toruloides. *Bioresour Technol*, **150C**, 266-270.

K. Bélafi-Bakó, F. Kovács, L. Gubicza, Hancsók, J. 2002. Enzymatic Biodiesel Production from Sunflower Oil by Candida antarctica Lipase in a Solvent-free System. *Biocatalysis and Biotransformation*, **20**(6), 437-439.

Karatay, S.E., Donmez, G. 2010. Improving the lipid accumulation properties of the yeast cells for biodiesel production using molasses. *Bioresour Technol*, **101**(20), 7988-90.

Kurosawa, K., Boccazzi, P., de Almeida, N.M., Sinskey, A.J. 2010. High-cell-density batch fermentation of Rhodococcus opacus PD630 using a high glucose concentration for triacylglycerol production. *J Biotechnol*, **147**(3-4), 212-8.

Kuttiraja, M., Krishna, S., Dhouha, A., Tyagi, R.D. 2015. A substrate-based approach for the selection of oil-bearing heterotrophs from nitrogen-deficient soil for lipid production. *Appl Biochem Biotechnol*, **175**(4), 1926-37.

Li Deng, Xuebing Xu, G., G., Haraldsson, Tianwei Tan, Wang, F. 2005. Enzymatic Production of Alkyl Esters Through Alcoholysis: A Critical Evaluation of Lipases and Alcohols. *JAOCS*, **82**(5), 341-347.

Li, L., Du, W., Liu, D., Wang, L., Li, Z. 2006. Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. *Journal of Molecular Catalysis B: Enzymatic*, **43**(1-4), 58-62.

Li, Q., Du, W., Liu, D. 2008. Perspectives of microbial oils for biodiesel production. *Appl Microbiol Biotechnol*, **80**, 749-756.

Li Y-h, L.B., Sun Y, Z-b Z, F-w B 2005. Screening of oleaginous yeasts for broad-spectrum carbohydrates assimilating capacity. *Chin J Biotechnol* **25**, 43-48.

Li, Y., Zhao, Z., Bai, F. 2007. High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture. *Enzyme 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**(19), 7581-6.

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**(4), 184-8.

Ling, J., Nip, S., Cheok, W.L., de Toledo, R.A., Shim, H. 2014. Lipid production by a mixed culture of oleaginous yeast and microalga from distillery and domestic mixed wastewater. *Bioresour Technol*, **173**, 132-9.

Liu, X., Mortensen, U.H., Workman, M. 2013. Expression and functional studies of genes involved in transport and metabolism of glycerol in Pachysolen tannophilus. *Microb Cell Fact*, **12**, 27-36.

Liu, Y., Wang, Y., Liu, H., Zhang, J. 2015. Enhanced lipid production with undetoxified corncob hydrolysate by Rhodotorula glutinis using a high cell density culture strategy. *Bioresour Technol*, **180**, 32-9.

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**(7), 2351-8.

Marilyn G Wiebe, Kari Koivuranta, Penttilä, M., Ruohonen, L. 2013. Lipid production in batch and fed-batch cultures of Rhodosporidium toruloides from 5 and 6 carbon carbohydrates. *AMB Express*, **3**(58), 1-9.

McCoy, M. 2005. An unlikely impact: growth of biodiesel has big implications for the oleochemical industry. *Chem Eng News*, **83**(8), 19-20.

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

Mhairi Workman, Philippe Holt, Thykaer, J. 2013. Comparing cellular performance of Yarrowia lipolytica during growth on glucose and glycerol in submerged cultivations. *AMB Express*, **3**(58), 1-9.

Mohan, G.V.S.S.V. 2014. Sustainable biodiesel production through bioconversion of lignocellulosic wastewater by oleaginous fungi. *Biomass Conv. Bioref.*

Munch, G., Sestric, R., Sparling, R., Levin, D.B., Cicek, N. 2015. Lipid production in the under-characterized oleaginous yeasts, Rhodosporidium babjevae and Rhodosporidium diobovatum, from biodiesel-derived waste glycerol. *Bioresour Technol*, **185**, 49-55.

Naganuma, T., Uzuka, Y., Tanaka, K. 1985. Physiological factors affecting total cell number and lipid content of the yeast, Lipomyces starkeyi *J Gen Appl Microbiol*, **31**, 29-37.

Oliveira, A.C., Rosa, M.F. 2006. Enzymatic Transesterification of Sunflower Oil in an Aqueous-Oil Biphasic System. *JAOCS*, **83**(1), 21-25.

Papanikolaou, S., Aggelis, G. 2002. Lipid production by Yarrowia lipolytica growing on industrial glycerol in a single-stage continuous culture. *Bioresour Technol*, **82**(1), 43-9.

Papanikolaou, S., Aggelis, G. 2011. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. *European Journal of Lipid Science and Technology*, **113**(8), 1031-1051.

Papanikolaou, S., Chatzifragkou, A., Fakas, S., Galiotou-Panayotou, M., Komaitis, M., Nicaud, J.-M., Aggelis, G. 2009. Biosynthesis of lipids and organic acids by Yarrowia lipolytica strains cultivated on glucose. *European Journal of Lipid Science and Technology*, **111**(12), 1221-1232.

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., Marc, I. 2002. Yarrowia lipolytica as a potential producer of citric acid from raw glycerol. *Journal of Applied Microbiology*, **92**, 737-744.

Patel, A., Pravez, M., Deeba, F., Pruthi, V., Singh, R.P., Pruthi, P.A. 2014. Boosting accumulation of neutral lipids in Rhodosporidium kratochvilovae HIMPA1 grown on hemp (Cannabis sativa Linn) seed aqueous extract as feedstock for biodiesel production. *Bioresour Technol*, **165**, 214-22.

Poli, J.S., da Silva, M.A., Siqueira, E.P., Pasa, V.M., Rosa, C.A., Valente, P. 2014. Microbial lipid produced by Yarrowia lipolytica QU21 using industrial waste: a potential feedstock for biodiesel production. *Bioresour Technol*, **161**, 320-6.

Ramalingam, S., Stephen, D., Mark, Z., Bajpai., R. 2010. Microbial lipids from renewable resources: production and characterization *J Ind Microbiol Biotechnol*, **37**, 1271-1287.

Ratledge, C., Wynn, J.P. 2002. The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms. *Advances In Applied Microbiology*, **51**.

Ruan, Z., Zanotti, M., Wang, X., Ducey, C., Liu, Y. 2012. Evaluation of lipid accumulation from lignocellulosic sugars by Mortierella isabellina for biodiesel production. *Bioresour Technol*, **110**, 198-205.

Rumbold, K., van Buijsen, H.J., Overkamp, K.M., van Groenestijn, J.W., Punt, P.J., van der Werf, M.J. 2009. Microbial production host selection for converting second-generation feedstocks into bioproducts. *Microb Cell Fact*, **8**, 64.

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 Biochemistry*, **46**(1), 210-218.

Shuib, S., Nawi, W.N., Taha, E.M., Omar, O., Kader, A.J., Kalil, M.S., Hamid, A.A. 2014. Strategic feeding of ammonium and metal ions for enhanced GLA-rich lipid accumulation in Cunninghamella bainieri 2A1. *ScientificWorldJournal*, **2014**, 173574.

Sriwongchai, S. 2014. Batch and Fed-Batch Cultivations of Oleaginous Bacterium Rhodococcus erythropolis for Lipid Production Using Glucose Medium with Auxiliary Nitrogen Source. *IPCBEE*, **70**(19), 99-103.

Stelmachowski, M. 2011. Utilization of glycerol, a by-product of the transestrification process of vegetable oils: A review. *Ecological Chemistry And Engineering S*, **18**(1), 9-30.

Streit, W.R., Entcheva, P. 2003. Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production. *Appl Microbiol Biotechnol*, **61**(1), 21-31.

Tan, H.W., Abdul Aziz, A.R., Aroua, M.K. 2013. Glycerol production and its applications as a raw material: A review. *Renewable and Sustainable Energy Reviews*, **27**, 118-127.

Thompson, J.C., He, B.B. 2006. Characterization of crude glycerol from biodiesel production from multiple feedstocks. *Appl. Eng. Agric*, **22**, 261-265.

Tsigie, Y.A., Wang, C.Y., Kasim, N.S., Diem, Q.D., Huynh, L.H., Ho, Q.P., Truong, C.T., Ju, Y.H. 2012. Oil production from Yarrowia lipolytica Po1g using rice bran hydrolysate. *J Biomed Biotechnol*, **2012**, 378384.

Tsigie, Y.A., Wang, C.Y., Truong, C.T., Ju, Y.H. 2011. Lipid production from Yarrowia lipolytica Po1g grown in sugarcane bagasse hydrolysate. *Bioresour Technol*, **102**(19), 9216-22.

Tsobanakis, P. 2007. Cargill Incorporate, presented at biofuels and feed stocks,Indonesia.

Uckun Kiran, E., Trzcinski, A., Webb, C. 2013. Microbial oil produced from biodiesel by-products could enhance overall production. *Bioresour Technol*, **129**, 650-4.

Vajpeyi, S., Chandran, K. 2015. Microbial conversion of synthetic and food waste-derived volatile fatty acids to lipids. *Bioresour Technol*, **188**, 49-55.

Valerie Dossat, Didier Combes, Marty, A. 2002. Lipase-catalysed transesterification of high oleic sunflower oil. *Enzyme and Microbial Technology* **30**, 90–94.

Walker, G.M., Chichester, J.R. 1997. Yeast Physiology and Biotechnology. *John Wiley and Sons*.

Wang, G.Y., Chi, Z., Song, B., Wang, Z.P., Chi, Z.M. 2012. High level lipid production by a novel inulinase-producing yeast Pichia guilliermondii Pcla22. *Bioresour Technol*, **124**, 77-82.

Wang, L., Du, W., Liu, D., Li, L., Dai, N. 2006. Lipase-catalyzed biodiesel production from soybean oil deodorizer distillate with absorbent present in tert-butanol system. *Journal of Molecular Catalysis B: Enzymatic*, **43**(1-4), 29-32.

Wei Du, Yuanyuan Xu, Liu, D. 2003. Lipase-catalysed transesterification of soya bean oil for biodiesel production during continuous batch operation. *Biotechnol. Appl. Biochem*, **38**, 103-106.

Wei, Y., Zheng, C., Ning, Z. 2008. Research progress of surfactants as fermentation accelerator. *Chem. Ind. Eng. Prog*, **27**, 983-989.

Woon-Yong, C., Sung-Ho Oh, Yong-Chang Seo, Ga-Bin Kim, Do-Hyung Kang, Shin-Young Lee, Kyung-Hwan Jung, Jeong-Sub Cho, Ju-Hee Ahn, Geun-Pyo Choi, Lee, H.-Y. 2011. Effects of Methanol on Cell Growth and Lipid Production from Mixotrophic Cultivation of Chlorella sp. *Biotechnology and Bioprocess Engineering* **16**, 946-955.

Wynn, J.P., Aidil bin Abdul Hamidt, Ratledge, C. 1999. The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. *Microbiology*, **145**, 1911-1917.

Wynn, J.P., Ratledge, C. 2005. Oils from microorganisms. *Bailey's industrial oil and fat products*, **6**(6).

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. *Biochemical Engineering Journal*, **65**, 30-36.

Xue, F., Gao, B., Zhu, Y., Zhang, X., Feng, W., Tan, T. 2010. Pilot-scale production of microbial lipid using starch wastewater as raw material. *Bioresour Technol*, **101**(15), 6092-5.

Yang, X., Jin, G., Gong, Z., Shen, H., Song, Y., Bai, F., Zhao, Z.K. 2014. Simultaneous utilization of glucose and mannose from spent yeast cell mass for lipid production by Lipomyces starkeyi. *Bioresour Technol*, **158**, 383-7.

Yang, X., Jin, G., Wang, Y., Shen, H., Zhao, Z.K. 2015. Lipid production on free fatty acids by oleaginous yeasts under non-growth conditions. *Bioresour Technol*, **193**, 557-62.

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**(1), 71-4.

Yousuf, A. 2012. Biodiesel from lignocellulosic biomass-prospects and challenges. *Waste Manag*, **32**(11), 2061-7.

Zhao, X., Kong, X., Hua, Y., Feng, B., Zhao, Z.K. 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.

Zhu, L.Y., Zong, M.H., Wu, H. 2008. Efficient lipid production with Trichosporon fermentans and its use for biodiesel preparation. *Bioresour Technol*, **99**(16), 7881-5.

Chapter II

Review Article

The recent trends in carbon capturing from classical carbon sources and waste by-products for lipid production- A review

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RÉSUMÉ

Les progrès récents dans la production d'huile microbienne ont ouvert une alternative prometteuse pour pétrole- diesel. L'amélioration de la production d'huile microbienne (SCO) a conduit à la réduction des gaz à effet de serre (GES) et, par conséquent, la réduction du réchauffement de la planète. Cela a amélioré aussi la sécurité énergétique de manière renouvelable et durable dans le long terme. Cependant, pour remplacer l'essence et le diesel, un processus industriel rentable et réussi pour la production d'huile microbienne n'a pas encore été développé jusqu'à présent. Dans le présent cas de figure, de nombreux organismes lipogéniques ayant une capacité d'accumulation des lipides de plus de 20 à 80% en poids / poids ont été rapportés dans la littérature. En dépit de la grande capacité d'accumulation de lipide par les microorganismes, la productivité globale des lipides a besoin d'une amélioration significative. Afin d'améliorer la productivité et la biomasse lipidique, plusieurs approches ont été utilisées. L'objectif principal de cette recherche est de trouver des sources de carbone et de nutriment peu cher pour la production de lipide. Deuxièmement, la génie génétique ou une approche d'ingénierie biochimique en vue de renforcer la capacité d'accumulation de lipides a été étudiée. Bien que les approches génétiques et biochimiques puissent accroître la teneur en lipides des cellules, le coût de la production d'huile microbienne réside encore dans le coût des matières premières (le carbone et d'autres éléments nutritifs). Pour cette raison, la présente étude a porté sur la compréhension et l'importance de la biochimie de la production de lipide à partir des différentes sources de carbone disponible pour la production de lipide microbien.

Mots clés : biodiesel, lipides, huile de cellule unique, le glycérol, Triacylglycérol.

Abstract

The recent advancement in the microbial oil production has opened a promising alternative for petrol- diesel. The improvement in microbial oil (SCO) production leads to the reduction of greenhouse gas (GHG) emission and thereby it reduces the global warming. It improves energy security in a renewable and sustainable manner for long-term. However, to replace the petrol-diesel a successful industrial process from microbial oil production has not been developed so far. In the present scenario, many lipogenic organisms with a capacity of lipid accumulation more than 20 to 80 %w/w were reported in the literature. In spite of high lipids accumulating capacity of microorganisms, the overall lipid productivity needs significant improvement. To enhance the lipid and biomass productivity, several approaches have been employed. The primary focus of the on-going research has been concentrated to find cheap carbon and nutrient sources for lipid production. Secondly, the genetic engineering or biochemical engineering approach to enhance the capacity of lipid accumulation has been studied. Though the genetic and biochemical approaches can enhance the lipid content of cells the cost of the microbial oil production still lies in the raw material cost (carbon and other nutrients). Due to this reason, the present review focused on understanding and importance of biochemistry of lipid production from different sources of carbon available for microbial lipid production.

Keywords; biodiesel, lipid, single cell oil, glycerol, Triacylglycerol.

1. INTRODUCTION

Rudolph Diesel's idea of using vegetable oil as fuel in diesel engine changed the domain of transportation in the 1890s. Consecutively, peanut oil as diesel fuel was demonstrated in World fair of 1900 by the French government. In earlier times pure vegetable oil was used for generating power for the locomotives in rural African countries (Huang et al., 2009). Discovery of crude oil led to industrialization and changed the fate of the world economy. Since the discovery of crude oil, it has been accredited as the main commodity serving the energy needs of the transportation and energy sector until date. Although alternative sources of energy (fuel ethanol, hydrogen) have been explored, an absolute alternative for petro fuel oil is only under developmental/demonstration stages. Rapid industrialization and urbanization have led to increasing demands for oil as a major source of energy and this uncontrolled use of hydrocarbon leads to the release of greenhouse gasses, eventually resulting in global warming. Due to these detrimental reasons, the release of greenhouse gasses into the environment is strictly regulated by different world organizations. Meanwhile, the use of fuel oil is unavoidable since it plays a crucial role in the present world. Rising demand for non-renewable fossil fuels leads to its depletion and is causing worldwide concern for the substitution of petroleum with effective renewable alternatives (Beligon et al., 2015). These renewable alternatives to fossil fuel must necessarily address the rapid depletion of oil reserves and also the increasingly negative impact on the environment. From an energy and environmental perspective, second and third generation fuels from renewable and sustainable resources are promising.

One of the approaches widely discussed for addressing these challenges is the bioconversion of biosolids or industrial wastes to lipids by microbial fermentation (Cheirsilp & Louhasakul, 2013). One of the primary issues when using industrial by-products for bioconversion is the acquiring of microbial hosts capable of tolerating batch-to-batch variations and the impurities found in this co-product (Choi et al., 2011; Fontanille et al., 2012). When screening for suitable microbial hosts for the bioconversion of industrial feedstock, a substrate-based approach rather than a product-oriented approach has been proposed as a way to improve the success of the process (Rumbold et al., 2009). Considering the same strategy of screening, microorganisms with natural abilities to use crude feedstock can be identified. This, in turn, will help to avoid the need to engineer microorganisms and adapt them to use these co-products.

Table 10 Lipogenic organisms and their corresponding lipid percentages

Organism	Substrate	Biomass (g/L)	Lipid %(w/w)	Reference
Y.lipolytica	Industrial glycerol	8.1	43	(Papanikolaou & Aggelis, 2002)
R toruloides	Glucose	11.2	62.2	(Wu et al., 2010)
C curvatus	Crude glycerol	39.2	52	(Liang et al., 2010)
R toruloides	Glucose	15.5	57.3	(Wu et al., 2011)
L starkeyi	Cellobiose + Xylose	25.2	52	(Gong et al., 2012)
P guilliermondii	inuline	20.4	60.6	(Wang et al., 2012)
R toruloides	Crude glycerol	35.3	46	(Esra et al., 2013)
R toruloides	Levoglucosan	6.8	39.7	(Lian et al., 2013)
M isabellina	Xylose	28.8	64.2	(Gao et al., 2013)
T fermentans	Sweet potato vines + glycerol	17.9	41.5	(Shen et al., 2013)
R glutinis	Glucose	86.2	26.7	(Zhang et al., 2014b)
L starkeyi	Glucose + Xylose	61.9	29.3	(Anschau et al., 2014)
L starkeyi	Glucose + mannose	22	58.3	(Yang et al., 2014)
Y.lipolytica	Glycerol	4.92	30.1	(Poli et al., 2014)
R babjevae	Crude glycerol	9.4	34.9	(Munch et al., 2015)
R diobovatum	Crude glycerol	12.0	63.7	
C curvatus	Acetate	80	60	(Beligon et al., 2015)
C curvatus	Fatty acids	13.3-17.1	65-75.3	(Yang et al., 2015)
C ehinulata	Glucose	15	46	(Fakas et al., 2009)
M isabellina	Glucose	27	44.6	(Fakas et al., 2009)
A awamori				(Mohan, 2014)
A niger	Crude glycerol			(André et al., 2010)
M isabellina	Xylose	28.8	64.2	(Gao et al., 2013)
L.starkeyi	Glucose	104.6	64.9	(Lin et al., 2011)
R erythropolis	Glucose	4.7	42.5	(Sriwongchai, 2014)
R opacus	Glucose	77.6	38	(Kurosawa et al., 2010)
Nocardia				(Alvarez & Steinbuchel, 2002)

However, several microbes have already been identified as potential candidates for producing lipids for biodiesel production, yet limited studies have been conducted and there is a need for improvement from an industrial perspective (Beligon et al., 2015). Similar to other biofuel generation, the bioconversion of carbon to lipids also has several bottlenecks to overcome. Bottlenecks such as identification of a potent strain for lipid accumulation, replacement of

expensive substrates, and replacement of nitrogen sources with low or zero cost organic waste materials and efficient lipid extraction methods must be addressed. On the other hand, the biochemical events catalyzed by ACL (ATP citrate lyase) play a crucial role in the biosynthesis of lipids in microbes. The role of ACL has been studied well by various authors (Ratledge & Wynn, 2002), but very little work has been carried out based on a biochemical approach to increase the activity of the enzymes, namely ACL, ACC (Acetyl-CoA Carboxylase), and malic dehydrogenase in microorganisms with respect to biodiesel application.

Technically biodiesel is defined as esters of fatty acids (FAME) produced by catalytic reaction of oil with short chain alcohols preferably mono chain alcohols (methanol or ethanol) a suitable alternative to petrodiesel fuel and acceptable for being environment friendly, ease of availability, transportation, direct use in diesel engines without any modification, reduction in greenhouse gases (GHGs), biodegradability, less volatile and non-toxic (Carraretto et al., 2004; Demirbas, 2009; Li et al., 2008).

2. Oil-bearing microbes and oleagenicity

Lipids are one of the important cell wall components and all the microorganisms synthesize necessary fractions of lipids to meet the requirements of cell wall synthesis. Organisms that accumulate more than 20% lipid of cell dry weight are known as oil-bearing microbes (Wynn & Ratledge, 2005). Microbes generate lipids under elevated carbon concentration with one or more nutrient-limited conditions and these are stored as triacylglycerides (TAG) in lipid bodies (Papanikolaou & Aggelis, 2011; Ratledge & Wynn, 2002). Though microbes are reported to store lipids; an appropriate fermentation process of microbial oil production is not yet used at an industrial scale. On the other hand plant oil followed by animal fat is widely used for biodiesel production. Algal oil and cooking oil are also used for the same to a certain extent. The use of plant oil or food grade oil has a negative impact on the socioeconomic sector and the cultivation of energy crops reduces the area of land available for cultivation of food crops (Damen; Xu et al., 2012). There are several problems associated with the use of animal fat (Yellow grease) and used cooking oil for biodiesel production, namely; high free fatty acid (FFA) content, sulfur content, food particles, collection, storage and others. Therefore, the urge in fuel grade oil production extended to the search for renewable and sustainable resources. Compared to plant and other oil resources, microbial oil is ideal for biodiesel production for being similar in

composition to TAG and high value added carboxylic acids such as Gama linolenic acid (GLA) can also be produced. Though all categories of microbes accumulate oil, in this review yeast has been emphasized due to its fast growth and high lipid accumulation capacity.

Although several heterotrophic microorganisms, including yeast and fungi, have been identified to accumulate comparable amount of lipids, only about 30 out of 600 organisms were reported to store more than 20% of TAGA (Ratledge & Wynn, 2002). The range of oil accumulation differs from 20 to 85% in different classes of organisms (Wynn & Ratledge, 2005). The oil-producing organism's genera include Basidiomycota (*Trichosporan*), Ascomycota (*Y. lipolytica*) and Zygomycota (*Mortirella)*. Table-10 lists the selected organisms reported accumulating lipids as energy reserve materials.

Oleagenicity of an organism depends on the presence of metabolism that can convert the extra carbon into TAGA by a series of biochemical events. The main difference of oil-bearing organisms from a non-oil harboring organism is the presence of ACL (ATP Citrate Lyase) (Beopoulos et al., 2009b). At the same time, there exist some organisms reported to have ACL and yet are incapable of accumulating lipids owing to the absence of consecutive enzymes in the TAG pathway. When other (ACC Acetyl-CoA Carboxylase and ME Malic enzyme) enzymes are inactive in the pathway the organisms synthesize (accumulate) citric acid due to the continuous accumulation of citric acid in the cytoplasm.

The typical pattern of lipid accumulation is expressed in Figure 6. At the onset of nitrogen limitation, lipid accumulation begins with the activation of lipogenic enzyme pool. The initial growth phase lasts for about 20h and then the organism undergoes a transition phase where excess carbon can be converted into citrate, which then results in lipid synthesis. Meantime the citric acid production also depends on the media composition and the C/N ratio during fermentation. That is, the fermentation can shift from citric acid to lipid production or only citric acid production or vice versa depends on the media composition and cultivation condition. In the initial steps of the process, while the enzyme isocitrate dehydrogenase is inactivated, AMP deaminase is activated. This brings about a rapid decrease in intracellular AMP. AMP deaminase catalyzes the following reaction (Beopoulos et al., 2009b; Ratledge & Wynn, 2002; Wynn & Ratledge, 2005).

$$AMP \longrightarrow IMP + NH^{+}_{4} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Activation of AMP deaminase results in scavenging the intracellular AMP. The reduction in AMP further affects the activation of ICD (isocitrate dehydrogenase. Thus the cell starts to accumulate the isocitrate and the isocitrate is equilibrated by aconitase to citric acid.

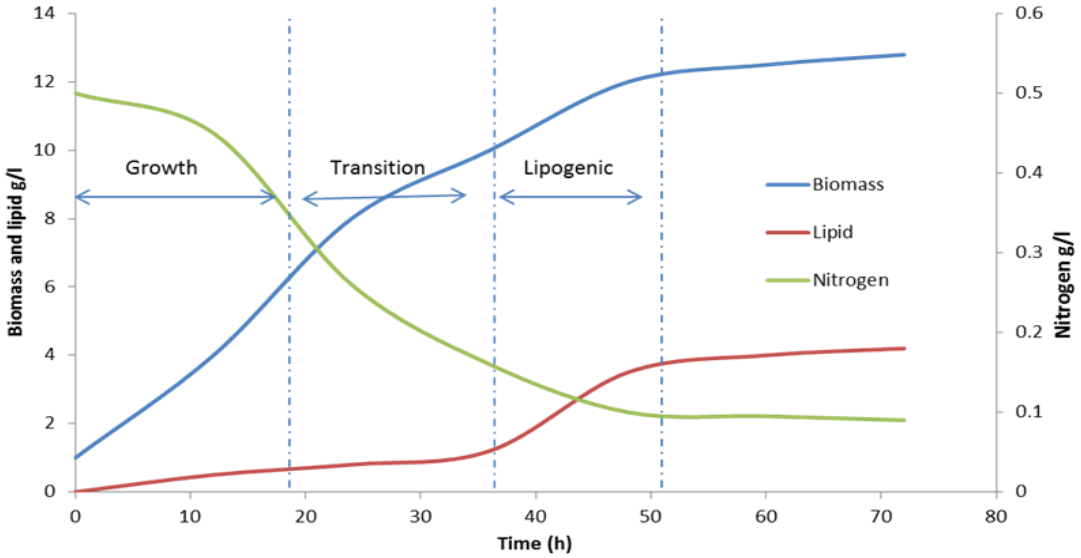

Figure 6 Stages of lipid accumulation in nitrogen deficient fermentation media (Kuttiraja 2015)

Understanding the biochemistry of TAG synthesis (under different culture conditions) holds the key to developing an economically viable process design for the production of microbial oil. It determines the amount of substrate that can be metabolized by the organism and the consecutive synthesis of TAG. Figure 7 explains the biochemical events involved in TAG synthesis from glucose and glycerol. The key enzymes involved in TAG synthesis are, ATP citrate lyase, Acetyl-CoA carboxylase (ACC), and malic enzymes. The other associated enzymes are AMP deaminase, Acyl-CoA oxidase, elongate, etc. (Ratledge & Wynn, 2002; Wynn & Ratledge, 2005). TAG synthesis begins when AMP deaminase activity increases. Due to insufficient supply of nitrogen for cell metabolism; AMP is cleaved by AMP deaminase to provide nitrogen for cell metabolism in mitochondria. This perturbs synthesis of α-ketoglutarate in the citric acid cycle. Since isocitrate dehydrogenase activity is perturbed, iso-citrate level in the mitochondria elevates; the increase in isocitrate levels is balanced by the enzyme aconitase, which equilibrates the isocitrate by converting it to citrate. Citrate is further transported to the cytoplasm where it accumulates.

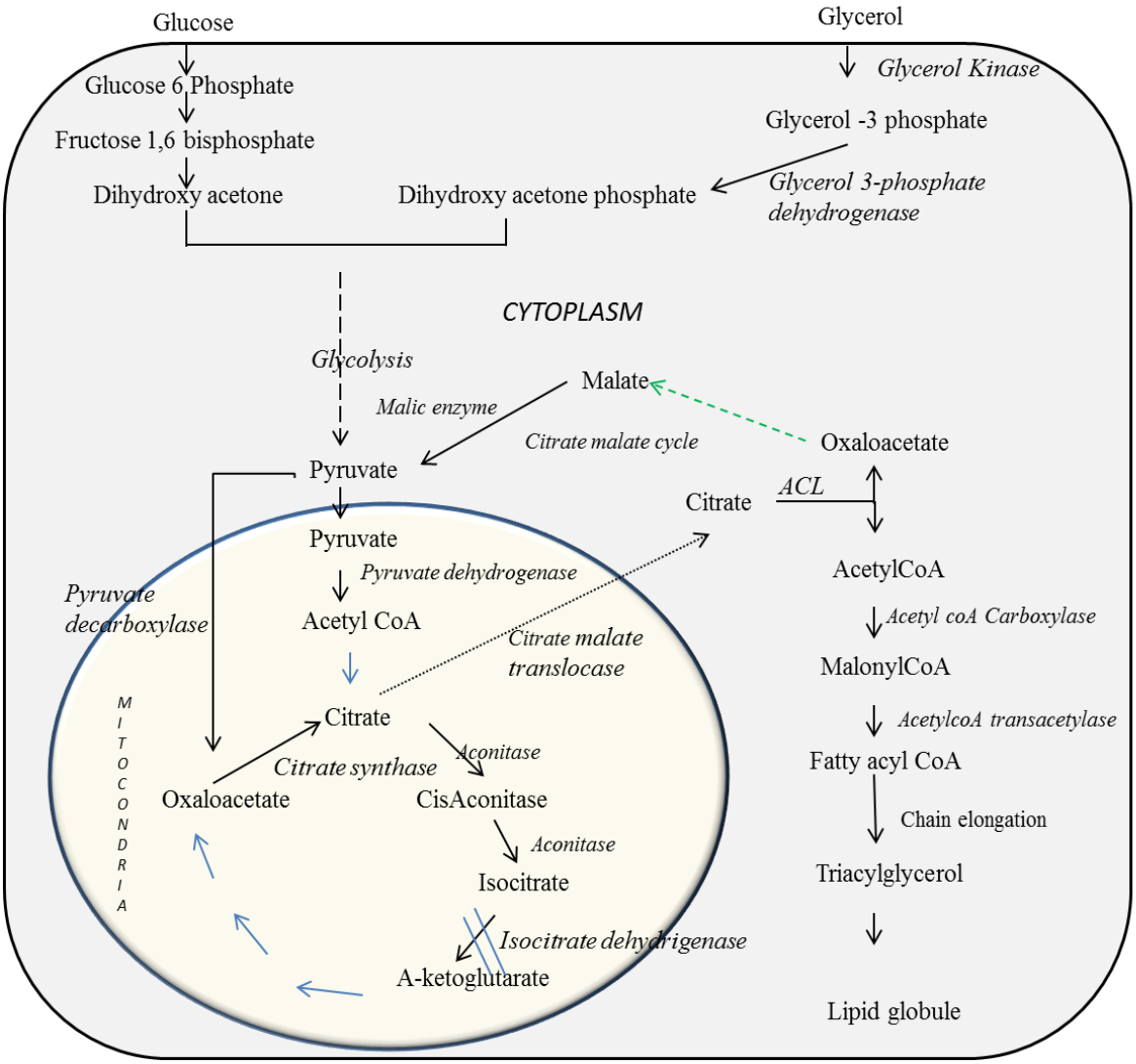

Figure 7 Glucose and Glycerol assimilation pathway

3.1. ACL (ATP Citrate lyase)

EC 2.3.3.8 ATP citrate lyase is one of the enzymes involved in the reverse TCA cycle. At the onset of nitrogen depletion, isocitrate is equilibrated via aconitase and transported to the cytosol by citrate / malate translocase. ATP citrate lyase is a cytosolic enzyme present in a wide range of microbes and is one of the major enzymes in oleaginous microbes (Ratledge & Wynn, 2002). Citrate is further processed into Acetyl CoA by ACL at the expense of one molecule of ATP.

$$\text{Citrate} + \text{CoA} + \text{ATP} \longrightarrow \text{Acetyl CoA} + \text{Oxaloacetate} + \text{ADP} + \text{Pi} \quad \ldots\ldots\ldots (2)$$

The presence of ACL does not make any organism completely oleaginous. Even though certain organisms possess ACL they accumulate less than 20% lipid of total dry cell mass and hence ACL can be called a precursor provider for lipogenesis (Acetyl-CoA). Lipid biosynthesis begins when Acetyl-CoA is reduced to malonyl-CoA by fatty acid synthesis (Wynn & Ratledge, 2005).

3.2. Malic Enzyme (ME)

Malic enzyme is the second key enzyme in lipogenesis. Acetyl Co-A is added to the fatty acid chain by fatty acid synthase. This requires two molecules of NADPH as a reducing power for the growth of TAG and is supplied mainly by ME. Although there are several pathways that lead to the generation of NADPH in oleaginous and non-oleaginous organisms, ME has been reported as the key enzyme that supplies NADPH towards lipid biosynthesis. The reaction involved in the release of NADPH is as follows:

$$\text{L-malate} + \text{NADP}^* \longrightarrow \text{pyruvate} + \text{NADPH} + CO_2 + H^+ \quad \ldots\ldots\ldots\ldots (3)$$

Malic enzyme is crucial to lipid synthesis even though it contributes only a part of the NADPH required for total cell metabolism. The complete inactivation or inhibition of ME totally arrests lipid synthesis (Ratledge & Wynn, 2002). This indicates that extended activity of ME can improve the biosynthesis period of lipid accumulation in lipogenic organisms. Previously it was thought that the lipogenicity is brought about by the ACL, but it was identified that the presence of ACL alone does not make the organism oleaginous (Wynn et al., 1999). Malic enzyme is tightly regulated by the genetic machinery of the organism and a continuous supply of ME increases lipid production, indicating that high degree of lipid accumulation in different organisms depends on the activity of ME. However, in most cases, ME activity is blocked shortly after the exhaustion of nitrogen in the media (Ratledge & Wynn, 2002). Indirect results by Beopolous *et al*. (2009) suggest that when the organism is in lipogenic phase it needs a certain amount of nitrogen (preferably more than a C/N ratio of 300) in order to maintain the lipogenic enzyme activity (Beopoulos et al., 2009b).

3.3. Acetyl-CoA Carboxylase (ACC)

ACC plays a key role in the synthesis and degradation of fatty acids and it is a multi-subunit enzyme in prokaryotes while in eukaryotes it is a multi-domain enzyme. ACC is a biotin-dependent enzyme and catalyzes the first carboxylation of acetyl-CoA to generate Malonyl-CoA,

which is regarded as the primary step in long chain fatty acid synthesis. In the first step, biotin-dependent carboxylase performs the carboxylation of the prosthetic group that is covalently linked to the biotin carboxyl carrier binding protein. In the next step carboxyltransferase (CT) domain catalyzes the transfer of carboxyl group from biotin to acetyl-CoA (Berg et al., 2002).

The carboxylation activity of ACC depends on the control of three different signals in eukaryotes, including glucagen, epinephrine, and insulin. Among these, insulin activates the carboxylase and favors the fatty acid synthesis while the other two have a negative impact on fatty acid synthesis. The level of citrate, palmityl CoA, and AMP also have an impact on ACC. Citrate level in the cell directly activates ACC while palmityl CoA and AMP inhibit fatty acid synthesis (Berg et al., 2002). The carboxylation activity of ACC is required biotin as the prosthetic group. In yeast, biotin is transported through active transport mechanism (Fisher et al., 2012). Moreover, in many organisms, it is an essential vitamin. The reaction catalyzed by ACC is represented below. Thus biotin is essential for long chain fatty acid synthesis (Carol et al., 1999; Streit & Entcheva, 2003).

HCO_3^- + Mg-ATP + Biotin-ACC $\longleftrightarrow$ Biotin-ACC- CO_2^- + Mg-ADP + Pi…….(4)

Biotin-ACC-CO_2^- + Acetyl CoA $\longleftrightarrow$ Biotin-ACC + Malonyl CoA……..(5)

4. Carbon sources for lipid production

4.1. Glucose

Glucose is the most abundant sugar present on earth and it is trapped as a polymer of cellulose in the form of lignocellulose in plant materials. The global need for glucose has steadily increased to $ 165 Million in 2010 and it is projected to increase to $356.5 million with a growth rate of 14.2% during the period of 2011 to 2016 .

Glucose is a simple monosaccharide and well-studied carbon source for any kind of microbial growth. In general, when glucose is used as the carbon source, the commercial yeast (*S. Cerevisiae*) and *E.coli* reaches a μ_{max} (maximum specific growth rate) of 0.4 h^{-1} (Chaudhary. et al., 2012; Walker & Chichester, 1997). Yeast possesses high affinity towards glucose; the main reason behind this is the entry of high concentration of glucose into the glycolytic pathway to form various metabolites along with ethanol. Though this strain has high ethanol tolerance and productivity, it fails to efficiently utilize other carbon sources such as glycerol, e.g. the μ_{max} was observed to be 0.02$h^{-1}$with glycerol as a carbon source (Liu et al., 2013).

The lipid production from glucose has been used for decades and has been appreciated as an ideal carbon source for lipid production. However, the high cost of glucose prohibits it use as a carbon source for mass lipid synthesis. There were recent studies showing a high biomass with considerable lipid accumulation in glucose supplemented fermentations. Biomass and lipid reached up to 28.1g/L with 62.4%w/w lipid accumulation, respectively with glucose supplemented fermentation (Zhu et al., 2008). However, when the mode of fermentation was changed from batch to fed-batch cultivation the biomass and lipid production was notably increased to 151.5g/L and 72.72g/L in the case of *Rhodosporidium toruloides* Y4 (Li et al., 2007a). This proves that the biomass growth highly depends on the initial substrate concentration ie, when the glucose concentration is high (over 100g/L) the cell growth is inhibited (Chang et al., 2013). It has been clear that many of the microbes are vulnerable when the carbon concentration exceeds the tolerable limit. Due to the osmotic imbalance, the microbial growth is significantly inhibited and this affects the biomass and lipid productivity even though an excess carbon source is important for lipid accumulation. However, the glucose utilization process varies according to the microbe, cultivation condition, and other physicochemical parameters. In a batch cultivation of *R toruloides* in glucose, resulted in 37g/L biomass with 49%w/w lipid accumulation. When the cultivation mode was changed from batch to fed-batch mode the biomass and lipid were improved in a range of 35 to 47g/L biomass and 50 to 75% lipid accumulation (Marilyn et al., 2013). On the other hand, a high-density cultivation method supplemented with glucose as carbon source and two stage fermentation was investigated by Lin *et al*. (Lin et al., 2011). The study reveals an initial high nutrient enrichment of biomass in the first stage and operation without auxiliary nutrients in the second stage. The biomass and lipid content reached 104.6g/L and 67.9g/L, respectively (Lin et al., 2011). Thus, this process suggested a new strategy to separate both biomass production and lipid accumulation in two stages and concomitantly considerably reducing the fermentation time to 40h. At the same time, other studies also support that the addition of low concentration of nitrogen during the late stage of fermentation also improves the lipid production (Shuib et al., 2014). In other words, the lipogenic enzyme ACL requires NH_4^+ ions for its activity, thus, it improves the AcetylcoA supply towards the lipid synthesis.

Recently, the cellular metabolism of *Y. lipolytica* was studied in detail where 20g/L glucose was used as the carbon source. A balanced growth was noticed until 16h of fermentation and there

was no product formation, only biomass and CO_2 was generated (Mhairi et al., 2013). On the other hand, *R. toruloides* CBS14 was reported to produce 76.1% (w/w) lipid when glucose was fed as the carbon source (Zhao et al., 2008). As mentioned above, it clearly indicates that the substrate preference varies in different microbes. When *Y. lipolytica* was used in glucose-based nitrogen deficient media with an initial glucose concentration of 30g/L; the citric acid accumulation dominated with a lipid yield of 0.2 to 0.33g/g of substrate consumed (Papanikolaou et al., 2009). This implies that along with substrate preference the concentration of substrate also plays a vital role in lipid accumulation. Meanwhile, oleaginous yeast strains are reported as capable of using a wide range of carbon sources, including glucose, xylose, arabinose, and mannose. After glucose, xylose is the preferable source and second largest plant-based carbon available on the planet. A high concentration of xylose (100g/L) resulted in 28.8g/L biomass and 18.5g/L lipid accumulation in *M isabellina* (Gao et al., 2013; Mhairi et al., 2013).

4.2. Sugars derived from agro-residues

Complexity in the availability of energy resources leads to the use of complex polymers source of fermentable sugars. The main source of complex polymers is the lignocellulosic biomass. The nature of the material makes the process difficult in terms of extracting the sugars from lignocellulosic biomass. Lignocellulosic biomass is made up of cellulosic polymer embedded in between hemicellulose and lignin (Ghosh & Ghose, 2003; Yousuf, 2012). There has been ample research reported with respect to the depolymerization of lignocellulosic biomass. However, the process economy depends on the efficiency of the process parameters such as pretreatment, saccharification, and fermentation. Associated with this is the release of the inhibitory materials into the fermentation stream, which reduces the fermentation ability of the process. Recently, hydrolysates from different agro residual materials were examined for lipid production. Among them, rice bran hydrolysate, wheat straw, tree leaves, tomato waste and others were used as a carbon source for lipid production. Table 11 represents the alternative carbon sources used for lipid production using different organisms.

A study conducted on rice straw as a raw material for lipid production by *T fermentans* resulted in 26.6g/L biomass and 11.5g/L lipid after 8 days of fermentation. The rice straw was initially pretreated with sulphuric acid followed by enzymatic saccharification. A poor yield of biomass and lipid was observed when the fermentation was conducted without detoxification of the

hydrolyzate (Huang et al., 2009). Further sugarcane bagasse was used for lipid production by Tsigie *et al* (Tsigie et al., 2012). The bagasse was pretreated with 2.5% HCl. The hydrolysate was detoxified and then fermented with *Y.lipolytica*. A final biomass and lipid concentration of 11.42g/L and 6.68 g/L respectively were reported (Tsigie et al., 2011). Based on the above-mentioned studies, it is clear that the detoxification of hydrolyzate may lead to enhance the biomass and lipid production by lowering the inhibition. Moreover, it has been reported that 2.5g/L furfural completely inhibits the growth of microbes. In the case of acetic acid generated during the pretreatment of lignocellulosic biomass (LCB) could inhibit the growth at a concentration of 3g/L (Galafassi et al., 2012). The other inhibitors associated with LCB pretreatment are hydroxymethylfurfural (5-HMF), formic acid, succinic acid, low molecular weight phenolics and other lignin degradation products.

Table 11 Microbial oil from lignocellulosic biomass by selected organisms

Organism	**Substrate**	**Biomass (g/L)**	**Lipid %(w/w)**	**Reference**
T fermentans	Rice straw	28.6	40.1	(Huang et al., 2009)
R glutinis	Starch waste	40	35	(Xue et al., 2010)
A oryzae	Wheat straw	6.7	8	(Lin et al., 2010)
C lipolytica	Molasses	NA	59.9	(Karatay & Donmez, 2010)
Y.lipolytica	Sugarcane bagasse	11.42	58.5	(Tsigie et al., 2011)
T cutaneum	Corn stover	15.44	23.5	(Huang et al., 2011)
M isabellina	Lignocellulosic	22.9	44.5	(Ruan et al., 2012)
R garminis	Corn stover	48	34	(Galafassi et al., 2012)
T cutaneum	Corn stover	15.44	23.5	(Huang et al., 2011)
R kratochvilovae	Hemp seed extract	15.1	55.56	(Patel et al., 2014)
R glutinis	Corncob hydrolyzate	70.8	47.2	(Liu et al., 2015)

Meanwhile, studies were reported based on non-detoxified lipid production with LCB hydrolyzates. Acid treated corn stover resulted in a 10.2g/L biomass and 8.8g/L lipid (Ruan et al., 2012). On the contrary, *R.glutinis* consumed furfural, acetic acid, and 5-HMF during fermentation for lipid production using un-detoxified alkali treated corncob. The hydrolyzate was richer in xylose than glucose. The batch study showed 15.1g/L biomass and 5.5g/L lipid.

Eventually, the intermediate addition of nitrogen showed a 4.6 fold increase in biomass (70.8g/L) and 6 fold increase in lipid (33.5g/L) (Liu et al., 2015). Interestingly, another study shows a positive effect of LCB born inhibitors on lipid production and at the same time the inhibitors were reported to improve lipid extraction as well (Kurosawa et al., 2010). Based on recent studies reported in the literature, it can be assumed that detoxification and neutralization of LCB hydrolyzate are economically not feasible and labor intensive. However, comparatively a significant amount of biomass and lipid has been produced with LCB hydrolyzate, still, the hurdles in pretreatment and saccharification technologies need ample improvement with respect to the economic viewpoint.

Other than lignocellulosic hydrolyzates various industrial effluents containing different sugar composition also were employed for lipid production using various lipogenic organisms. Table 12 represents the other carbon sources used for lipid production. Acetate is one of the single carbon molecules and can be assimilated by microbes when it is present in low concentrations (>5g/L). In a fed-batch study, 80g/L biomass with 60%w/w lipid content was achieved when the C/N ratio feed was maintained at 300. Moreover, other volatile fatty acids (VFA) such as propionate and butyrate were also tested for lipid production with *Y.lipolytica*. On the other hand, the control of fermentation (pH, DO) while using VFA or organic acid remains highly tedious compared to other sugars. It has been reported that a VFA concentration more than 5g/L could strongly inhibit the yeast growth. The inhibitory effect of weak acid was brought by the dissociation constant (it affects the cell physiology by altering the membrane proteins thereby affects the membrane transport) of the particular acid. In other words, the undissociated form of weak organic acids easily penetrates the cell membrane and dissociates at cytoplasm and brings the intracellular pH down. Hence, the pH of the medium plays a crucial role in using these substrates (Beligon et al., 2015).

Table 12 Other carbon sources

Organism	Substrate	Biomass (g/L)	Lipid %(w/w)	Reference
C curvatus	Acetate	80	60	(Beligon et al., 2015)
Y.lipolytica	Acetic acid	31	40	(Fontanille et al., 2012)
R toruloides and C pyrenoidosa	Distillery and domestic mixed wastewater	7.25	63.4	(Ling et al., 2014)
Cryptococcus albidus	Volatile fatty acids	1.1	28.3	(Vajpeyi & Chandran, 2015)

4.3. Glycerol as carbon source

In the present decade, the development of bio-diesel industry has opened wide availability of glycerol for different applications including biotransformation to various value added products. Until date, the main sources of feedstock for biodiesel production used are vegetable oil, yellow grease, plant oils, etc. Every 100kg of biodiesel generates 10kg of glycerol as a major by-product (Ayoub & Abdullah, 2012). The blooming of the biodiesel industry throughout the world generates a surplus amount of crude glycerol available for different applications. The low purity and the source of raw materials used in the trans-esterification reactions make the purification of crude glycerol very laborious and further affect its application in food and pharmaceutical industry. The world biodiesel production estimated to reach approximately 2.73 billion kg in 2020. (CRFA, 2014). Moreover, every year the biodiesel industry grows considerably at the rate of 17% (from 2007 to 2012). Apart from biodiesel industry, glycerol is produced from the ethanol and soap industry (Amin Inhm et al., 2010). Therefore, glycerol can be considered a potential raw material for biodiesel production.

The rapid development of biodiesel industry results in an additional burden to the industries in terms of handling the crude glycerol (Thompson & He, 2006). As mentioned earlier, the purification of glycerol obtained from biodiesel industry is tedious with a high cost. The small scale industries cannot afford purification processes. However, the global turnover of crude glycerol increased due to the rapid development in biodiesel industries, which causes instability of glycerol price (Ayoub & Abdullah, 2012). The price for glycerol was stable for the last few decades and purified glycerol was priced $ 0.27 US to $ 0.41 per kg. This was expected to drop

below $0.2 and related to the production/ utilization of different value-added materials from crude glycerol (Petersen et al., 2004).

Based on the available data from DOE (Department of Energy) (US), glycerol is classified as a building block for value-added chemicals such as propylene glycol, 1,3 PDO, diacids, propyl alcohol, dialdehydes, epoxides etc. (Petersen et al., 2004). Glycerol of higher purity and with fewer prices is available from the petrochemical industry when compared to the crude glycerol of biodiesel industries. An adequate amount of glycerol is available for chemical and pharmaceutical markets with high purity and stable price for the past decades leading to the surplus availability of crude glycerol from bio-based industries. Due to this reason, the price of crude glycerol declined from $0.25 (in 2004) to 0.05 /lb (in 2006). The continuous demand for biodiesel shifts the glycerol market more versatile in terms of price reduction and the demand for glycerol also considerably dropped (Ayoub & Abdullah, 2012).

Table 13 Worldwide glycerol applications**(Stelmachowski, 2011)**

Product	Value (%)
Food	23-25
Cosmetics	37-40
Tobacco products	9-10
Polyurethane	7-10
Pharmaceuticals	6-8
Alkyl resin	3-9
Other products	1-5

Two billion pounds or 0.906 billion kg of glycerol worth one billion dollars is produced annually throughout the world (McCoy, 2005) and is used to produce various products (Table 13). The major contributors of crude glycerol are Europe, US, and South East Asian countries. The present scenario of environmental policies in countries such as United States, European Union, South East Asia, Canada and South America led to the implementation of production of renewable and sustainable energy. It has been reported that the fatty acid and soap industry based glycerol supply dominated the market until 2003 only to be replaced by biodiesel derived glycerol in the year 2008 (Tsobanakis, 2007). The glycerol production from different industries has increased considerably in the past few years (Table 14) and though the glycerol has a wide

range of application, its overproduction has the industries thinking about alternative ways to use or dispose of the glycerol.

Table 14 Glycerol Production industry wise(Tsobanakis, 2007)

Source / Year	Global production in metric tons/ year		
	2003	**2008**	**2010**
Soaps	188	146	83
Fatty acids	333	438	521
Biodiesel	167	521	1583
Chemical	63	0	0
Fatty alcohols	104	167	250
Others	63	0	21

4.4. Problems associated with glycerol valorization by microbes

As mentioned before glycerol is the main by-product of biodiesel, soap, petrochemical, ethanol, and fat industries. The purity of glycerol obtained is always questionable. The high purity of glycerol from petrochemical industry makes this fraction useable in different food and pharmaceutical applications. On the other hand, the glycerol released from the biodiesel industry combined with the impurities/ hazardous materials such as high catalyst content, the presence of alcohols (mainly methanol and/or ethanol), heavy metals, and high free fatty acid makes it more complicated for the microbial fermentation (Gonzalez-Pajuelo et al., 2005; Thompson & He, 2006). Since the purification of crude glycerol makes an additional burden for small and medium scale biodiesel industries, it lacks the possible use in food and pharmaceutical applications. However, the soap industry based glycerol lacks its concern since soap is replaced hugely by different kinds of detergents (Ayoub & Abdullah, 2012). Due to this reason, the current crude glycerol production mainly depends on biodiesel industry, which is very clear from Table 14.

Table 15 Physical and chemical character of Crude glycerol (based on our lab data)

Parameters	Elements	Weight %
Physical	Relative density kg/m^3	1102-1280
	Viscosity at RT* (cP)	7-8.5
	Glycerol content	12-83
	Color (APHA)	Dark
	Ash	1.5-4
	Soap	3.0-27.3
	Methanol	1.5-35
	pH	4-11 (depends on catalyst)

**RT- room temperature*

The raw material used for biodiesel production can vary based on availability; Europe uses rapeseed oil whereas the US uses canola oil. Restaurant waste oil and yellow grease also are important commodities in the biodiesel industry (Thompson & He, 2006). Due to the high degree of difference in the feedstock used for biodiesel production, the final composition of crude glycerol also varies. Apart from variation in feedstock the chemical process used for transesterification process, the efficiency of the transesterification, free fatty acid content in the feedstock oil also determine the final quality of crude glycerol (Papanikolaou et al., 2008).

The typical composition of glycerol derived from biodiesel industry is presented in Table 15. Glycerol concentration in crude glycerol is reported to vary from 35 to 95% and it solely depends on the method of biodiesel production. Thompson *et al*. (Thompson & He, 2006) characterized glycerol composition obtained from different feedstocks such as sunflower oil 30% (w/w), mustard seeds 62% (w/w), soy oil 67.85% (w/w) and vegetable oils 76% (w/w) (Thompson & He, 2006). The other common components reported in association with the above components are (w/w) phosphorus 1.05 to 2.36%, potassium 2.2 to 2.33%, sodium 0.9 to 0.11% and calcium, mercury and arsenic are present in trace amounts (Table 16) (Tan et al., 2013).

Table 16 Elemental Analysis of crude glycerol from different feedstocks (conc.in ppm) (Thompson & He, 2006)

Elements in ppm and feedstock's	Ida gold	Pac Gold	Rapeseed	Canola	Soybean	Crambe
Calcium	11.7	23	24	19.7	11	163.3
Potassium	BDL	BDL	BDL	BDL	BDL	216.7
Magnesium	3.9	6.6	4	5.4	6.8	126.7
Phosphorus	25.3	4	65	58.7	53	136.7
Sulfur	21.0	16	21	14	BDL	128
Sodium	1.17	1.23	1.06	1.07	1.20	1.1
Nitrogen	0.04	0.04	0.05	0.05	0.04	0.06

(BDL-Below detectable limit)

4.5. Municipal sewage sludge as carbon and nutrient source

Industrial waste materials such as cheese whey, slaughterhouse wastes, pulp, and paper industry, and municipal solid and liquid waste materials have been evaluated in recent years as carbon and energy source for the production of value-added products. Wastewater treatment sludge (WWS) is considered as a raw material (to a certain extent) for value added product formation (Mondala et al., 2012; Seo et al., 2013). Biodiesel from WWS is a new area of research where significant improvement needs to be brought. WWS composed of the multi-nutrient medium, which also contains a considerable amount of lipids (fat grease, oils, and microbial phospholipids). Very few studies have been conducted in the past decade regarding WWS for biodiesel applications. In a study conducted by Dufreche et al, (Dufreche et al., 2007) 27.4% w/w of lipid was extracted from primary and activated sludge. Due to the complex nature of WWS, availability of carbon for cell growth and lipid accumulation (availability of free carbon for microbes) is restricted. To enhance the microbial growth and lipid production addition of external carbon such as glucose or glycerol was investigated and the addition of glucose improved the overall biomass and lipid production. However, the addition of external carbon and nitrogen sources may lead to a higher cost of production. To avoid this issue, a pretreatment was employed by researchers to solubilize the possible sludge complex carbon by acid or alkali. *T oleaginous* was used as the biocatalyst to

use the sludge nutrients to produce lipid in pre-treated WWS. The lipid production was noted to be completed within 42h of fermentation and the total lipid content of the cells was 39%w/w with a biomass concentration of 20.1g/L (Zhang et al., 2014a).

5. Carbon source comparaison (Glucose Vs Glycerol)

5.1. Glucose

Glucose is transported into the cell by facilitated diffusion with the help of integral proteins in yeast cells. Hexose sugars are the most abundant renewable energy resource on the earth but are trapped as a complex material called lignocellulose. Apart from lignocellulose other simple sugar resources are mainly the food crops. So as not to affect the yield of food crops several alternative strategies have been investigated to derive free sugars from lignocellulosic materials in order to produce energy reserve materials. This process consumes high energy and involves relatively toxic chemical treatment that affects the environment.

During the microbial utilization of glucose, it directly enters the glycolytic pathway and is converted to pyruvate by the action of glycolytic enzymes. One molecule of glucose through glycolysis generates four ATP molecules and 2NADH molecules that are reduced molecules possessing high energy. Since two molecules of ATP are used in the glycolytic pathway, the net energy recovery from glycolysis is two molecules each of ATP and NADH. The two molecules of pyruvate generated during glycolysis enter into the Krebs cycle (Citric acid cycle) as Acetyl-CoA formed by the enzyme pyruvate dehydrogenase. At the end of the Krebs cycle, the oxidation of pyruvate produces two ATP, eight NADH, and two FADH2 molecules. In the case of lipid production, the citric acid cycle is perturbed at the isocitrate level; such that citrate molecules are transported to the cytoplasm and converted to Acetyl CoA**.** Thus the generated Acetyl CoA is further used for TAG synthesis in lipogenic organisms.

5.2. Glycerol

Glycerol is transported into the cells via two different routes; affinity transport system (facilitated diffusion) and high-affinity symport system (active transport). In the presence of glucose, glycerol is transported into the cells by facilitated diffusion, whereas in the presence of non-fermentable sugars such as acetate and ethanol uptake of glycerol is mediated by the active transport system. Once the molecule is transported into the cell it can be assimilated by two different pathways; phosphorylation and oxidation (Figure 8).

While comparing the growth of microbes on glucose or glycerol, the specific growth rate was higher in the case of glycerol ($0.3h^{-1}$) than glucose ($0.24h^{-1}$). However, the combination of both glucose and glycerol resulted in a specific growth rate of $0.38h^{-1}$. Further the study shows the higher requirement of O_2 in glycerol assimilation than glucose. This indicates the organism consumes the glycerol by phosphorylation pathway at initial phase (Mhairi et al., 2013).

6. Crude glycerol and lipid accumulation

The simple nature of glycerol renders it as an excellent carbon source for the production of various value added products. In the present decade, the availability of glycerol for various industrial applications augmented considerably due to the endeavor of the bio-diesel industry. Glycerol consumption by yeast, bacteria, and fungi is well evaluated (Table 10). Propionic acid, butanol, succinic acid and dihydroxyacetone are a few other compounds reported being produced from crude glycerol (Asad-ur-Rehman. et al., 2008).

In current scenario crude glycerol from different industries holds the key to renewable energy production. Due to the high degree of variation of glycerol consumption, the microbes need to adapt to the new inhibitors as well as the different concentrations of glycerol considering the high carbon requirement for lipid production. *Y. lipolytica* belonging to Candida species has been reported by various authors for the production of citric acid using glycerol as the sole carbon source (Papanikolaou et al., 2002). However, *Y. lipolytica* possesses a high potential to produce lipids from crude glycerol having different inhibitory compounds. As mentioned earlier, the variation of substrate tolerance differs from organism to organism. *Y. lipolytica* is reported to tolerate a crude glycerol concentration of 120g/L, whereas *C. curvatus* showed growth inhibition when the glycerol concentration exceeded 64g/L (Papanikolaou & Aggelis, 2002). Glycerol also has been evaluated for lipid production using different microbes. The widely accepted high lipid accumulating microbes among yeasts includes *Rhodotorula glutinis*, *Cryptococcus albidus*, *Lipomyces starkeyi*, *Candida curvata*, and *Y. lipolytica*, which have been well evaluated in crude glycerol-based media (Christophe et al., 2012; Meng et al., 2009).

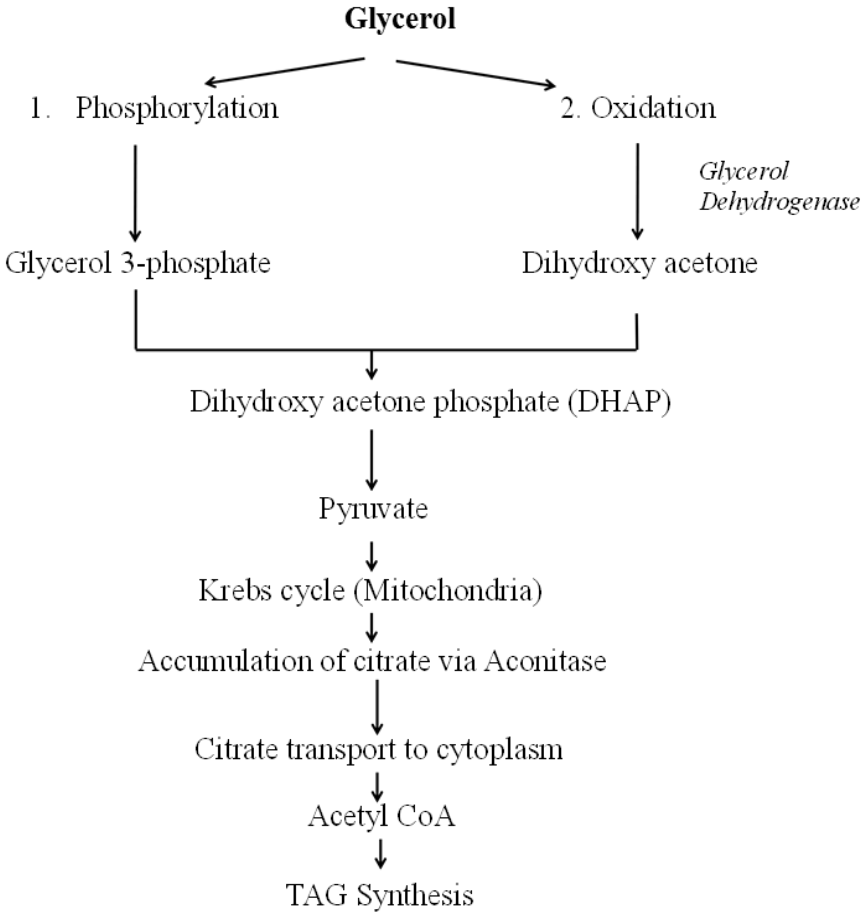

Figure 8 Glycerol utilization pathways

Many studies have been carried out during past decade reporting numerous fungal isolates producing value added lipids (Wynn & Ratledge, 2005). Even though the final goal was not achieved, several independent variables were noted as critical for lipid production. Compared to bacterial and yeast strains, fungal strains have numerous advantages and disadvantages in using glycerol and other substrates. Most of the fungal strains are capable of consuming glycerol even at elevated concentration levels. On the other hand, during fermentation at large scale, high shear rate, clogging and mat formation on the walls of the probes makes fungal based lipid production more complicated than yeast and bacteria derived lipids (Azocar et al., 2010). Fungal species have slower growth rates and most of the fungal species are reported to produce industrially important polyunsaturated oils. The most reported fungal species are *Mucor sp., Mortierella sp.,* etc. On comparing the three categories of organisms, lipid accumulation is comparable for

example; *Rhodococcus 87% w/w, Rhodotorula, cryptococcus and Lipomyces above 60% w/w and Mortierella sp. 65% w/w* as reported (Alvarez et al., 1996; Beligon et al., 2015; Gao et al., 2013; Saenge et al., 2011; Yang et al., 2014). In the case of *Mortierella* species, the oil is rich in Gama linolenic acid (GLA). Though fungi grow at a slower rate the biomass accumulation exceeds that of other organisms in certain cases. A biomass accumulation of 28.1g/L with a lipid content of 62.4%w/w was reported in the case of *T. fermentans* (Zhu et al., 2008).

7. Inhibition of cell growth and lipid accumulation by components present in crude glycerol

Though crude glycerol is an excellent carbon source for lipid production, however, the impurities associated with crude glycerol are known to be inhibitory if they are present at high concentrations. Apart from other inhibitors glycerol itself is inhibitory at high concentrations depending upon the tolerance level of microbes (Liang et al., 2010). There are several research reports on different organisms with a wide range of glycerol tolerance, for example; *R. glutinis* can tolerate a glycerol concentration of 8.5% w/v above which it inhibits the growth rate of the organism. Whereas, in the case of *Y. lipolytica*, glycerol concentration even at 10% w/v exhibits natural growth (Beopoulos et al., 2009a; Papanikolaou & Aggelis, 2002). In the case of *Cryptococcus* glycerol concentration varied from 40 to 60 g/L. When crude glycerol is used as a carbon source it is accompanied by the impurities associated with it, which may be inhibitory or sometimes even beneficial to microbial growth and lipid production. The major components associated with crude glycerol are the catalyst (acid or alkali), soap, methanol, heavy metals and FFA. The most commonly prevalent inhibitor is methanol, but it is acceptable as a carbon source for cell growth in some algal species and in some yeast strains. The methanol concentration in the crude glycerol varies from 1.5 to 35 % (v/v) depending upon the downstream process of biodiesel followed by the methanol recovery process employed. A methanol concentration of 1% v/v improved the overall lipid production by 1.2% in *Chlorococcum sp*. Liang *et al.* (Liang et al., 2009) reported that the use of methanol by *Cryptococcus* strain in fed-batch cultivation, 23.6g of methanol was added to the media, decreased the amount of methanol (4.8g/L). About 11.7g/L methanol was expected to be present in the fermentation media, but only 4.7g/L was observed at the end of fermentation (Liang et al., 2010). Though the loss of methanol by evaporation was not recorded by the authors considering the fact that to a certain level the organism resists methanol (Woon-Yong et al., 2011) the assimilated methanol may have been diverted for cell proliferation.

As mentioned earlier soap formation in trans-esterification reaction depends on the catalyst used in the reaction. When alkalis such as NaOH or KOH are used, the oleic acid present in lipid fraction is converted to sodium or potassium oleate. Though it is an excellent surfactant, when its concentration exceeds 2g/L in the media it inhibits proliferation of *Schizochytrium limacinum* when employed for DHA (Docosahexaenoic acid) production (Denver et al., 2008). A positive effect of sodium oleate on biomass and lipid production even at a concentration of 4g/L has been reported by Xu et al (Xu et al., 2012). The fact behind the inhibition or non-inhibition of microbial growth with regard to the presence of soap depends on the interaction of soap with the cell wall membrane. Inhibition may occur due to the complex reaction of surfactant (soap) with the cell membrane, whereas the positive effect may be due to the enhancement of flow of nutrition from the surroundings into the cell interior owing to the modification of its cell wall permeability (Wei et al., 2008). As is already known, the separation of biodiesel needs the adjustment of pH followed by settling of glycerol and other impurities. The settling process favors the accumulation of glycerides and some amount of FAME in the glycerol fraction. Among the FAME methyl oleate is the most common fraction and found in most of the biodiesel derived glycerol samples. Methyl oleate is noted to improve lipid accumulation in *R. toruloides* (Xu et al., 2012). It has been well understood that in nature some organisms are capable of assimilating hydrophobic substrates, such as alkanes, fatty acids, oils, and hydrocarbons (Fickers et al., 2005). In the case of *Y. lipolytica* both the pathways for substrate assimilation de novo and ex de novo (hydrophobic substrates) are reported and well-studied and is a well-known molecular tool for yeast modification (Beopoulos et al., 2009b).

Another class of compounds found in crude glycerol is salted (NaOH). There are reports stating that the presence of these compounds improves lipid production rather than inhibiting growth. Usually, the concentration of salts in the crude glycerol varies from 5 to 7% w/w and aids in microbial growth (Xu et al., 2012). The addition of sodium chloride from 4 to 16 g/L improves lipid yield by 12 to 40% (Xu et al., 2012). The elemental analysis of crude glycerol from different feedstocks is presented in Table 16.

8. Sustainability of glycerol as carbon source for lipid synthesis

Based on the above literature review it is evident that in terms of energy value and abundance, glucose is the main energy molecule suitable for microbial fermentation. However, the availability and cost of glucose render the process of lipid synthesis un-economical.

Nevertheless, glycerol has gained huge attention in the world market and its overproduction has led to surplus availability at low cost for different value added commodity productions. Glycerol is also considered as a simple and preferable carbon source by different organisms and due to this glycerol has been listed as one among the most valuable building blocks. In most cases, the impurities present in the crude glycerol act as nutrients in the fermentation process and thus reduce the burden of glycerol based biodiesel industries.

As reported and evaluated by different authors the feedstock cost for biodiesel comprises 60 to 75% of total production cost (Hughes & Benemann, 1997). An economically viable process can be achieved only by employing a highly potential strain capable of using cheaper carbon sources. According to the current market value of soybean oil based biodiesel, a gallon of biodiesel costs $2.94 (0.78US$/L), which is slightly less than the market price of biodiesel at $3.02 (0.8US$/L) (Yazdani & Gonzalez, 2007). Bio-refineries and agro-industries can benefit substantially from the use of waste streams generated from the process if it is used for the synthesis of value-added products. The rapid growth in biodiesel industry has resulted in the production of a large amount of glycerol as a by-product, which is declining its market value as described in the previous sections. At the current price (5.5 cents/kg) of crude glycerol, it is highly competitive with other sugar materials available in the market. From a metabolic point of view, glycerol produces equivalent (net energy as ATP) energy and its transport into the cell by simple diffusion reduces the consumption of energy (ATP) (Yazdani & Gonzalez, 2007).

9. Factors affecting lipid accumulation

The availability of crude glycerol from biodiesel industry opened a new opportunity to generate a considerable improvement in renewable energy production. Though the carbon source is available in surplus, the rest of the process depends on the other parameters, which determine the efficiency of lipid accumulation in oleaginous organisms (Wynn & Ratledge, 2005). The main factors include C/N molar ratio, inoculum concentration and age, the supply of micro and macro nutrients, pH and temperature (Li Y-h, 2005). Most importantly, the process efficiency depends on the capability of the organism to assimilate the given carbon source at a low nutrient level and the overall capacity to accumulate lipid. It has been noted that different organisms have different levels of oil accumulation properties (Wynn & Ratledge, 2005).

9.1. C/N ratio

The nature of nitrogen source (inorganic or organic) is crucial to biomass and lipid accumulation. Supplementation of inorganic nitrogen source improves lipid accumulation, on the other hand, the supply of organic nitrogen source improves the biomass and lipid accumulation to a certain extent(Kalam et al., 2014).

The C/N ratio is a well-studied subject. The study shows that an increase in carbon along with high initial C/N ratio results in better lipid accumulation (Beopoulos et al., 2009a). In the case of *L. starkeyi* a lipid content of 68% w/w was attained when the C/N ratio was 150, whereas the lipid content was decreased to 40% w/w when the C/N ratio was 60. It has been proven that employing *Y. lipolytica* the initial C/N molar ratio of 80 to 120 significantly favors citric acid accumulation in the early stage along with a low-level of lipid accumulation. This phenomenon correlates well with a carbon concentration in the media, i.e. a higher carbon concentration inhibits ACL due to which citrate accumulates in the medium instead of being diverted to TAG synthesis (Beopoulos et al., 2009a; Wynn & Ratledge, 2005). The critical nitrogen concentration required for citric acid production in *Y. lipolytica* has been reported to be10^{-3} mol l^{-1} and it is important to keep the threshold value, exceeding which the citrate accumulation reduces and thereby affects the total lipid production too. In the case of *R. glutinis* an increase in the initial C/N molar ratio of 150 to 350 improves the overall yield of lipid from 0.25 to 0.40 g/g of glucose consumed in a batch culture. However, an increase in the C/N ratio beyond 350 significantly affects lipid synthesis owing to deficiency of nitrogen for lipogenic enzyme activity (Beopoulos et al., 2009a; Cescut, 2009). For example, the ACL requires ammonium ion for activation. As mentioned in previous sections, a high C/N ratio during lipogenic phase improves lipid production instead of a nitrogen-free scenario at lipogenic phase.

9.2.Trace elements

The inorganic compounds (micronutrients) also have a strong influence on lipid accumulation. In most of the cases, these elements act as cofactors for enzyme activity. Magnesium, ammonium, zinc, iron, and phosphate are some of the well explored inorganic compounds. Naganuma *et al.* revealed that a deficiency of NH_4^+, K^+, Mg, PO_4-, SO_4^{2}-, Zn^2+, Fe^3+, or Mn^2+ has a strong effect on biomass accumulation, whereas elements such as NH_4^+, K^+, Ca^+, Zn_2^+, Fe_3^+, and Mn_2^+ influence lipid accumulation (Naganuma et al., 1985).

9.3. Impact of pH and DO

The effect of pH and dissolved oxygen (DO) were reported as the main indicators of growth and lipid production phase (Yen & Zhang, 2011). When the organism was in exponential growth phase the variation in pH was noted to be less significant in lipogenic organisms. The transition phase in *Y. lipolytica* is known to produce a considerable amount of citric acid and other organic acids. The pH of the medium drastically drops due to the secretion of these organic acids into the external medium (Beopoulos et al., 2009a). It is well known that the transport mechanism of carbon and nutrient sources depends on cell wall permeability; the reduction in pH interferes with the cellular transport mechanism. Overproduction of organic acids may also inhibit the intracellular enzymes required for lipid synthesis. In order to know the effect of pH on cell growth and lipid accumulation in *R. glutinis*, pH controlled and pH uncontrolled experiments were carried out. In the pH controlled reactor, the pH was decreased significantly from 6.5 to 4.3 in the initial 48h of fermentation. The lipid content in the pH controlled experiment was 6% higher than that of the experiments with uncontrolled pH. Substrate utilization also is better under controlled pH conditions (Saenge et al., 2011).

DO is another critical factor, the growth phase requires high DO whereas the lipogenesis phase requires less DO. It has been reported that during the lipogenic phase of *Y. lipolytica* there is a steep decrease in O_2 utilization and continuous supply of O_2 had an increase in the DO level in fermentation media. A low level of DO (15 %) improved the total lipid accumulation in *Mucor sp. RRl001* and *Candida lipolytica* (Ahmed et al., 2009). Meanwhile, high level of DO (80%) leads to citric acid production (Bati et al., 1984). The effect of DO on cell growth and lipid accumulation was tested at different levels by Yen et al. 2001 (Yen & Zhang, 2011) and it was observed that a low level of DO enhances lipid accumulation but inhibits cell growth whereas a higher level of DO enhances biomass accumulation rather than lipid accumulation. In conclusion, a two stage DO controlled experiment was conducted by the authors wherein initial DO was maintained at 60%± 10 for about 70h and the DO was reduced to 25% ±10 until the end of fermentation (180h). It improved the total biomass concentration of 36.2g/L to 45.4 g/L, but the lipid content dropped from 62±7% to 42 ±3%w/w of cell dry biomass(Yen & Zhang, 2011).

10. Mode of fermentation

Batch, fed-batch and continuous mode of fermentation have been studied to cultivate oil-bearing microbes using different carbon sources. Glycerol and glucose based studies are well reported for the high-density cultivation of these organisms.

10.1. Batch studies

Carbon and other essential nutrients were supplemented initially along with fermentation medium. The initial principal parameter (C/N ratio) to boost or trigger lipogenesis was adjusted. In all cases, a carbon source is supplemented in excess to meet the need for excess carbon at lipogenic phase. In the batch mode of cultivation, substrate utilization is limited due to which the final biomass production is also limited, whereas the lipogenic mechanism begins with the increase in C/N ratio of the medium. Due to a balanced growth at the initial stage of fermentation, the available nitrogen is assimilated and nitrogen paucity arises during the later period of the fermentation. During growth, assimilated carbon is mainly utilized for cell proliferation; therefore lipid free biomass accumulates in the medium. At high C/N ratio growth depletes and the assimilated carbon is routed towards FFA synthesis by activation of a pool of lipogenic enzymes present in the organism. In batch mode, nutrient limitation is crucial (C/N ratio) for lipid production. Hence the initial C/N ratio optimization in batch fermentation requires more attention and, in general, a C/N molar ratio of 80 to 350 is believed to be suitable for lipid synthesis (Beopoulos et al., 2009a), however, this may vary depending upon the organism and initial carbon concentration. However, the initial high glycerol concentration in batch studies has a negative impact on biomass growth. For this reason in many cases, the initial glycerol concentration was restricted within 20 to 60g/L. However, the low initial substrate concentration may impact on the lipid production due to rapid depletion of carbon source, which results in low carbon availability during lipid synthesis phase of fermentation(Beopoulos et al., 2009b; Ratledge & Wynn, 2002).

10.2. Fed-batch approach

Unlike batch process, fed-batch process supplies carbon and nutrients in a controlled manner. In order to achieve a balanced in cell growth and lipid production, the flow of carbon and nitrogen are monitored and supplied accordingly. In other words, the C/N ratio of the fermentation medium has been adjusted according to different stages of fermentation (growth, transition and lipogenic phases) (Ramalingam et al., 2010). For lipid synthesis, the lipogenic phase is

considered most crucial. Hence, if the molar ratio of carbon and nitrogen in the fermentation medium is not optimal, metabolism may shift to different organic acid synthesis. Based on earlier reports, C/N molar ratio of 20 Cmol/Nmol^{-1} prevents citric acid production in *Y. lipolytica*. A precise control of C/N ratio is advised for higher lipid production by fed-batch mode (Beopoulos et al., 2009a).

The fed-batch approach has been known to result in an increase in biomass productivity and lipid content. A fed-batch experiment conducted by Li et al (Li et al., 2007b) proved that a biomass concentration of 151.5g/L with a lipid content of 48.0% w/w was achieved in 25 days in flask fermentation. The experiments were repeated at reactor level under controlled conditions and 106g/L biomass with 67.55%w/w lipid content was attained at 134h (>6 days) of fermentation. In both cases, glucose was fed at a rate of 20g/L/day. According to the studies of Saenge et al (2011) (Saenge et al., 2011), fed-batch fermentation was performed with yeast *Rhodotorula glutinis* to produce lipid using glycerol as carbon source. The aim of the fed-batch process was to avoid substrate inhibition and enhance the production of lipid by feeding additional substrate. The fermentation with an initial working volume of 800mL was first operated in a batch mode. The initial glycerol concentration and C/N ratio were 9.5% (w/v) and 85, respectively. Then 50mL of crude glycerol with the optimal C/N ratio (85) for lipid content was added every 12 h up to 48 h of fermentation time to maintain the glycerol concentration at an optimum level of 9.5% (w/v). Feeding the substrate in fed-batch mode increased the biomass, and lipid content, up to 10.05 g/L, and 60.70%, respectively. The glycerol consumption by microbes was greater in fed-batch fermentation. The final lipids concentration in the fed-batch was 8.36 g/L, which was about twice of the batch culture (4.33 g/L) (Saenge et al., 2011).

10.3. Continuous process

Continuous process refers to the assimilation of carbon and other nutrients at a constant rate depending upon microbial growth and dilution rate of cultivation medium. The concentration of the substrates within the bioreactor is maintained steadily and depending on the dilution rate as well; the actual C/N ratio of the fermentation medium remains constant unlike in batch process. Similar to batch fermentation the C/N ratio of the fresh medium needs to be higher than initial C/N ratio in order to obtain high lipid accumulation by continuous fermentation. Due to steady state cultivation, C/N ratio in the fermentation medium is always higher than that of fresh medium supplemented. The high dilution rate may affect the metabolic balance of lipid

synthesis. Hence, dilution rate is critical in continuous fermentation (Huijberts & Eggink, 1996). Papanikolaou and Aggelis reported that *Y. lipolytica* was capable of producing a huge quantity of lipids when cultured on raw glycerol under nitrogen-limited continuous process (Papanikolaou & Aggelis, 2002). In a highly aerated continuous process, lipid production is favored at low dilution rates, and the highest lipid productivity achieved was 0.12 $g.l^{-1}$ h^{-1} at the lowest studied dilution rate of 0.03 h^{-1}. Increased dilution rates resulted in increased cell mass yield, but with a lower lipid fraction. Furthermore, fatty acid composition of the lipids was not affected by dilution rate.

11. Summary and conclusion

The global growth in biodiesel industry has led to the surplus availability of crude glycerol in the world glycerol market. The glycerol price has decreased tremendously owing to its surplus availability. Though the reports are available for combustion of glycerol to generate energy in an easy manner, the energy yield is low compared to conventional fuels and combustion is difficult at lower and higher temperatures. Glycerol's high viscosity renders it difficult to spray at low temperature. Due to these reasons, the microbial conversion of glycerol into different value added products seem to be a feasible and environment-friendly approach to using glycerol. Although several organisms are reported to produce lipids from glycerol, an economically viable process is presently far from reality. Several organisms have been reported to produce 20-85% lipids of their dry biomass, however, the microbial oil production has been controlled by multiple process parameters such as glycerol utilization, tolerance of high concentration of glycerol, tolerance against crude glycerol born inhibitors, fermentation time, biomass concentration, lipid concentration. Hence, a complete industrial process for lipid production is yet in R&D stage.

In order to develop a stable process, the factors mentioned above must be addressed and a thorough research of physical and biochemical parameters and genetic engineering approach must be conducted so as to understand the mechanism of glycerol utilization by different organisms. Yeasts gained more attention over filamentous fungi owing to fast growth rate, ease of handling, efficient lipid accumulation, and knowledge of the basic biochemistry of lipid synthesis, etc. In general, the future of microbial oil for bioenergy application holds great potential and several potential organisms have been identified for the same although the process development is still under laboratory scale. To overcome the bottlenecks associated with the

process optimizing of culture parameters and strain improvement in terms of high biomass and lipid production must be prioritized.

12. Future perspectives

At the present stage, the lipid production from microbial fermentation reached its threshold to become an industrial reality. However, the major concerns with the production cost strictly depend on the carbon and operation cost. Based on the available literature, the main research needed is in finding very cheap (low or zero cost) substrate to produce lipid, which will lead to a successful process. The waste streams from municipal waste, agricultural residues, and food industry effluents could serve as carbon and nutrient sources for microbial growth and lipid production. Even though high biomass and lipid were produced with pure substrate the overall specific product formation still needs improvement. Intermediate nutrient supply (mainly nitrogen) and other co-factors for lipogenic enzymes may lead to high production rates and virtually medium engineering may avoid the genetic modification of strains.

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(1), 108.

Alvarez, H.M., Mayer, F., Fabritius, D., Steinb chel, A. 1996. Formation of intracytoplasmic lipid inclusions by Rhodococcus opacus strain PD630. *Arch Microbiol*, 165, 377-386.

Alvarez, H.M., Steinbuchel, A. 2002. Triacylglycerols in prokaryotic microorganisms. *Appl Microbiol Biotechnol*, 60(4), 367-76.

Amin Inhm, Mohammad A W, Markom M, Peng L C, N., H. 2010. Analysis of deposition mechanism during ultrafiltration of glycerin-rich solutions. *Desalination* 261, 313-320.

André, A., Diamantopoulou, P., Philippoussis, A., Sarris, D., Komaitis, M., Papanikolaou, S. 2010. Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid. *Industrial Crops and Products*, 31(2), 407-416.

Anschau, A., Xavier, M.C., Hernalsteens, S., Franco, T.T. 2014. Effect of feeding strategies on lipid production by Lipomyces starkeyi. *Bioresour Technol*, 157, 214-22.

Asad-ur-Rehman., Wijesekara, R.G., 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), 1072.

Ayoub, M., Abdullah, A.Z. 2012. Critical review on the current scenario and significance of crude glycerol resulting from biodiesel industry towards more sustainable renewable energy industry. *Renewable and Sustainable Energy Reviews*, 16(5), 2671-2686.

Azocar, L., Ciudad, G., Heipieper, H.J., Navia, R. 2010. Biotechnological processes for biodiesel production using alternative oils. *Appl Microbiol Biotechnol*, 88(3), 621-36.

Bati, N., Hammond, E.G., Glatz, B.A. 1984. Biomodification of fats and oils: trialswith Candida lipolytica,. *J. Am. Oil Chem. Soc*, 61, 1743-1746.

Beligon, V., Poughon, L., Christophe, G., Lebert, A., Larroche, C., Fontanille, P. 2015. Improvement and modeling of culture parameters to enhance biomass and lipid production by the oleaginous yeast Cryptococcus curvatus grown on acetate. *Bioresour Technol*, 192, 582-91.

Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.L., Molina-Jouve, C., Nicaud, J.M. 2009a. Yarrowia lipolytica as a model for bio-oil production. *Prog Lipid Res*, 48(6), 375-87.

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

Berg, J.M., Tymoczko, J., Stryer, L. 2002. Acetyl Coenzyme A Carboxylase Plays a Key Role in Controlling Fatty Acid Metabolism. *Biochemistry,NCBI Bookshelf*, 5.

Carol, Z.B., Anne, C.-S., John, C.W., Grover, L.W. 1999. The Biotin Domain Peptide from the Biotin Carboxyl Carrier Protein of Escherichia coli Acetyl-CoA Carboxylase Causes a Marked Increase in the Catalytic Efficiency of Biotin Carboxylase and Carboxyltransferase Relative to Free Biotin*. *The journal of biological chemistry*, 247(45), 31767–31769.

Carraretto, C., Macor, A., Mirandola, A., Stoppato, A., Tonon, S. 2004. Biodiesel as alternative fuel: experimental analysis and energetic evaluations. *Energy*, 29, 2195-2211.

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

Chang, Y.-H., Chang, K.-S., Hsu, C.-L., Chuang, L.-T., Chen, C.-Y., Huang, F.-Y., Jang, H.-D. 2013. A comparative study on batch and fed-batch cultures of oleaginous yeast Cryptococcus sp. in glucose-based media and corncob hydrolysate for microbial oil production. *Fuel*, 105, 711-717.

Chaudhary., N., Ngadi, M.O., Simpson, B. 2012. comparison-of-glucose-glycerol-and-crude-glycerol-fermentation-by-escherichia-coli K12. *J Bioproces Biotechniq*(S-1).

Cheirsilp, B., Louhasakul, Y. 2013. Industrial wastes as a promising renewable source for production of microbial lipid and direct transesterification of the lipid into biodiesel. *Bioresour Technol*, 142, 329-37.

Choi, W.J., M.R, H., Chan , W.H., Yeo, S.S. 2011. Ethanol production from biodiesel-derived crude glycerol by newly isolated Kluyveracryocrescens. *Applied Microbial biotechnology* 89(4), 1255-1264.

Christophe, G., Kumar, V., Régis, N., Geneviève, G., Pierre, F., Ashok, P., Carlos, R., Soccol,, Christian, L. 2012. Recent Developments in Microbial Oils Production: a Possible Alternative to Vegetable Oils for Biodiesel Without Competition with Human Food? *Brazilian archives of biology and technology*, 55(1), 29-46.

CRFA. 2014. From biofuels to bioeconomy. *Canadian renewable fuel Association*

Damen, B. Bioenergy and food security in ASEAN:An FAO perspective.

Demirbas, A. 2009. Progress and recent trends in biodiesel fuels. *Energy Conversion and Management*, 50(1), 14-34.

Denver, J.P., Rafael, A.G., Zhiyou, W. 2008. Producing docosahexanoic acid-rich algae from biodiesel derived-crude glycerol. *j.Agric. Food Chem*, 56, 3933–3939

Dufreche, S., Hernandez, R., French, T., Sparks, D., Zappi, M., Alley, E. 2007. Extraction of Lipids from Municipal Wastewater Plant Microorganisms for Production of Biodiesel. *Journal of the American Oil Chemists' Society*, 84(2), 181-187.

Esra, U.K., Antoine, T., Colin, W. 2013. Microbial oil produced from biodiesel by-products could enhance overall production. *Bioresour Technol*, 129, 650-4.

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 and Bioenergy*, 33(4), 573-580.

Fickers, P., Benetti, P.H., Wache, Y., Marty, A., Mauersberger, S., Smit, M.S., Nicaud, J.M. 2005. Hydrophobic substrate utilisation by the yeast Yarrowia lipolytica, and its potential applications. *FEMS Yeast Res*, 5, 527-543.

Fisher, D.J., Fernandez, R.E., Adams, N.E., Maurelli, A.T. 2012. Uptake of biotin by Chlamydia Spp. through the use of a bacterial transporter (BioY) and a host-cell transporter (SMVT). *PLoS One*, 7(9), e46052.

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.

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

Gao, D., Zeng, J., Zheng, Y., Yu, X., Chen, S. 2013. Microbial lipid production from xylose by Mortierella isabellina. *Bioresour Technol*, 133, 315-21.

Ghosh, P., Ghose, T.K. 2003. Bioethanol in India: recent past and emerging future. *Adv. Biochem. Eng. Biotechnol*, 85(1-27), 1.

Gong, Z., Wang, Q., Shen, H., Hu, C., Jin, G., Zhao, Z.K. 2012. Co-fermentation of cellobiose and xylose by Lipomyces starkeyi for lipid production. *Bioresour Technol*, 117, 20-4.

Gonzalez-Pajuelo, M., Meynial-Sallesb, I., Mendes, F., Andrade, J.C., Vasconcelos, I., Soucaille, P. 2005. Metabolic engineering of Clostridium acetobutylicum for the industrial production of 1,3-propanediol from glycerol. *Metabolic Engineering*, 7(5), 329-336.

Huang, C., Zong, M.H., Wu, H., Liu, Q.P. 2009. Microbial oil production from rice straw hydrolysate by Trichosporon fermentans. *Bioresour Technol*, 100(19), 4535-8.

Huang, X., Wang, Y., Liu, W., Bao, J. 2011. Biological removal of inhibitors leads to the improved lipid production in the lipid fermentation of corn stover hydrolysate by Trichosporon cutaneum. ***Bioresour Technol*****, 102(20), 9705-9.**

Hughes, E., Benemann, J. 1997. Biological fossil CO2 mitigation. ***Energy Convers Manag*****, 38, 467-437.**

Huijberts, P.A.E.P.M.á.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.**

Kalam, A., Abul, Abu, Y., Atia, F., Md, M., Hasan, Md, R., Karim, Asraful, J. 2014. Production of Microbial Lipids from Rice Straw Hydrolysates by Lipomyces starkeyi for Biodiesel Synthesis. ***Journal of Microbial & Biochemical Technology*****, S8(01).**

Karatay, S.E., Donmez, G. 2010. Improving the lipid accumulation properties of the yeast cells for biodiesel production using molasses. ***Bioresour Technol*****, 101(20), 7988-90.**

Kurosawa, K., Boccazzi, P., de Almeida, N.M., Sinskey, A.J. 2010. High-cell-density batch fermentation of Rhodococcus opacus PD630 using a high glucose concentration for triacylglycerol production. ***J Biotechnol*****, 147(3-4), 212-8.**

Li, Q., Du, W., Liu, D. 2008. Perspectives of microbial oils for biodiesel production. ***Appl Microbiol Biotechnol*****, 80, 749-756.**

Li Y-h, L.B., Sun Y, Z-b Z, F-w B 2005. Screening of oleaginous yeasts for broad-spectrum carbohydrates assimilating capacity. ***Chin J Biotechnol*** **25, 43-48.**

Li, Y., Zhao, Z., Bai, F. 2007a. High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture. ***Enzyme and Microbial Technology*****, 41(3), 312-317.**

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

Lian, J., Garcia-Perez, M., Chen, S. 2013. Fermentation of levoglucosan with oleaginous yeasts for lipid production. ***Bioresour Technol*****, 133, 183-9.**

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(19), 7581-6.**

Liang, Y., Sarkany, N., Cui, Y. 2009. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. ***Biotechnol Lett*****, 31(7), 1043-9.**

Lin, H., Cheng, W., Ding, H.T., Chen, X.J., Zhou, Q.F., Zhao, Y.H. 2010. Direct microbial conversion of wheat straw into lipid by a cellulolytic fungus of Aspergillus oryzae A-4 in solid-state fermentation. ***Bioresour Technol*****, 101(19), 7556-62.**

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(4), 184-8.**

Ling, J., Nip, S., Cheok, W.L., de Toledo, R.A., Shim, H. 2014. Lipid production by a mixed culture of oleaginous yeast and microalga from distillery and domestic mixed wastewater. ***Bioresour Technol*****, 173, 132-9.**

Liu, X., Mortensen, U.H., Workman, M. 2013. Expression and functional studies of genes involved in transport and metabolism of glycerol in Pachysolen tannophilus. ***Microb Cell Fact*****, 12, 27-36.**

Liu, Y., Wang, Y., Liu, H., Zhang, J. 2015. Enhanced lipid production with undetoxified corncob hydrolysate by Rhodotorula glutinis using a high cell density culture strategy. ***Bioresour Technol*****, 180, 32-9.**

Marilyn, G.W., Kari, K., Merja, P., Laura, R. 2013. Lipid production in batch and fed-batch cultures of Rhodosporidium toruloides from 5 and 6 carbon carbohydrates. ***AMB Express*****, 3(58), 1-9.**

McCoy, M. 2005. An unlikely impact: growth of biodiesel has big implications for the oleochemical industry. ***Chem Eng News*****, 83(8), 19-20.**

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

Mhairi, W., Philippe, H., Jette, T. 2013. Comparing cellular performance of Yarrowia lipolytica during growth on glucose and glycerol in submerged cultivations. ***AMB Express*****, 3(58), 1-9.**

Mohan, G.V.S.S.V. 2014. Sustainable biodiesel production through bioconversion of lignocellulosic wastewater by oleaginous fungi. ***Biomass Conv. Bioref.***

Mondala, A.H., Hernandez, R., French, T., McFarland, L., Santo Domingo, J.W., Meckes, M., Ryu, H., Iker, B. 2012. Enhanced lipid and biodiesel production from glucose-fed activated sludge: Kinetics and microbial community analysis. ***AIChE Journal*****, 58(4), 1279-1290.**

Munch, G., Sestric, R., Sparling, R., Levin, D.B., Cicek, N. 2015. Lipid production in the under-characterized oleaginous yeasts, Rhodosporidium babjevae and Rhodosporidium diobovatum, from biodiesel-derived waste glycerol. ***Bioresour Technol*****, 185, 49-55.**

Naganuma, T., Uzuka, Y., Tanaka, K. 1985. Physiological factors affecting total cell number and lipid content of the yeast, Lipomyces starkeyi ***J Gen Appl Microbiol*****, 31, 29-37.**

Papanikolaou, S., Aggelis, G. 2002. Lipid production by Yarrowia lipolytica growing on industrial glycerol in a single-stage continuous culture. ***Bioresour Technol*****, 82(1), 43-9.**

Papanikolaou, S., Aggelis, G. 2011. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. ***European Journal of Lipid Science and Technology*****, 113(8), 1031-1051.**

Papanikolaou, S., Chatzifragkou, A., Fakas, S., Galiotou-Panayotou, M., Komaitis, M., Nicaud, J.-M., Aggelis, G. 2009. Biosynthesis of lipids and organic acids by Yarrowia lipolytica strains cultivated on glucose. ***European Journal of Lipid Science and Technology*****, 111(12), 1221-1232.**

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 and Bioenergy*****, 32(1), 60-71.**

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., Marc, I. 2002. Yarrowia lipolytica as a potential producer of citric acid from raw glycerol. ***Journal of Applied Microbiology*****, 92, 737-744.**

Patel, A., Pravez, M., Deeba, F., Pruthi, V., Singh, R.P., Pruthi, P.A. 2014. Boosting accumulation of neutral lipids in Rhodosporidium kratochvilovae HIMPA1 grown on hemp (Cannabis sativa Linn) seed aqueous extract as feedstock for biodiesel production. ***Bioresour Technol*****, 165, 214-22.**

Petersen, T.W.a.G., Bozell, A.A.a.J., Holladay, J., White, J., Manheim, A. 2004. Top Value Added Chemicals from Biomass Volume I—Results of Screening for Potential Candidates from Sugars and Synthesis Gas. 1.

Poli, J.S., da Silva, M.A., Siqueira, E.P., Pasa, V.M., Rosa, C.A., Valente, P. 2014. Microbial lipid produced by Yarrowia lipolytica QU21 using industrial waste: a potential feedstock for biodiesel production. ***Bioresour Technol*****, 161, 320-6.**

Ramalingam, S., Stephen, D., Mark, Z., Bajpai., R. 2010. Microbial lipids from renewable resources: production and characterization ***J Ind Microbiol Biotechnol*****, 37, 1271-1287.**

Ratledge, C., Wynn, J.P. 2002. The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms. ***Advances In Applied Microbiology*****, 51.**

Research, B. 2012. Continuous Glucose Monitoring (CGM): Technologies and Global Markets.

Ruan, Z., Zanotti, M., Wang, X., Ducey, C., Liu, Y. 2012. Evaluation of lipid accumulation from lignocellulosic sugars by Mortierella isabellina for biodiesel production. ***Bioresour Technol*****, 110, 198-205.**

Rumbold, K., van Buijsen, H.J., Overkamp, K.M., van Groenestijn, J.W., Punt, P.J., van der Werf, M.J. 2009. Microbial production host selection for converting second-generation feedstocks into bioproducts. ***Microb Cell Fact*****, 8, 64.**

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 Biochemistry*, 46(1), 210-218.

Seo, Y., Lee, I., Han, J. 2013. Cultivation and lipid production of yeast Cryptococcus curvatus using pretreated waste active sludge supernatant. *Bioresour Technol*, 135, 304-8.

Shen, Q., Lin, H., Zhan, J., Wang, Q., Zhao, Y. 2013. Sweetpotato vines hydrolysate induces glycerol to be an effective substrate for lipid production of Trichosporon fermentans. *Bioresour Technol*, 136, 725-9.

Shuib, S., Nawi, W.N., Taha, E.M., Omar, O., Kader, A.J., Kalil, M.S., Hamid, A.A. 2014. Strategic feeding of ammonium and metal ions for enhanced GLA-rich lipid accumulation in Cunninghamella bainieri 2A1. *ScientificWorldJournal*, 2014, 173574.

Sriwongchai, S. 2014. Batch and Fed-Batch Cultivations of Oleaginous Bacterium Rhodococcus erythropolis for Lipid Production Using Glucose Medium with Auxiliary Nitrogen Source. *IPCBEE*, 70(19), 99-103.

Stelmachowski, M. 2011. Utilization of glycerol, a by-product of the transestrification process of vegetable oils: A review. *Ecological Chemistry And Engineering S*, 18(1), 9-30.

Streit, W.R., Entcheva, P. 2003. Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production. *Appl Microbiol Biotechnol*, 61(1), 21-31.

Tan, H.W., Abdul Aziz, A.R., Aroua, M.K. 2013. Glycerol production and its applications as a raw material: A review. *Renewable and Sustainable Energy Reviews*, 27, 118-127.

Thompson, J.C., He, B.B. 2006. Characterization of crude glycerol from biodiesel production from multiple feedstocks. *Appl. Eng. Agric*, 22, 261-265.

Tsigie, Y.A., Wang, C.Y., Kasim, N.S., Diem, Q.D., Huynh, L.H., Ho, Q.P., Truong, C.T., Ju, Y.H. 2012. Oil production from Yarrowia lipolytica Po1g using rice bran hydrolysate. *J Biomed Biotechnol*, 2012, 378384.

Tsigie, Y.A., Wang, C.Y., Truong, C.T., Ju, Y.H. 2011. Lipid production from Yarrowia lipolytica Po1g grown in sugarcane bagasse hydrolysate. *Bioresour Technol*, 102(19), 9216-22.

Tsobanakis, P. 2007. Cargill Incorporate, presented at biofuels and feed stocks,Indonesia.

Vajpeyi, S., Chandran, K. 2015. Microbial conversion of synthetic and food waste-derived volatile fatty acids to lipids. *Bioresour Technol*, 188, 49-55.

Walker, G.M., Chichester, J.R. 1997. Yeast Physiology and Biotechnology. *John Wiley and Sons*.

Wang, G.Y., Chi, Z., Song, B., Wang, Z.P., Chi, Z.M. 2012. High level lipid production by a novel inulinase-producing yeast Pichia guilliermondii Pcla22. *Bioresour Technol*, 124, 77-82.

Wei, Y., Zheng, C., Ning, Z. 2008. Research progress of surfactants as fermentation accelerator. *Chem. Ind. Eng. Prog*, 27, 983-989.

Woon-Yong, C., Sung-Ho Oh, Yong-Chang Seo, Ga-Bin Kim, Do-Hyung Kang, Shin-Young Lee, Kyung-Hwan Jung, Jeong-Sub Cho, Ju-Hee Ahn, Geun-Pyo Choi, Lee, H.-Y. 2011. Effects of Methanol on Cell Growth and Lipid Production from Mixotrophic Cultivation of Chlorella sp. *Biotechnology and Bioprocess Engineering* 16, 946-955.

Wu, S., Hu, C., Jin, G., Zhao, X., Zhao, Z.K. 2010. Phosphate-limitation mediated lipid production by Rhodosporidium toruloides. *Bioresour Technol*, 101(15), 6124-9.

Wu, S., Zhao, X., Shen, H., Wang, Q., Zhao, Z.K. 2011. Microbial lipid production by Rhodosporidium toruloides under sulfate-limited conditions. *Bioresour Technol*, 102(2), 1803-7.

Wynn, J.P., Aidil bin Abdul Hamidt, Ratledge, C. 1999. The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. *Microbiology*, 145, 1911-1917.

Wynn, J.P., Ratledge, C. 2005. Oils from microorganisms. *Bailey's industrial oil and fat products*, 6(6).

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. *Biochemical Engineering Journal*, 65, 30-36.

Xue, F., Gao, B., Zhu, Y., Zhang, X., Feng, W., Tan, T. 2010. Pilot-scale production of microbial lipid using starch wastewater as raw material. *Bioresour Technol*, 101(15), 6092-5.

Yang, X., Jin, G., Gong, Z., Shen, H., Song, Y., Bai, F., Zhao, Z.K. 2014. Simultaneous utilization of glucose and mannose from spent yeast cell mass for lipid production by Lipomyces starkeyi. *Bioresour Technol*, 158, 383-7.

Yang, X., Jin, G., Wang, Y., Shen, H., Zhao, Z.K. 2015. Lipid production on free fatty acids by oleaginous yeasts under non-growth conditions. *Bioresour Technol*, 193, 557-62.

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

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(1), 71-4.

Yousuf, A. 2012. Biodiesel from lignocellulosic biomass-prospects and challenges. *Waste Manag*, 32(11), 2061-7.

Zhang, X., Yan, S., Tyagi, R.D., Surampalli, R.Y., Valero, J.R. 2014a. Ultrasonication aided in-situ transesterification of microbial lipids to biodiesel. *Bioresour Technol*, 169, 175-80.

Zhang, Z., Zhang, X., Tan, T. 2014b. Lipid and carotenoid production by Rhodotorula glutinis under irradiation/high-temperature and dark/low-temperature cultivation. *Bioresour Technol*, 157, 149-53.

Zhao, X., Kong, X., Hua, Y., Feng, B., Zhao, Z.K. 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.

Zhu, L.Y., Zong, M.H., Wu, H. 2008. Efficient lipid production with Trichosporon fermentans and its use for biodiesel preparation. *Bioresour Technol*, 99(16), 7881-5.

Chapter-III

Research Article-1

A substrate-based approach for the selection of oil bearing heterotrophs from nitrogen deficient soil for lipid production

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RÉSUMÉ

Dans cette étude, neuf isolats de levure hétérotrophes ont été testés pour leur capacité à assimiler le glycérol brut et successivement le convertir en Try Acyl Glycerides (TAG). Tous les organismes ont d'abord été essayés sur un milieu de sélection à base de glycérol brut et ceux qui ont produit des globules de lipide ont été évalués après pour la production de lipide. La coloration Sudan Black B de huit isolats a montré des globules de lipide. Ces souches ont été étudiées après sur différents rapports de C/N. L'identification moléculaire a révélé que les isolats appartiennent au même genre que *Candida et Yarrowia*. Parmi ces isolats, SKY7 (*Y.lipolytica*) a produit jusqu'à 42,04 ± 0,11% de lipide W/W) avec un rapport C/N de 100 et un temps de fermentation de 72 heures. Les autres souches ont produit 5,82 ± 0,4 à 34,57 ± 0,44% de lipide (W/W). Le profil lipidique GC-FID a montré que le lipide produit par les souches avait une ressemblance étroite avec l'huile végétale et pourrait servir comme matière première pour la production du biodiesel. Le test BIOLOG des isolats a révélé un large spectre d'utilisation du carbone.

Mots clés : La production de lipide, le glycérol brut, biomasse, Biologie

Abstract

In this study, nine heterotrophic yeast isolates were tested for their ability to assimilate crude glycerol and consecutively conversion to Try Acyl Glycerides (TAGA). All the organisms were initially screened on crude glycerol-based selection media and those producing lipid globules were further evaluated for lipid production. Sudan black B staining of eight isolates showed lipid globules. These strains were further studied at different C/N ratio. The molecular identification revealed that the isolates belonged to the genera of *Yarrowia* and *Candida*. Among these isolates SKY7 *(Y.lipolytica*) produced up to 42.04±0.11% of lipid w/w) with a C/N ratio of 100 and fermentation time of 72h. The other strains produced 5.82±0.4 to 34.57±0.44% lipid (W/W). The GC-FID lipid profile showed that the lipid produced by the strains had close resemblance with vegetable oil and could serve as a feedstock for biodiesel production. BIOLOG test of the isolates revealed a wide spectrum of carbon utilization.

Keywords. Lipid production, crude glycerol, Biomass,Biolog

Introduction

Glycerol is generated as a major by-product of the biodiesel industry and has become one of the important renewable feedstocks for biodiesel production (1). Apart from biodiesel industry, saponification, alcoholic beverage production units, and petrochemical industries are the other main sources of glycerol (2). Considering a large global production of glycerol, it has become one of the low-cost feedstock for various applications such as lipid production by heterotrophic microbes, 1,3-propanediol production by several prokaryotic microbes (3). At present scenario eukaryotic microbes have been investigated to produce several values added products from crude glycerol generated from various industries. Among which lipid fermentation using heterotrophic eukaryotic microbes gained more attention encompassed by researchers and several attempts were made to produce lipids(4), food grade pigments(5), flavors, and poly-hydroxy-alkanoates (6).

Despite the microbial lipids promise for future energy security, at present the vegetable oils, used cooking oil and animal fats are used as the main sources for biodiesel production (7). Due to food versus fuel conflict, the cost of biodiesel production from fresh vegetable oil is expensive. Though used cooking oil and animal fat are utilized, availability and collection are the main challenges. Above all the composition of used cooking oil plays a key role in transesterification process. The presence of high free fatty acid content results in high soap formation and water content reduces the conversion efficiency and quality of biodiesel(8) . Due to the above-stated reasons, the microbial oil production stands on the top. Characteristically microbial oil consists similar composition profile as plant based oil and there is no food versus fuel conflict (9). Meanwhile, the cost of microbial oil production is considerably reduced due to the use of organic waste, which offers clean energy along with environmental clean-up with low cost.

Among heterotrophic microbes, yeast strains are considered efficient in lipid production when compared to filamentous fungi and they have several advantages such as fast growth rate, high rate of lipid accumulation, wide spectrum carbon utilization, and low shear rate during fermentation (10). At present, the strains from the genera of *Yarrowia, Cryptococcus, Lypomysis, Rodoturella, Rhodococcus,* and *Candia* have been explored for Try Acyl Glycerides (TGA) production(11) . On the other hand, in recent years, new strains belonging to the above genera producing higher content of TGA were reported by various authors (12, 13). Though several studies were reported for lipid production using lipogenic microbes, the commercial level

process is in its infancy. Therefore, the aim of the current study is to identify potent oil bearing heterotrophic microbial strains from carbon-rich environments and utilizing those isolates to convert glycerol to lipids. In the present study 9 glycerol, positive environmental yeast strains were isolated from wood shavings and virgin forestry samples from Quebec region of Canada and the isolates were further investigated on biodiesel industry base crude glycerol for TGA production.

1. Materials and methods

1.1 sample collection

Soil samples were collected from lake shores, forest areas-(soil under rotten woods) and soil deposited with wood shaving or with pulp and paper waste from different regions in the province of Quebec. Nearly 50 soil samples were collected, numbered and preserved at 4ºC for further studies. Crude glycerol used in the studies was collected from biodiesel producing company in Canada.

1.2 Screening of oleaginous microbial strains

From the collected samples 1g of each sample was aseptically transferred into an Erlenmeyer flask containing 25mL of glycerol enriched medium (GEM) with following composition: 20 g/L crude glycerol, 1.0g/L$(NH_4)_2SO_4$, 1g/L KH_2PO_4, 0.5g/L MgSO4.7H2O, 0.2g/L yeast extract. The samples were incubated for 24h at 28^0C with an agitation rate of 180 rpm (revolution per minute). Then the enriched samples were serially diluted using (10^3 to 10^5 dilution) 0.8% saline solution and spread plated on selection media. The selection media used had the following composition. 2% (w/v) crude glycerol source, 0.3g/L yeast extract, 1g/L KH_2PO_4, 0.5g/L $MgSO_4$, 50mg/L chloramphenicol and 20g/L agar (14). The plates were inoculated at 28^0C for 72h. Based on the morphological differences, fungal and yeast colonies were separated from the mother plate and the cultures were purified to single culture by subsequent subculturing. All the analytical chemicals and reagents used in the study were purchased from VWR, Fisher Scientific and Sigma-Aldrich, Canada unless specified otherwise.

The pure colonies were further grown on GEM for 72h. Samples of 0.1mL were withdrawn (for each strain) and microscopic analysis was performed using Sudan black B. Smear of each microbe was prepared, air dried and heat fixed on a clean slide and the entire smear was flooded with Sudan black solution (0.3 g of the powdered stain in 100 ml of 70% ethanol) and allowed

the slide to remain undisturbed at room temperature for 10 minutes. Excess stain was drained off followed by washing with 8% (v/v) acetic acid solution and counterstained with safranin for 30 seconds and washed off the unbound stain with distilled water, air dried and examined under phase contrast microscope with oil immersion. Strains showing fat globules were further subcultured and preserved in glycerol stocks for further studies.

1.3 Biochemical characterization and Identification based on BIOLOG.

BIOLOG provides the metabolic fingerprint of individual organisms based on the carbon source utilization. The isolated heterotrophic yeast and fungal strains were further purified and evaluated. A pure inoculum was prepared based on the protocol provided by the manufacturer (BIOLOG Inc. USA). Then the inoculum was transferred to the 96 well plate coated with different substrates in each well. The plates were incubated at 28^0C for 72h and the plates were read in the micro station at each 12 h intervals until the isolate provided the species level identification(15) . The negative plates were discarded and the culture was re-sub cultured into pure form and the assay was performed again as described above.

1.4 DNA isolation and PCR (Polymerase Chain Reaction) amplification of Internal Transcribed Spacer region (ITS)

The pure cultures were inoculated in YPD broth (50ml) and incubated for 48h at 28^0C with an agitation rate of 150rpm. Each well-grown culture was centrifuged and washed with sterile distilled water and transferred into a sterile falcon tube. The DNA extraction was performed according to the method described by Zhou et al(16) . The 18s, rDNA region was amplified for each strain using the primers ITS-F (5-TCCGTAGGTGAACCTGCG-3) and ITS-4 (5-TCCTCCGCTTATTGATATGC-3) (17). The reaction was carried out as follows. PCR master mixture 12.5 µl (Promega USA), forward primer 2.5 µl, and reverse primer 2.5 µl (Invitrogen USA), template DNA 3µl and the volume was adjusted to 25 µl using nuclease-free water. The PCR product was run on a 1% (w/v) agarose gel and the amplicons were excised and the DNA was purified using Qiagen gel extraction kit. The purified amplicons were sequenced and BLASTED (Basic Local Alignment Search Tool) on NCBI (National Center for Biotechnology Information).

1.5 Inoculum preparation and fermentation

A loop full of each pure culture was inoculated into 10 mL YPD broth and incubated for 48h. The well-grown inoculum was aseptically transferred into the fermentation medium and 10% of the inoculum was used in all the cases. All fermentation experiments were conducted in duplicate in 1L Erlenmeyer's flask with 200 mL working volume and all the cultures were screened at two different C/N ratio. (C/N ratio 100:1, 200:1). The media was supplemented with 100g/L crude glycerol, 2.7g/L KH_2PO_4, 1.0g/L $MgCl_2$, 1.0g/L $Na_2H_2PO_4$ and yeast extract to achieve required C/N ratio. The medium was sterilized in an autoclave at 121^0C for 15 minutes. The sterilized medium in each flask was inoculated with individual pure culture and incubated at 28^0C with an agitation rate of 180 rpm for 120h; samples were withdrawn at every 24h intervals. The collected samples were stored at 4^0C until used for further analysis.

1.6 Biomass Estimation

A Sample of fermentation broth (25mL) was transferred into a clean centrifuge tube and centrifuged at 8000 rpm for 10 minutes. The supernatant was collected into a clean tube and the biomass (pellet) was transferred into a pre-weighed aluminum pan and placed at 105^0C in a hot air oven. After achieving a constant weight the biomass concentration was calculated as described below. The supernatant portion was further used for glycerol estimation.

$$Biomass\ \left(\frac{g}{L}\right) = -\frac{W0 - W1}{Volume\ of\ smple}$$

Where W0, the weight of the aluminum pan, W1, the weight of sample with an aluminum pan.

1.7 Extraction of lipids and lipid profiling with Gas Chromatography

25 mL of fermentation broth was transferred to a clean centrifuge tube and it was centrifuged at 8000 rpm for 10 minutes. The biomass pellet was collected and washed twice with distilled water and used for lipid extraction. To extract the lipids the biomass was transferred to a 20mL bead beater (Biospec bead beater model : HBB908) vessel and adequate amount of 0.5mm zirconium beads were added followed by addition of 15mL of chloroform and methanol (2:1 ratio) and the extraction was carried out for 3 minutes at room temperature. The supernatant was collected by filtering the mixture using 0.2 µm Whatman filter paper (Whatman, Kent, England) aided with a vacuum filtration apparatus. The extraction was repeated with 1:1 chloroform and methanol mixture and the supernatants were pooled in a pre-weighed glass tube. To the supernatant 1/3

volume of distilled water was added and then centrifuged at 4000rpm in order to obtain a phase separation. The upper phase containing water and methanol was discarded and the lower part (containing lipid in chloroform) was concentrated under reduced nitrogen pressure. After complete removal of chloroform, the residual weight was recorded. Fatty acids profiling was determined by GCcoupled with FID (Flame ionization detector) after converting lipid into methyl ester derivatives as previously described (18). Lipid extract (20 µL) was heated for 90 min at 80ºC (in an oil bath) in the presence of 1 ml of 2.5% (v/v) solution of sulfuric acid in methanol containing 50 mg of heptadecanoic acid as an internal standard.

$$Lipid\ \left(\frac{g}{L}\right) = -\frac{T0 - T1}{Volume\ of\ smple}$$

Where T0, the weight of the testtube, W1, the weight of lipid with test tube.

1.8 Residual glycerol estimation

The residual glycerol in the medium was determined by the spectrophotometric method described by Paolo Bondioli et al (19). All the samples were centrifuged and the supernatants were collected in clean falcon tubes. The samples were diluted to get a glycerol concentration of 3.75-15mg/L. Calibration was made with pure glycerol (Sigma-Aldrich). From each diluted sample, 0.5mL solution was pipetted out into a clean test tube and 1.5 ml of 50% v/v ethanol solution was added. To this solution, 1.2 ml of sodium periodate solution (20 mM) was added and after mixing 1.2 mL of acetylacetone solution (0.5mM) was added. The mixture was placed in a pre-heated water bath at 70^0C for one minute and then it was immediately cooled. The OD (Optical Density) was recorded at 410 nm using a spectrophotometer (Varian Cary 500. USA).

2 Results and discussion

2.1 Selection of glycerol positive microbes

The growth of various strains on GEM medium was observed from 12h and the cultures were isolated based on their growth rate (fast or slow growing). Only the colonies visible within 36h were selected for further studies. The glycerol positive yeast and fungal strains were separated and named as follows SKY, SKY7, SKY8, SKY10, SKY28, SKY11, A1, SKY2, and N1, and the fungal strains were named as follows SKF5, SKF9.

2.2 Sudan Black B staining and selection of lipid accumulating microbes

All the above strains were grown on nitrogen deficient media with high glycerol concentration. Due to the limitation in nitrogen, the assimilated glycerol is converted to citric acid inside the mitochondria. The continuous accumulation of citric acid in mitochondria was transported to the cytoplasm where ATP citrate lyase (ACL) cleaves the citric acid into Acetyl Co-A and it enters into fatty acid synthesis pathway, resulting in the production of fat globules in the cytoplasm. Sudan Black B specifically binds with neutral lipids and reflects black to brown spots under a light microscope. In SKY7, N1, SKY8, SKY28 considerably large black spots were observed and in other cases was scattered. Therefore, these strains (with large black spots) were selected for further study.

2.3 Biochemical characterization (BIOLOG)

Metabolic activities of the isolates were examined using Biolog System. As an indicative study to select the suitable organisms for lipid production, the metabolic fingerprint of the isolate plays a vital role (20). Based on the data retrieved from the Biolog system (Table 17) the above isolates were initially screened specifically for glycerol, cellobiose, xylose, and glucose utilization along with the capability of exhibiting resistance towards the inhibitory compounds present in the biodiesel industry derived crude glycerol. The strains named SKY7, SKY28, SKY, SKY8, SKY10, SKY-11, and SKY2, were found to assimilate glycerol and cellobiose in a wide range and shows resistance to acetic acid, propionic acid, and succinic acid. On the other hand A1 strain revealed a wide range of metabolic activity towards 33 different substrates present in the microtiter plate but in the case of N1 it was negative for glycerol utilization and exhibited poor metabolic activity even after 72h of incubation and thus made this strain non-suitable for lipid production in a media containing crude glycerol as sole source of carbon. The detailed substrate utilization behavior of the strains is summarized in Table 17. From the metabolic fingerprint, it is evident that the isolates (except N1) could be potential organisms for lipid production or other value added products production using crude glycerol as a sole carbon source. Moreover, the results also helped to evaluate the carbon sources other than glycerol or glucose and thus reduce the effort for screening the strains for other carbon sources.

Table 17 BIOLOG substrate utilization spectrum of isolates

Sl.No	Substrate	SKY7	SKY 8	SKY	SKY28	A1	N1	SKY 2	SKY10	SKY11
1	Acetic acid	±	±	-	±	±	-	-	-	-
2	Formic acid	-	-	-	-	-	-	-	+	-
3	Propinonic acid	±	-	-	±	-	±	±	-	±
4	Succinic acid	+	+	+	+	+	+	-	+	±
5	Succinicacid Methyl Ester	+	+	+	+	+	-	±	+	-
6	L-Aspartic acid	+	+	+	+	+	-	-	+	-
7	L-Glutamic acid	+	+	+	+	+	-	-	+	-
8	L-Proline	+	+	+	+	+	-	±	+	±
9	D-Gluconic acid	±	+	+	-	+	-	±	+	±
10	Dextrin	±	+	+	+	+	+	+	+	+
11	Inulin	+	+	+	+	+	+	+	+	+
12	Cellobiose	-	+	+	-	+	±	+	+	+
13	Genitobiose	±	+	+	-	+	+	-	+	±
14	L-Maltose	-	+	+	-	+	+	-	-	±
15	D-Maltose	-	+	+	-	+	+	+	+	±
16	Maltotriose	-	±	+	-	+	-	-	+	±
17	D-Melibiose	-	±	+	-	+	-	-	+	-
18	Palatinose	-	+	+	-	+	-	-	+	±
19	D-Raffinose	-	-	+	-	+	-	-	+	-
20	Stachyose	-	-	+	-	+	-	-	+	-
21	Sucrose	-	+	+	-	+	-	-	+	+
22	Trehalose	-	+	+	-	+	-	+	+	+
23	D -(+)-Turanose	-	+	+	-	+	-	-	±	±
24	N-Acetyl-D-Glucosamine	+	+	+	+	+	+	-	+	±
25	a-D-Glucose	+	+	+	+	+	+	+	+	+
26	D-Galactose	±	+	+	-	+	-	-	+	-

27	D-Gsicose	+	+	+	±	+	-	±	+	+
28	L-Sorbose	-	+	+	±	+	±	-	+	-
29	Salicin	-	±	+	-	+	-	+	+	+
30	D-Manitol	-	+	+	±	+	-	±	+	±
31	D-Sorbitol	±	+	+	±	+	-	±	+	+
32	D-Arabitol	-	+	+	+	+	-	±	+	±
33	Xylitol	-	+	±	-	+	-	±	+	+
34	Glycerol	+	+	+	+	+	-	±	+	+
35	Tween 80	+	+	±	+	+	+	-	+	±

2.4 Molecular characterization of the isolates

The ITS region of the fungal genome is the most widely sequenced segment, which is considered as a molecular signature to identify organisms on the species level. ITS region of the fungal genome (rDNA) includes 18s, 5.8S, and 28S which codes for rRNA and is highly variable and conserved. Due to its versatility in nature the variations among the species can be differentiated effectively. PCR amplification yields a 400 to 650BP sequence. The BLAST results show that SKY7 and SKY28 were 99% sequence similarity with *Yarrowia lipolytica* and other isolates were showing 99 % similarity with *Candida membranious*. The sequences were submitted in Genebank (NCBI) and the accession numbers are as follows KF908249, KF908250, KF908251, KF908252, KF908253, KF908254, KF908255, and KF908256. The closest similar sequences were retrieved from the Genebank repository and the pairwise alignment was conducted using ClustalW (EMBL). The phylogenetic relations were revealed and represented in Figure 9.

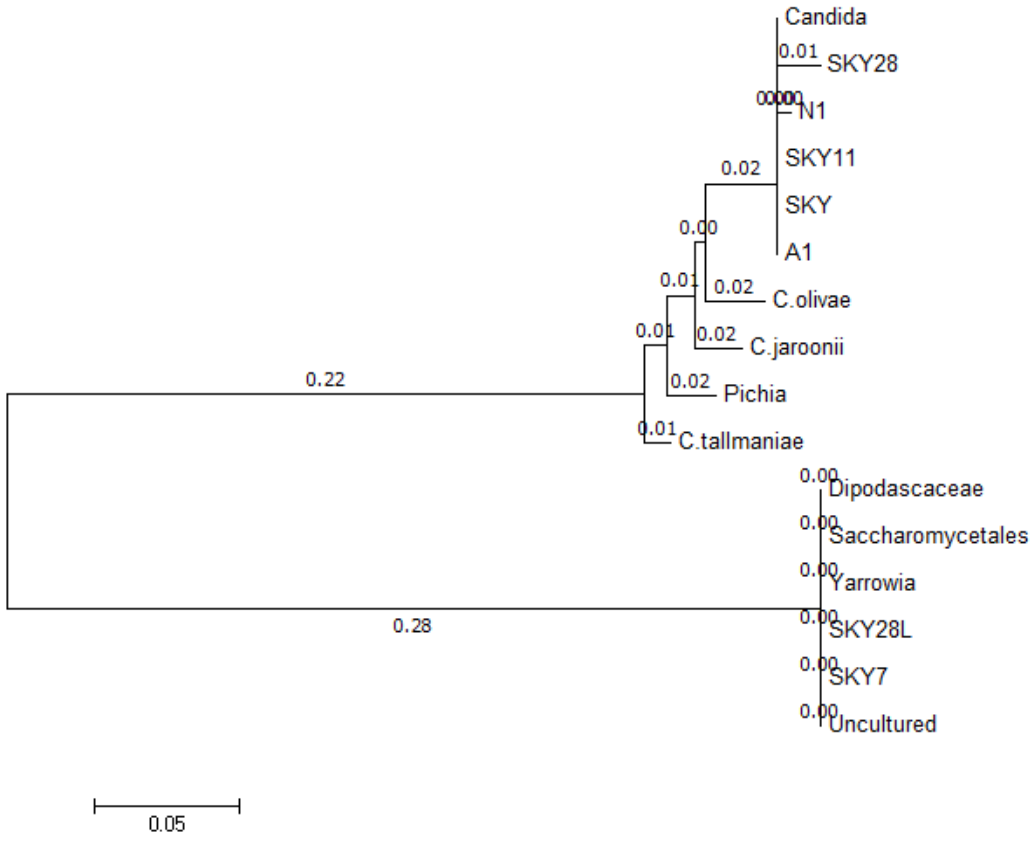

Figure 9 Phylogenetic tree of isolates showing evolutionary relationship with other closely related species

2.5 Effect of C/N ratio and crude glycerol concentration on growth and lipid accumulation.

The crude glycerol as a by-product of biodiesel production usually contains methanol, soap, salts, moisture, free fatty acids and a small amount of fat-soluble compounds. In the present study, the glycerol concentration in the samples was analyzed by chemical oxidation method and the original concentration of glycerol in the sample was estimated as 84.5% (w/w) (21).

In order to check the integrity of all the isolates for the lipid production, the organisms were grown in crude glycerol-based fermentation media supplemented with yeast extract as nitrogen source at two different initial C/N ratio (100, 200). In all cases, the initial glycerol concentration was maintained at 100g/L since the lipid accumulation occurs at high carbon concentration (22). Though the initial glycerol concentration is high the growth was not inhibited except in the case of N1 since it is not a glycerol utilize. The biomass produced by each isolate at both C/N ratios in 72 and 120 hours fermentation time are presented in the Tables 18 &19 respectively. The values

of kinetic parameters (Yx/s- Yield of biomass produced per gram of substrate, Yp/s Yield of lipid produced per gram of substrate, Yp/x Yield of lipid produced per gram of biomass, μ_{max}- Specific growth rate) for each strain are presented in Tables 18 & 19. In all cases, the maximum specific growth rate was significantly decreased when fermentation time increased. Due to the decrease in specific growth rate, the Yx/s was significantly decreased. This phenomenon is critical in the case of lipid accumulation since lipid accumulation accelerates when the specific growth rate depletes. The strains produced a comparable amount of biomass and at the same time, the impurities present in the biodiesel industry derived crude glycerol did not show any significant impact on growth since the concentrations were lower (23). In general, the crude glycerol derived from biodiesel contains methanol, salts, free fatty acids, and trace amounts of heavy metals. Based on the catalytic process the amount of above-stated compounds varies. Among these, the amount of methanol varies from 3 to 15%. In the present study, the crude glycerol used contains 3% of methanol. During the fermentation due to the dilution of crude glycerol, the final concentration of methanol reaches 0.3% (w/v) and which is below the inhibitory limit (24). Maximum biomass accumulation was observed in the case of SKY 11 was 18.47±0.13g/L followed by 16.28±0.5g/L for A1 isolate at 120h of incubation with a C/N ratio of 100. The biomass concentration for other strains varied from 5.04±0.23g/L to 12.56±0.02g/L (Tables 18&19). Meanwhile, in the case of C/N ratio 200 the biomass concentration was moderately low, which could be explained due to the deficiency of nitrogen in the medium. Exceptionally, in the case of SKY7 *(Yarrowia lipolytica),* the biomass produced decreased when the C/N ratio was increased from 100 to 200. *Y. lipolytica* is a known industrially accepted microorganism for the production of citric acid and a similar kind of biomass accumulation was observed by Makri et al (25). On the other hand, low pH of the fermented broth is an indicator of the secretion of acidic metabolites (organic acid). In this case, the pH was decreased from 6.5 to 4.2, which could probably be due to the excretion of citric acid into the fermentation broth.

Table 18 Biomass and lipid production at 100 C/N molar ratio

Sl no	Strain	Age (h)	Biomass (g/L)	Lipid % (w/w)	Glycerol Concentration (g/L)	μ max (h^{-1})	Yp/s (g/g)	Yx/s (g/g)	Yp/x (g/g)
1	SKY	72	9.42 ± 0.11	18.11±0.30	85.75±0.23	0.084	0.062	0.346	0.179
		120	11.72±0.21	19.48±0.66	65.54±0.51	0.052	0.023	0.116	0.2
2	SKY7	72	7.53±0.27	42.04±0.11	59.83±0.33	0.037	0.071	0.158	0.45
		120	6.99±0.04	37.24±0.98	50.44±0.21	0.023	0.034	0.073	0.459
3	SKY 8	72	8.52±0.59	21.73±0.47	74.630.26	0.037	0.052	0.227	0.23
		120	12.56±0.02	23.53±0.26	63.46±0.39	0.026	0.029	0.117	0.244
4	SKY 10	72	9.56±0.08	18.26±0.52	70.67±0.18	0.039	0.05	0.261	0.19
		120	11.85±0.04	23.15±0.16	62.34±0.82	0.025	0.027	0.11	0.241
5	SKY 11	72	13.23±0.06	5.82±0.40	71.67±0.42	0.092	0.021	0.386	0.055
		120	18.47±0.13	8.69±0.29	56.34±0.26	0.058	0.015	0.181	0.084
6	SKY 28	72	4.45±0.16	26.28±1.28	76.68±0.34	0.022	0.025	0.071	0.32
		120	5.44±0.52	26.97±0.91	52.35±0.33	0.014	0.013	0.04	0.322
7	A 1	72	13.70±0.32	18.50±0.12	81.65±0.85	0.045	0.025	0.362	0.013
		120	16.28±0.50	6.24±0.64	60.62±0.59	0.029	0.013	0.15	0.012
8	N1	72	2.15±0.04	29.24±1.32	98.3±0.4	0.026	0.095	0.284	0.333
		120	5.04±0.23	17.91±0.31	94.23±0.73	0.024	0.01	0.054	0.187
9	SKY A2	72	9.33±0.04	26.33±0.67	73.14±0.11	0.039	0.074	0.264	0.28
		120	9.25±0.05	19.46±0.42	68.38±0.14	0.024	0.018	0.085	0.207

Table 19 Biomass and lipid production at 200 C/N molar ratio

Sl no	Strain	Age (h)	Biomass (g/L)	Lipid % (w/w)	Glycerol conc (g/L)	μ max (h^{-1})	Yp/s (g/g)	Yx/s (g/g)	Yp/x (g/g)
1	SKY	72	9.56±0.08	7.64±0.13	88.49±0.93	0.084	0.027	0.348	0.077
		120	10.57±0.34	19.99±0.47	69.43±0.27	0.051	0.021	0.103	0.2
2	SKY7	72	5.31±0.43	26.53±1.12	67.83±0.24	0.032	0.036	0.121	0.294
		120	6.27±0.23	25.29±0.56	58.44±0.19	0.021	0.016	0.057	0.275
3	SKY 8	72	11.07±0.99	17.02±0.44	78.63±0.34	0.042	0.057	0.316	0.18
		120	12.53±0.21	25.01±0.35	68.46±0.62	0.026	0.031	0.117	0.262
4	SKY 10	72	8.58±0.21	16.57±0.76	76.67±0.27	0.038	0.041	0.234	0.177
		120	10.27±0.08	20.70±0.54	66.34±0.33	0.024	0.021	0.095	0.219
5	SKY 11	72	12.21±0.16	11.34±0.72	78.67±0.3	0.091	0.041	0.356	0.114
		120	16.36±0.04	9.85±0.37	60.34±0.56	0.057	0.16	0.16	0.099
6	SKY 28	72	4.73±0.11	21.10±0.38	77.77±0.51	0.023	0.03	0.105	0.268
		120	5.94±0.23	26.52±1.18	59.47±0.13	0.016	0.015	0.049	0.315
7	A 1	72	11.73±0.04	10.48±0.39	85.65±0.25	0.042	0.025	0.308	0.083
		120	12.38±0.48	13.85±0.33	63.27±0.22	0.026	0.013	0.115	0.117
8	N1	72	1.77±0.06	34.57±0.44	99.36±0.31	0.024	0.143	0.335	0.426
		120	3.61±0.04	13.45±0.37	97.03±0.2	0.02	0.005	0.032	0.148
9	SKY A2	72	11.35±0.01	27.26±0.10	79.14±0.96	0.042	0.105	0.369	0.286
		120	11.71±0.21	22.10±0.30	70.38±0.28	0.025	0.025	0.109	0.232

The organisms, which can grow on nitrogen deficient soil with high carbon content, are known to accumulate various intracellular storage reserve materials. In most of the prokaryotic organisms, the pathway is directed towards different polymeric substances production and in the case of eukaryotic TGA was observed as storage compound. The organisms, which can accumulate more than 20% of storage lipids in their dry biomass, are classified as the oleaginous

organisms. Even though several organisms have been isolated from the soil samples in the present study, only those who grew on crude glycerol were selected for lipid production. For lipid production, nutrient (nitrogen, phosphorus, sulfur, etc.) limitation plays a vital role in diverting the flow of excess carbon into various storage compounds. In the present study limitation of nitrogen was investigated. As the amount of nitrogen depletes in the media, the citrate accumulation inside the mitochondria increases, which is finally transported to the cytoplasm (26). ATP citrate lyase (ACL) is believed to be the primary key enzyme involved in the fatty acid biosynthetic pathway. Though this enzyme was present in several organisms, the cascade of fatty acid synthesis was blocked due to the absence of the consecutive enzymes in the free fatty acids (FFA) synthesis (7). As reported by various researchers (27, 28), the higher lipid production was observed at the elevated carbon concentration with a limited supply of nitrogen, considering this fact the study was conducted with an initial glycerol concentration of 100g/L with a C/N ratio of 100 and 200. The maximum lipid accumulation observed in the case of the isolated SKY7 *(Y.lipolytica)* was 42.04±0.11% (w/w) with a C/N ratio of 100 and followed by N1 34.57%±0.44 (w/w) at C/N ratio 200 and in 72h. On the other hand, *Y.Lipolytica* was reported to produce 45% (w/w) lipid at a glycerol concentration of 102g/L with a C/N ratio of 100 (29). Meanwhile the other isolates accumulated lipid, which varied from 5.82±0.4 to 26.97±0.91% (w/w) with C/N ratio of 100. On the contrary, when C/N ratio was increased to 200, the lipid accumulation also reduced significantly and this could be attributed to the imbalance of carbon concentration during the lipogenic phase. As presented in Table 18, *C.mabraniencis (SKY A2)* is reported for the first time for a lipid accumulation of 27.26% (w/w) at a C/N ratio 200 and fermentation time 72h in the present study.

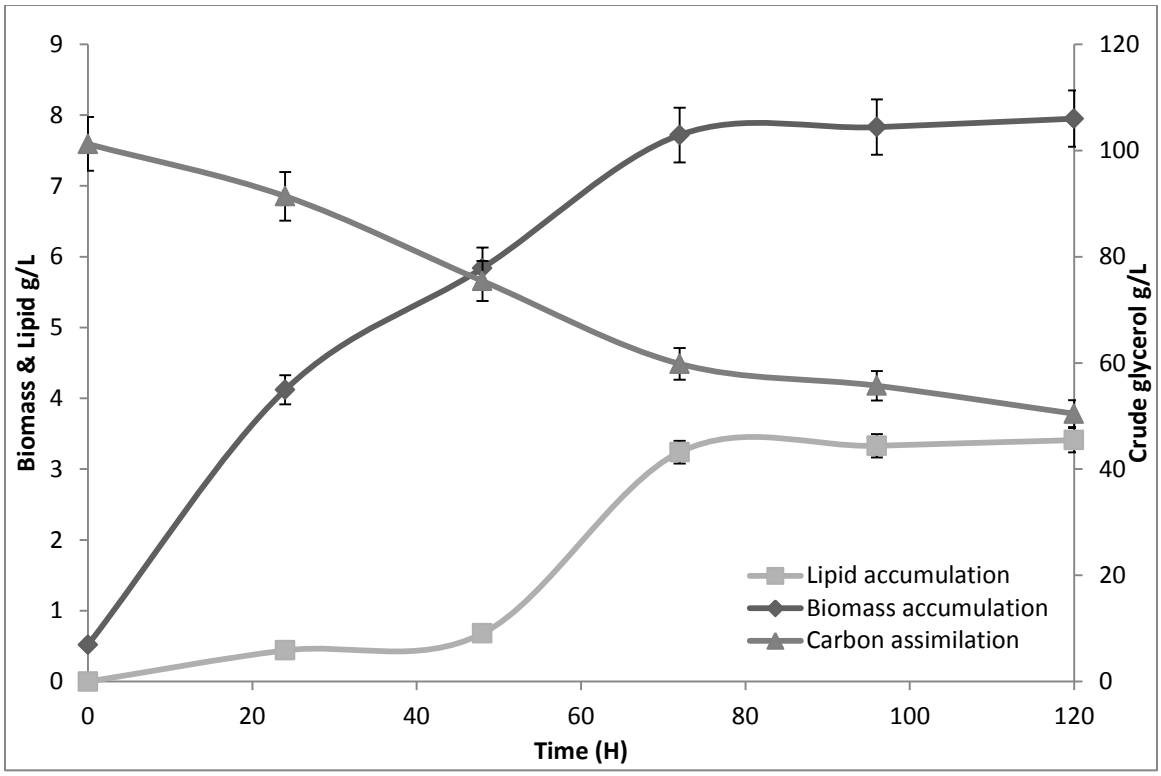

Figure 10 Glycerol assimilation with respect to biomass and lipid accumulation by SKY7

When comparing Yp/s the strains N1, SKY 2, and SKY7 seems a potential candidate for lipid production, but while comparing Yp/x SKY7 has a significant magnitude of improvement with respect to other isolates. Due to above reasons, SKY7 was selected for further studies and Figure 10 presents the biomass, lipid production with respect the glycerol assimilation and fermentation time. From figure 10, it is clear that the lipogenic phase accelerated from 48h to 72h.

The glycerol utilization profile of all the isolates shows a similar trend. Figure 10 represents the glycerol assimilation by the strain *Y.lipolytica* (SKY7). It is observed that the glycerol concentration was reduced from 101.2g/L to 50.4g/L in the case of SKY7 and in all other cases the glycerol concentration was reduced in a range between 50.44-68.38g/L in the case of C/N ratio 100 and 59.4 to 97.03 g/L in the case of C/N 200 (Tables 18 & 19). It is clear that the glycerol assimilation was significantly low after a fermentation period of 72h at the same time the biomass and lipid concentration stagnated. It was reported that most of the oil producing strains assimilate the substrate at a slow rate (30).

2.6. Fatty acid composition

Figure 11 (Chromatogram) represents the lipid produced by SKY7, which is a similar lipid profile observed in the case of SKY, SKY10, SKY 2, SKY11, A1, N1 and SKY 28. It is evident that myristic acid (C 14:0), palmitic acid (C 16: 0) stearic acid (C18:0), linolenic acid (C18:3) and linoleic acid (C18:3) are the main components produced (Table 20). C: 18:1 and C: 18:2 were the major components in the case of SKY7. The above characteristic is observed in lipid profile of *Y. lipolytica* by various authors(26) . These lipids have resemblance with vegetable oil. The presence of polyunsaturated fatty acids (PUFA) makes the isolate (*Y. lipolytica*) as an industrially important organism.

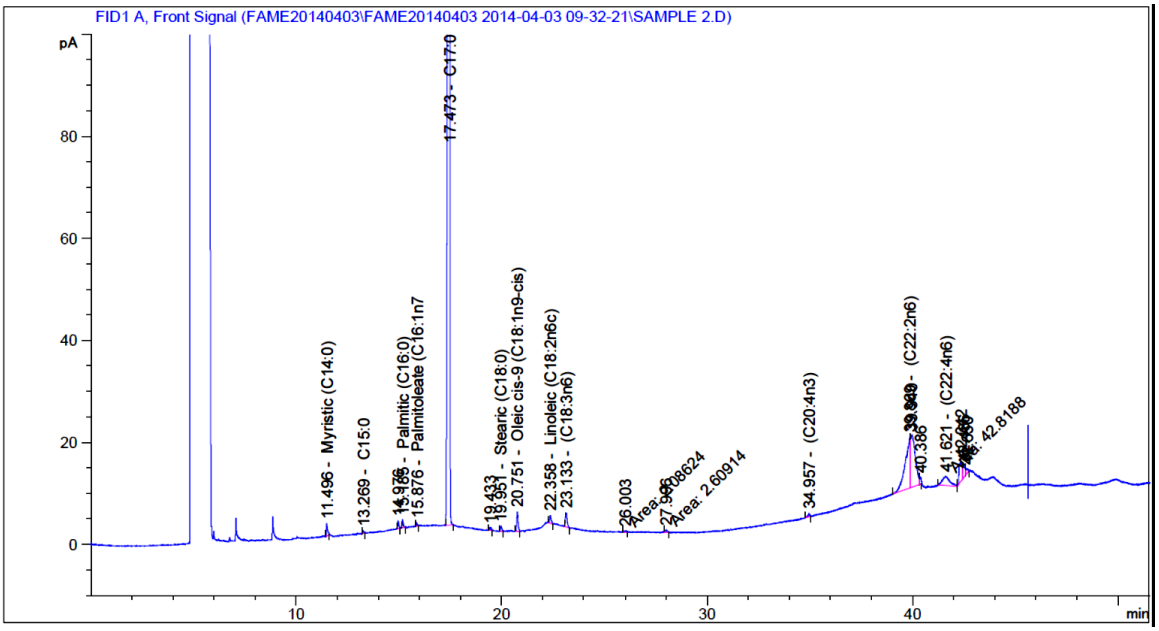

Figure 11 GC-FID profile of TGA produced by SKY7 (Y.lipolytica)

Table 20 Lipid profile of INRS Isolates (percentage of total lipid extracted)

SAMPLE	14:1	16:0	16:1	18:0	18:1	18:3	18:3(N-6)	3 (N-6)
SKY	2.8	25.5	0	8.2	47.6	9.1	0.9	1.5
SKY7	0.6	0	0	3.5	29.2	22.0	0	0
SKY8	4.9	0	1.6	0.6	20.6	18.3	0	0
SKY10	2.4	32.1	1.6	3.5	0.4	3.5	1.5	0
SKY11	0.5	0.6	2.4	2.3	1.3	0.5	0	0
SKY28	2.1	31.0	8.8	9.7	6.2	15.2	0	0
A1	1.5	18.2	45.6	10.6	0.8	9.2	0	0
N1	1.7	15.1	7.0	5.0	37.8	8.4	0	0
SKY2	2.1	35.8	5.6	2.4	0.7	8.3	0	0

3 Summary and Conclusion

Nine newly isolated heterotrophic yeast strains were screened for lipid production. Among all SKY7 *(Y.lipolytica)* was considered to be a potent candidate for lipid production using biodiesel industry derived crude glycerol as the carbon source. Compared to other isolates, *Y.lipolytica* possesses additional characteristics such as citric acid production along with lipid accumulation, which could improve the process economy. The lipid accumulation reached up to 42.04±0.11% (w/w) with an incubation time of 72h at C/N ratio 100. However, still the process needs to be improvised with regard to achieving higher biomass and a higher concentration of lipid accumulation. Since the lipid produced from above isolates was comparable in composition with the vegetable oils, it could be used for biodiesel generation. On the other hand, the citric acid concomitantly generated could be utilized by the food industry since the organism is safe to use at the industrial level.

Acknowledgement

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References

1. Almeida, J. R., Favaro, L. C. and Quirino, B. F. (2012) Biotechnol Biofuels, **5,** 48.
2. Papanikolaou, S. and Aggelis, G. (2002) Bioresour Technol, **82,** 43-49.
3. Sheikh, H. N. B. M. A. H. U. F. M. A. (2008) Acid and Base Catalyzed Transesterification of Animal Fats to Biodiesel. Iran. J. Chem. Chem. Eng., **27,** 41-48.
4. Huijberts, P. A. E. P. M. á. G. N. M. and Eggink, G. (1996) Appl Microbiol Biotechnol, **45,** 575-579.
5. Mantzouridou, F., Naziri, E. and Tsimidou, M. z. (2008) Industrial Glycerol as a Supplementary Carbon Source in the Production of -Carotene by Blakeslea trispora. J Agric Food Chem, **56,** 2668-2675.
6. Mothes, G., Schnorpfeil, C. and Ackermann, J. U. (2007) Engineering in Life Sciences, **7,** 475-479.
7. Papanikolaou, S. and Aggelis, G. (2009) Lipid Technology, **21,** 83-87.
8. Lin, Y.-H., Luo, J.-J., John Hwang, S.-C., Liau, P.-R., Lu, W.-J. and Lee, H.-T. (2011) Biomass and Bioenergy, **35,** 2217-2223.
9. Gwendoline Christophe, V. K., Régis Nouaille, Geneviève Gaudet, Pierre and Fontanille, A. P., Carlos Ricardo Soccol and Christian Larroche. (2012) Recent Developments in Microbial Oils Production: a Possible Alternative to Vegetable Oils for Biodiesel Without Competition with Human Food? Brazilian archives of biology and technology, **55,** 29-46.
10. Azocar, L., Ciudad, G., Heipieper, H. J. and Navia, R. (2010) Appl Microbiol Biotechnol, **88,** 621-636.
11. Ageitos, J. M., Vallejo, J. A., Veiga-Crespo, P. and Villa, T. G. (2011) Appl Microbiol Biotechnol, **90,** 1219-1227.
12. Anschau, A., Xavier, M. C., Hernalsteens, S. and Franco, T. T. (2014) Effect of feeding strategies on lipid production by Lipomyces starkeyi. Bioresour Technol, **157,** 214-222.
13. Chang, Y.-H., Chang, K.-S., Hsu, C.-L., Chuang, L.-T., Chen, C.-Y., Huang, F.-Y. and Jang, H.-D. (2013) A comparative study on batch and fed-batch cultures of oleaginous yeast Cryptococcus sp. in glucose-based media and corncob hydrolysate for microbial oil production. Fuel, **105,** 711-717.
14. Kitcha, S. and Cheirsilp, B. (2011) Energy Procedia, **9,** 274-282.
15. Kornelia, S., Ute, W., Holger, H., Wen-Tso, L. and Larry, F. (1998) Analysis of BIOLOG GN Substrate Utilization Patterns by Microbial Communities. Applied and Environmental Microbiology,, **64,** 1220–1225.
16. Zhou, J., Bruns, M. A. and Tiedje, J. M. (1996) Applied and Enviroinmetal Microbiology, **62,** 316-322.

17. Sequerra, J., Marmeisse, R., Valla, G., Normand, P., Capellano, A. and Moiroud, A. (1997) Mycological Research, **101,** 465-472.
18. Browse, J., McCourt, P. J. and Somerville, C. R. (1986) Analytical Biochemistry, **152,** 141-145.
19. Bondioli, P. and Della Bella, L. (2005) European Journal of Lipid Science and Technology, **107,** 153-157.
20. Garland, J. L. and Mills, A. L. (1991) Applied and Enviroinmetal Microbiology, **57,** 2351-2359.
21. Bondioli, P. and Della, B., Laura. (2005) An alternative spectrophotometric method for the determination of free glycerol in biodiesel. European Journal of Lipid Science and Technology, **107,** 153-157.
22. Asad-ur-Rehman., Wijesekara, R. G., S., , Nomura, N., Sato, S., and 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.
23. Taccari, M., Canonico, L., Comitini, F., Mannazzu, I. and Ciani, M. (2012) Bioresour Technol, **110,** 488-495.
24. Chatzifragkou, A. and Papanikolaou, S. (2012) Appl Microbiol Biotechnol, **95,** 13-27.
25. Makri, A., Fakas, S. and Aggelis, G. (2010) Bioresour Technol, **101,** 2351-2358.
26. Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J. L., Molina-Jouve, C. and Nicaud, J. M. (2009) Prog Lipid Res, **48,** 375-387.
27. Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J. L., Molina-Jouve, C. and Nicaud, J. M. (2009) Yarrowia lipolytica as a model for bio-oil production. Prog Lipid Res, **48,** 375-387.
28. Ratledge, C. and Wynn, J. P. (2002) The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms. Advances In Applied Microbiology, **51**.
29. Papanikolaou, S. and Aggelis, G. (2009) Biotechnological valorization of biodiesel derived glycerol waste through production of single cell oil and citric acid byYarrowia lipolytica. Lipid Technology, **21,** 83-87.
30. Jacob, Z. and Krishnamurthy, M. (1990) Journal of the American Oil Chemists, **67**.

Chapter-IV-Partie-I

Research Article 2

Elucidating the effect of glycerol concentration and C/N ratio on Lipid Production by *Yarrowia lipolytica* SKY7

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RÉSUMÉ

La forte demande de l'énergie renouvelable et l'augmentation de la production du biodiesel ont menés à une disponibilité excédentaire du glycérol brut. Due à cette raison l'addition de la valeur ajoutée d'une base bio du glycérol à différents produits est étudiée, et parmi celles-ci le lipide microbien est attrayant. L'étude présente est consacrée à trouver la concentration du glycérol optimale et le rapport C/N pour produire un maximum de lipide en utilisant *Y.lipolytica SKY7* comme biocatalyseur. La concentration du glycérol (34,4 to168.2) et le rapport C/N (25 à 150) ont été sélectionné pour investigation afin de maximiser la production de lipide. La concentration initiale du glycérol 112.5g/L de glycérol, le rapport molaire de C / N de 100 et une supplémentation de 5% v / v d'inoculum a été jugée optimale pour la production de la biomasse et des lipides. Sur la base des paramètres optimaux ci-dessus, la concentration en lipides de 44,6% p/p et une concentration de biomasse de 14.08g / litre ont été atteintes. Dans le cas de la concentration du glycérol, le maximum de Yp/s (0.192), Yx/s (0,43) a été observée lorsque la concentration du glycérol initiale était 112.5g/l avec un rapport molaire C/N de 100 et un volume d'inoculum de 5% v/v. l'assimilation du glycérol a augmenté aussi avec l'augmentation de la concentration du glycérol à faible rapport C/N, la consommation du glycérol a été élevée (79.43g/l on C/N 25) tandis que la consommation du glycérol a diminué quand le Rapport C/N a été élevé à 150 (40.8g/l).

Mots clés: *Y.lipolytica*, glycérol brut, Biodiesel, lipides microbiens et and ratio C/N

Abstract

The high demand for renewable energy and increased biodiesel production lead to the surplus availability of crude glycerol. Due to the above reason the bio-based value addition of crude glycerol into various bioproducts are investigated, among them microbial lipids are attractive. The present study is dedicated to find the optimal glycerol concentration and C/N ratio to produce lipid using *Y.lipolytica* SKY7. The glycerol concentration (34.4 to168.2g/L) and C/N ratio (25 to 150) was selected to investigate to maximize the lipid production. Initial glycerol concentration 112.5g/L, C/N molar ratio of 100 and with 5%v/v inoculum supplementation was found to be optimum for biomass and lipid production. Based on the above optimal parameters, lipid concentration of 43.8 %w/w with a biomass concentration of 14.8g/L was achieved. The maximum lipid yield (Yp/s) 0.192 g/g, biomass yield (Yx/s) 0.43g/g was noted when the initial glycerol concentration was 112.5g/L with C/N molar ratio 100 and inoculum volume 5% v/v. The glycerol uptake was also noted increased with the increase in glycerol concentration. At low C/N ratio (C/N 25), the glycerol consumption was found to be high, whereas the glycerol consumption was observed to decrease when the C/N ratio was raised to 150 (40.8g/L).

Keywords: *Yarrowia lipolytica*, Crude glycerol, Biodiesel, Microbial Lipid, and C/N ratio

1. Introduction

Biofuels can be described as the high energy molecules produced by microbes through carbon fixation. These are biodegradable, nontoxic, and carbon neutral (1). The recent advancement of microbial-based triacylglycerol (TAG) production made a revolutionary change in biodiesel production. At present biodiesel available in the market has been produced based on first generation biofuel technology. This includes the sugar, carbohydrates, edible oils, used cooking oils, animal fat, tallow, etc.(2-4). The current US biodiesel market analysis (monthly) shows 122 million gallons of biodiesel has been produced in June 2015, which is expected to grow further (5). Meanwhile, the global biodiesel market have been expected to expand to 140 billion liters at the end of 2016, which promises a substantial surplus availability of crude glycerol in the world market (6). In the case of the Canadian biodiesel market, about 400 million liters of biodiesel has been reported to be produced at the end of 2014 (7) . This shows 40 million liters of crude glycerol production every year. Furthermore, a rapid growth in biodiesel market resulted in the low price of crude glycerol (8.50 to 10 US CTS/LB) in the beginning of 2014 (8). Due to the low cost and surplus availability of crude glycerol, the research on third-generation biofuels and value-added products was boosted in recent decades.

Biodiesel from crude glycerol using microbes as a biocatalyst is well established. Crude glycerol has been used to produce various value added products such as SCO (single cell oil), PHA (Polyhydroxyalkanoates), sugar alcohols, β-carotene, organic acids, etc. (9-13). Among them, SCO from crude glycerol became one of the popular due to the high demand of energy in transportation and strict environmental protection policies. SCO can be produced by microbes in heterotrophic or autotrophic mode. In autotrophic mainly algae were employed to produce lipid using sunlight/CO_2/glycerol as a carbon source (14). In the heterotopic mode mainly yeast, filamentous fungi or bacteria were used. Among them, yeast was preferred over filamentous fungi due to its unicellular nature and capability of growing faster than fungi. The main yeast cultures used for SCO production were *Rhodotorula, Yarrowia, Candida, Cryptococcus, Lypomyces, Rhodosporidium, Mortirella, and Mucor,* (15-17). *Yarrowia lipolytica* has becomes one of the important SCO producers due to its heterotrophic nature and multiple product formations and is regarded as the model organism to understand the lipid synthesis (18).

In the case of lipid production by heterotrophic microbes the cultivation condition play a crucial role. The culture parameters such as carbon concentration, carbon to nitrogen ratio (C/N molar ratio) and other macro and microelements were investigated in the past decade. In a batch cultivation, a 30.1% lipid accumulation (1.27g/L) was attained in a media supplemented with 10%w/w crude glycerol by Poli et al (2). This study compared the pure glycerol with crude glycerol and found no significant difference when crude glycerol was used for lipid production (2). However, when *Y.lipolytica* was cultivated in a continuous culture with nutrient limited media, the biomass increased to 60g/L with a volumetric lipid productivity of 0.43g/L/h (19). Based on the literature survey, it has been found that the carbon concentration is a critical factor that controls the biomass and lipid production. A high initial carbon concentration inhibits the biomass production. At the same time, a low glycerol concentration during the lipid production phase significantly affects the volumetric lipid production. Due to the above reasons the present study engaged to illustrate the impact of glycerol concentration and C/N (Carbon/ Nitrogen) ratio on biomass and lipid production using *Y.lipolytica* SKY7.

Optimizing the glycerol concentration (carbon) is one of the important parameters to achieve high biomass and lipid content. Due to the variation in glycerol composition derived from biodiesel industries, the concentration of glycerol in crude glycerol solution varies from 15 to 80% w/w. The other major components are soap, free fatty acids, methanol, catalyst, and water. Among them, FFA and soap are known for its inhibitory effects on biomass and lipid accumulation. In spite of high carbon concentration, C/N ratio regarded as the controller of biochemical events of lipid production since the limitation of external nitrogen activates the AMP-deaminase and the intracellular AMP degraded to release NH_4^+ ions. The low intracellular AMP reverses the citric acid cycle and accumulates citrate in mitochondria and then it is transported to the cytoplasm (20, 18). Thus studying the impact of C/N ratio could improve the biomass and lipid production.

2. Materials and methods

2.1 Culture and inoculum

A pure culture of *Y.lipolytica* SKY7 was stored in YPD (yeast extract, peptone, and dextrose) plates at 4°C. The culture was activated by inoculating a loop full of culture in YPD broth and incubated for 24h at 28°C in a shaking incubator (180rpm). The activated culture was further

used for the inoculum preparation. The inoculum for the current study was prepared in YPG broth (yeast extract, peptone, and crude glycerol). The prepared YPG broth was sterilized by autoclaving at 121°C for 20 minutes and after the sterilization 10%v/v of the activated culture of *Y.lipolytica SKY7* was inoculated aseptically and the culture was incubated for 24h at 28°C with an agitation of 180rpm.

2.2 Crude glycerol characterization

The composition of the crude glycerol was analyzed before the fermentation. The glycerol content in the present crude glycerol was measured according to the method described by Bondioli and Della Bella (21). Appropriately diluted crude glycerol samples were mixed with 1:1 ethanol-water solution and then 1.2mL of 20mM sodium periodate solution was added. After well-mixing, 1.2mL of 200mM acetylacetone solution was added. The resulting mixture was incubated at 70°C for 1 minute. The yellow-colored compound developed by this reaction was measured at 410nm using a UV-Visible spectrophotometer (Varian Cry 500- Agilent Technologies, Santa Clara, CA, USA). A calibration standard was made with pure glycerol procured from Sigma-Aldrich Canada.

The density of glycerol was measured by weighing 5ml of crude glycerol by a conventional gravimetric method. The methanol content was measured by distilling the crude glycerol at 60°C using an automated rotary evaporator. The obtained methanol was measured gravimetrically and recorded. The pH was measured by dissolving 1g of glycerol in 10mL of distilled water and the pH was measured with a galvanometric pH meter. The FFA and catalyst content was measured by adjusting the pH to 1 by adding 85% orthophosphoric acid and thus the resulting mixture was centrifuged at 5000rpm for 10 minutes. Due to the density difference three distinct layers formed. The top dark red layer composed of FFA and soap and the middle turbid layer represents the catalyst and other insoluble matters and the bottom layer with glycerol and water.

2.3 Effect of glycerol concentration on biomass and lipid production

The impact of glycerol concentration on biomass and lipid accumulation was investigated in the range of 34.43g/L to 168.25g/L. Experiments were carried out in duplicates in 2L Erlenmeyer flask with a working volume of 500mL. The C/N ratio was adjusted to 100 with required amount of yeast extract powder. The fermentation medium was formulated with crude glycerol,

Na_2HPO_4 1g/L, KH_2PO_4 2.7g/L, $MgSO_4.7H_2O$ 0.5g/L, and yeast extract (varied according to the C/N ratio). The prepared medium was sterilized by autoclaving at 121°C for 15 minutes. The sterile medium was inoculated with 15 ml (10%v/v) of the well grown pure culture of *Y.lipolytica* SKY7. The flasks were incubated at 28°C with an agitation rate of 180rpm for a period of 120h. Samples were collected at 0h, 72h, and 120h. The collected samples were analyzed for biomass lipid and residual glycerol.

2.4 Evaluation of C/N ratio

In all the experiments 100g/L of crude glycerol was used as the carbon source. The C/N ratio was adjusted from 25 to 150 using yeast extract as the source of nitrogen. The C/N ratio was calculated based on; the carbon present in the glycerol (39.1%w/w) and the nitrogen present in the commercially available yeast extract approximately (12%w/w). The other medium components remained the same as mentioned in the previous section. The flask containing 500ml of the medium was inoculated with 15mL of inoculum (10%v/v) and incubated at 28°C with an agitation rate of 180rpm for a period of 120h. The samples were collected at 0h, 72h, and 120h and were analyzed for biomass, lipid, and residual glycerol.

2.5 Influence of inoculum concentration on biomass and lipids

An actively growing inoculum has a huge impact on biomass and lipid accumulation hence it is one of the factors that determines the final biomass and lipid accumulation. In the present study 2.5 to 10 %v/v inoculum was studied. The pre-inoculum and the fermentation media were prepared as explained in previous sections. The flasks were inoculated with well-grown pre-culture (36h) of a *Y.lipolytica* SKY7. The culture was incubated for 120h at 28°C with an agitation of 180rpm. The samples were withdrawn at 0h, 72h and 120h and the biomass and lipid and residual glycerol were analyzed.

2.6 The importance of trace elements on growth and lipid accumulation

Trace elements have a high influence on biomass and lipid accumulation. In order to assess the influence of trace elements in biomass and lipid accumulation, a trace element solution was prepared with the following composition and was studied in a range from 1 to 10ml/L. Composition of trace element solution (g/L) $FeSO_4.7H_2O$ 4g, $CaCl_3$ 3.6g, $ZnSO_4.7H_2O$ 0.75g, $CuSO_4.5H_2O$ 0.13g, $MnSO_4.H_2O$ 0.5g, $CoCl_2.6H_2O$ 0.13g, $Na_2MoO_4.2H_2O$ 0.17g. Samples were

withdrawn at 0h, 72h, and 120h and analyzed for biomass, lipid accumulation, and residual glycerol.

2.7 Quantification of biomass and lipid

20 ml of fermented broth was used for biomass and lipid quantification. The collected samples were centrifuged at 4000rpm for 10 minutes and the supernatant was collected in a clean tube. The biomass was washed with distilled water for two times and the cell pellets were collected by centrifugation. For biomass quantification, the samples were transferred into a clean pre-weighed aluminum pan and it was kept at 105°C until it reaches a constant weight. For lipid extraction, a modified method of Folch et al., (22) was employed. Wet sample was mixed with 2:1 chloroform-methanol mixture and to that zirconium beads (0.7mm) were added and the cells were disrupted by bead beater for 3 minutes (Biospecs-USA) and the samples were filtered using Whatman filter paper with a pore size of 0.45µm. The solid sample was resuspended in 1:1 chloroform-methanol mixture and the procedure was repeated. The resulting filtrate was pooled into a pre-weighed glass tube and concentrated under reduced nitrogen pressure and the samples were further dried at 60°C in a hot air oven until the sample reaches a constant weight.

2.8 Characterization of lipid

25 mg of lipid was trans-esterified using 1mL of 2.5%v/v sulphuric acid in methanol. Transesterification was carried out at 80^0C for 2h in a preheated oil bath. The FAME was collected by adding 0.5mL of hexane in transesterified mixture and the samples were centrifuging at 2000rpm for 5 minutes. Decahexanoic acid was used as the internal standard. The samples were further characterized by GC equipped with flame ionization detector (FID).

3 Results and discussion

3.1 Characterization of crude glycerol

The composition of the crude glycerol used for the present study is presented in Table 21. The glycerol concentration was 78.3% w/w, with other components methanol, soap, free fatty acids, and catalyst. The pH of the crude glycerol was 6.8. The pH may vary according to the catalyst used for the trans-esterification process. Based on the pH of the current glycerol it can be assumed that the catalyst used was a base. The near acidic pH (6.8) reduces the addition (NaOH) or adjustment of pH during the fermentation since the final fermentation pH is 6.5. The

impurities present in the biodiesel industry driven crude glycerol were mainly methanol, soap, minerals, and catalyst. These impurities can influence the growth and metabolite production in either way. In a study conducted by Chatzifragkou et al., 2012 reported positive effects on certain metabolite production (23).

Table 21 Composition of crude glycerol

Components	Concentration (%w/w)
Glycerol	78.3 %(w/w),
Methanol	1.28 %(w/w),
Soap	2.4 %(w/w),
Water	17.54 %(w/w),
Catalyst	0.12 %(w/w),
pH	6.8

3.2 Impact of glycerol concentration on biomass and lipid production

Four different concentrations (34.4, 52.3, 112.5 and 168.2g/L) of glycerol were studied and the C/N ratio was maintained 100 in all conditions. The comparison of biomass production and glycerol consumption on each glycerol concentrations are given in Figure 12A & B. Biomass concentration of 16.1g/L was obtained on 52.3g/L glycerol supplements experiment and followed by 14.2g/L in 112.5g/L glycerol supplemented experiments. Whereas in the case of 34.4g/L glycerol supplemented experiment the biomass concentration reached 11.7g/L and in the case of 168.2g/L glycerol supplemented experiments it was 11.04g/L. In the case of glycerol consumption, 30.1, 34.1, 48.6 and 32.5 g/L of glycerol was assimilated by *Y.lipolytica* SKY7 on 34.4, 52.3, 112.5 and 168.2g/L glycerol supplemented experiments respectively. From the experimental data, it has been concluded that there could be two reasons for low biomass concentrations in 34.4g/L and 168.2g/L glycerol supplemented experiments. Primarily the available nitrogen content in the fermentation media can influence the biomass concentration. When 34.4g/L glycerol was studied the amount of nitrogen added to the media was 0.135g/L in order to achieve a C/N ratio 100 (13.5g of carbon in 34.4 gram of glycerol). Hence, the low

biomass production in 34.4g/L glycerol supplemented studies could be due to the low nitrogen concentration in the medium. The second reason could be the inhibition of high substrate concentration in the case of 168.2g/L glycerol supplemented experiments. From figure 12A, it can be observed that when glycerol concentration increased the biomass concentration also increases up to the glycerol concentration of 52.3g/L. However, it declined when the glycerol concentration was increased to 112.5 and 168.2g/L. In the case of 168.2 g/L, 0.6g/L available nitrogen was supplemented in the media. However, due to high glycerol concentration, the growth was comparatively lower than in other conditions. To confirm the above results the specific growth rates were plotted (Figure 13) and it showed a declining trend from the early stage of fermentation in all conditions and this can be due to minimal nutrient supplementation and high substrate concentration.

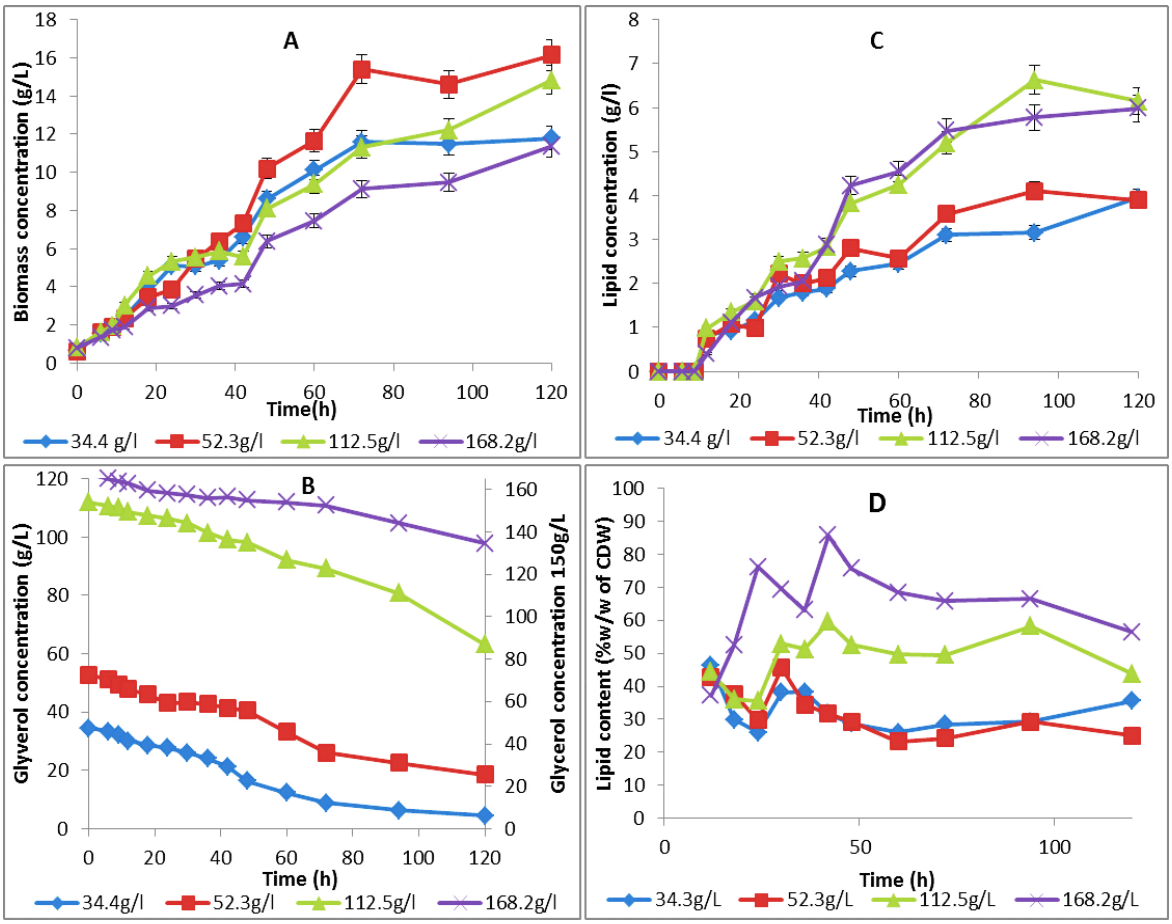

Figure 12 (A) biomass concentration obtained on different glycerol concentration, (B) the glycerol consumed on each fermentation and (C) lipid concentration and (D) % of lipid per gram of cell dry weight (CWD)

Figure 14A represents the Yx/s (yield of biomass/ gram of glycerol consumed) shows a higher substrate to biomass conversion at the beginning of fermentation (0 to 24h) in the case of 34.4 and 112.5g/L glycerol supplemented experiments, whereas it was slightly different in the case of 52.3 and 168.2g/L glycerol supplemented experiments. In the case of 52.3g/L glycerol supplemented experiments, the maximum Yx/s (0.78g/g) was achieved at 48h and in 168.2g/L (0.44g/g) it was at 60h. The specific growth rate shows (Figure 13) that at higher glycerol concentration a lower the specific growth obtained. The experiment with 52.3g/L glycerol showed a slightly higher specific growth rate than 34.4g/L and 112.5g/L glycerol concentration. In the case of 34.4g/L glycerol supplemented experiments the original concentration of nitrogen supplied is low compared to 52.3g/L and 168.2g/L glycerol supplemented experiments. Due to the low concentration of available nitrogen, cell growth was also affected. In the case of 112.5

and 168.2g/L glycerol supplemented experiments, even though the nitrogen concentration is higher than for the 52.3g/L glycerol supplemented experiment the high glycerol concentration inhibits the growth. From this observation, it can be concluded that the glycerol concentration from 52.3 to 112.5g/L could be optimal for biomass production. Moreover, the recent reports on *Y.lipolytica* cultivated on the crude glycerol showed either low or approximately similar biomass production (6.7g/L, (2), 19.14g/L (24) and 12g/L (25)) in batch fermentations. From the literature reports and the experimental data on *Y.lipolytica* SKY7, it has been clear that this stain has great potential to produce biomass and TAG in a minimal media.

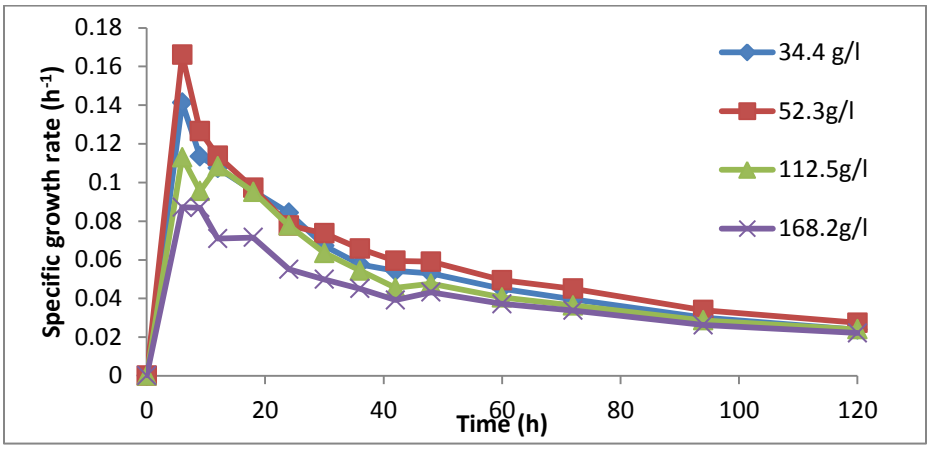

Figure 13 Variation in specific growth rate upon different glycerol concentrations

Figure 12C shows the variation in lipid concentration observed at different glycerol concentrations. The maximum lipid concentration (6.6g/L) was obtained in 112.5g/L glycerol supplemented experiment. The lower concentration of lipid (3.95g/L) was observed in 34.4g/L glycerol containing experiment. The experimental data shows that , when the glycerol concentration in the medium was increased the lipid concentration also increased. However, when the glycerol concentration is increased above 112.5g/L the lipid concentration tends to drop due to a low biomass concentration even though the lipid content was higher in the cells (Figure 12D).

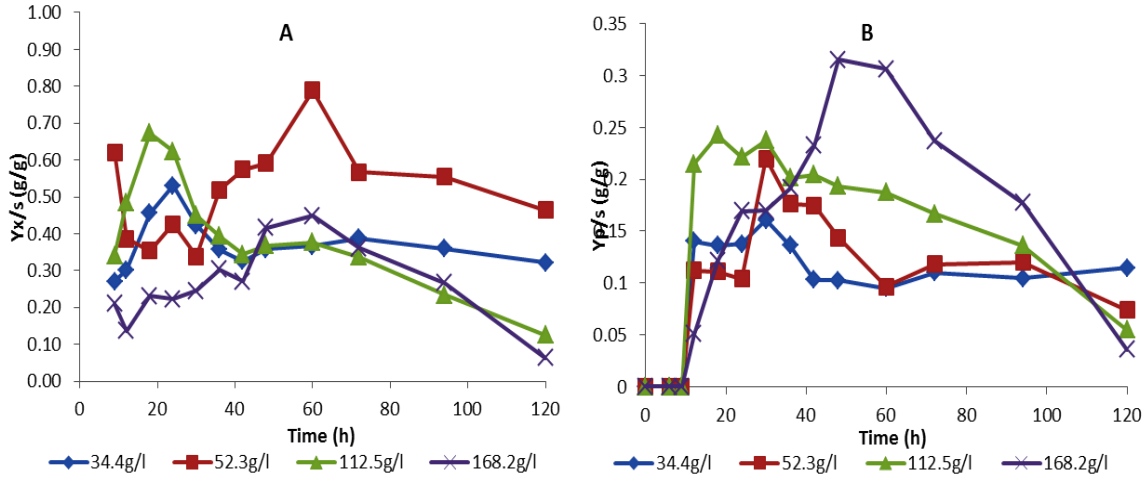

Figure 14 (A) yield of biomass, (B) yield of lipid under different glycerol concentrations

Comparisons of lipid yield are presented in Figure 14B. A low yield of 0.161g lipid /g glycerol (30h) was observed when the glycerol concentration was 34.4g/L. The yield of lipid (Yp/s) was noted to be increased with increase in glycerol concentration. In the case of 52.3g/L glycerol, the Yp/s was 0.22g/g at 30h, which was noted to decline with fermentation time. However, the maximum yield of lipid (0.243g/g) was attained at 18h in 112.5g/L glycerol supplemented experiment. From 18h onwards the Yp/s decline. In the case of 168.2g/L glycerol supplemented study the Yp/s reached a maximum at 48h (0.315g/g). When considering the above four conditions the yield of lipid was higher in the case of 112.5g/L glycerol supplemented experiment within 18h of fermentation. This indicated that the lipid production occurred at the early stage of fermentation. This could be due to the low nutrient concentration of the medium. When the medium is poor the growth decelerates at an early stage and the cells start to produce lipids. In the first three cases, the lipid production was noted to decline after 18 to 30h of fermentation. Whereas in the case of 168.2g/L glycerol supplemented experiment the decline in yield was observed after 48h. This could be due to the inhibition of growth by high glycerol concentration. Due to a slow growth of the cells, the available nitrogen could be utilized slowly thus the lipid yield was higher at 48h. Based on Yp/s and total concentration of lipid, glycerol concentration of 112.5g/L could be better for lipid and biomass production.

3.3 Impact of C/N ratio on biomass and lipid production

Based on the above studies conducted on glycerol concentration, 112.5g/L glycerol concentration was chosen for evaluating the C/N ratio. Figure 15A compares the difference in

biomass obtained in four experimental conditions. The biomass concentration was noted to decrease with respect to the increase in C/N ratio. Comparatively higher biomass (22.6g/L) was obtained in C/N ratio of 25 and the low biomass (13.5g/L) was in C/N ratio 150. Thus, when the C/N ratio increases the available nitrogen content of the fermentation medium decreases and it directly affects the biomass production. To support the above fact, the specific growth rate was calculated and presented in Figure 16. In the case of C/N ratio 25 and 50, the specific growth rate was identical throughout the fermentation. However, in the case of C/N ratio 100 and 150 the specific growth rates were significantly lower compared to the other two conditions. This confirms that the C/N ratio affects the overall growth and thus it reduces the biomass generation in high C/N ratios. Moreover, the most recent studies on the influence of C/N ratio on biomass and lipid production by *Y. lipolytica* also confirmed the C/N ratio 75 to 100 could be the optimum. A study conducted by Dobrowolski et al (2016) with *Y. lipolytica* A101 showed a biomass production of 8.46±0.23g/L in the case of C/N 75 and 7.97±0.23g/L on C/N100(26). However, in the case of *Y. lipolytica* SKY7 the biomass concentrations was found to be 16. 37±0.1g/L at 120h of fermentation

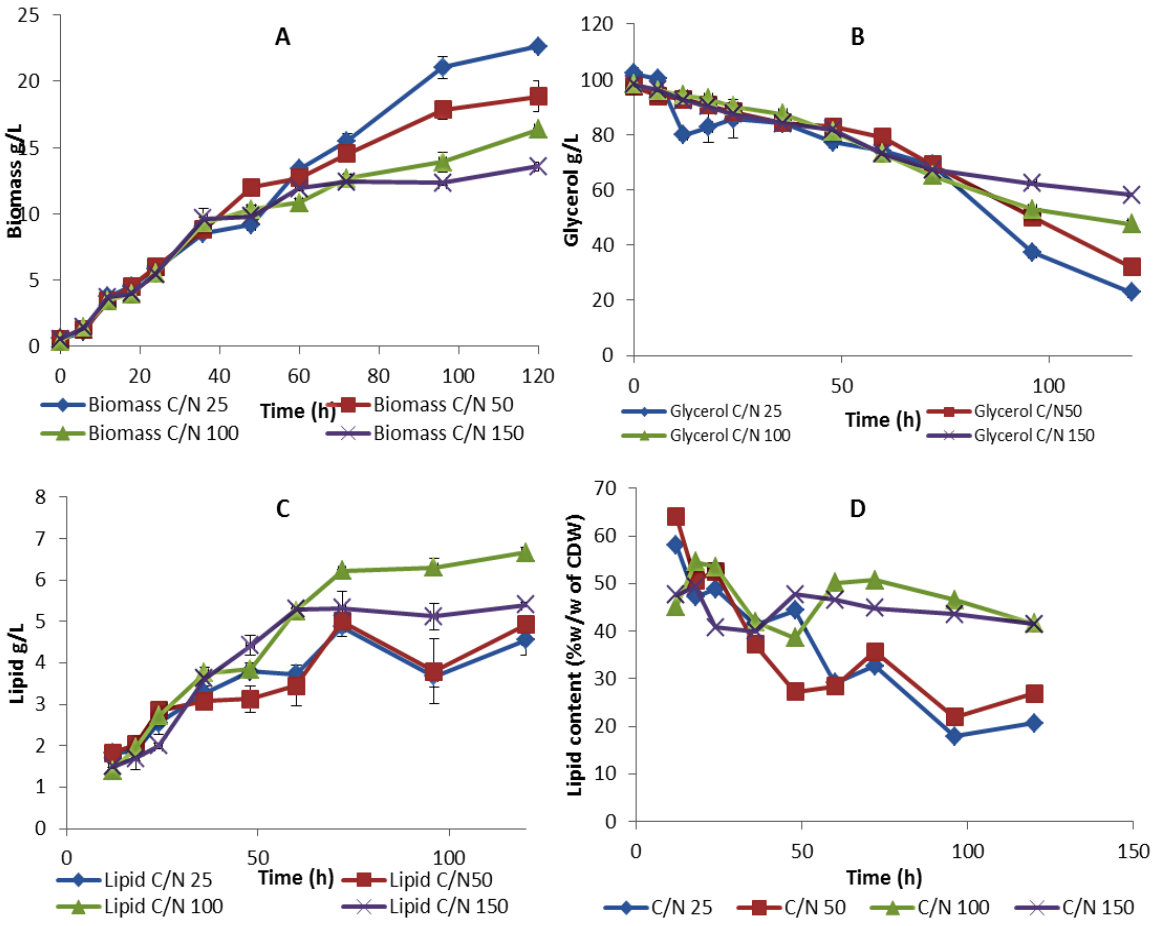

Figure 15 (A) Biomass concentration, (B) Glycerol concentration and (C) lipid concentration and (D) % of lipid per gram of cell dry weight (CDW) obtained on different initial C/N ratio

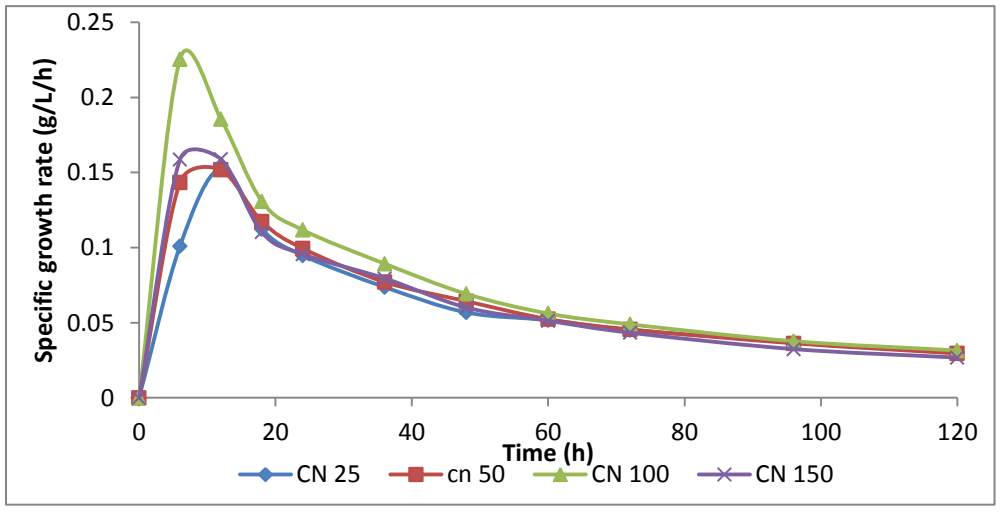

Figure 16 Difference between the specific growth rates obtained at different C/N molar ratios

The yield of biomass is presented in Figure 17A. It reveals a contradicting observation. The biomass yield was low in the case of C/N 25 and 150. However, the Yx/s was high in the case of C/N 50 and 100. The above observations reveal that more substrate was converted into biomass in the case of 50 and 100 C/N ratios. In the case of C/N 25, the assimilated substrate was converted into biomass and other metabolites. To ensure the above observation, the substrate consumption rate (dg/dt) was calculated and presented in Figure 18. The substrate consumption was noted to be high in the case of C/N 25 followed by C/N 150. On the other hand, the substrate consumption rate shows a gradual increase in C/N 50 and 100. The monotonous increase in substrate consumption and an increase in biomass and lipid yield (Figure 15A and 17B) in C/N 100 indicate that most of the assimilated substrate could be converted into biomass at initial fermentation time and lipid and less byproduct formation at a later stage of fermentation.

Figure 15B represents the lipid produced on each condition. The maximum lipid was produced when the C/N ratio was 100 (6.66g/L) and the low lipid concentration was in C/N 25 (4.55g/L). In the case of C/N 100, 50 and 25 the maximum lipid concentration was reached at 72h and in C/N 150 it was at 60h of fermentation. Further, the lipid content (Figure 15D) of the cells also showed a higher lipid content in the case of C/N ratio 100 and 150. This confirms further that the C/N ratio above 50 will enhance the lipid production by altering the cellular metabolism towards lipid synthesis.

The lipid yield (Yp/s, Figure 17B) shows higher substrate to lipid conversion at C/N 100 and this could be due to a low utilization of substrate (glycerol). In C/N 50 and 100, the Yp/s was approximately similar throughout the fermentation, whereas the low substrate to lipid conversion was found in C/N ratio 25. Based on these observations, C/N 100 yielded a comparatively high concentration of lipid even though, the yield (Yp/s) was higher in C/N 150. This allows concluding that C/N 100 could be suitable for lipid production in *Y.lipolytica* SKY7.

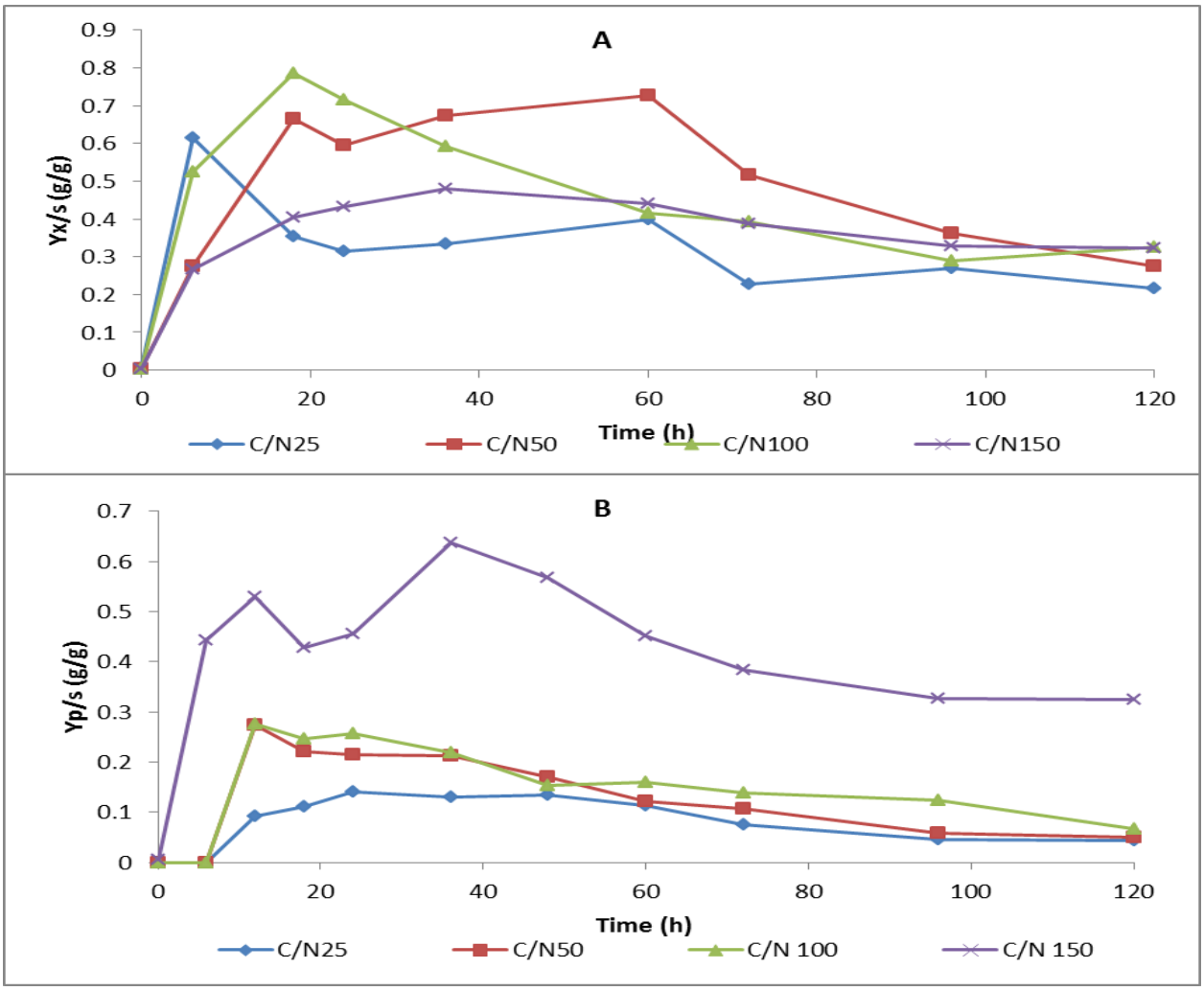

Figure 17 (A) Biomass yield and (B) lipid yield at various C/N molar ratios

3.4 Importance of inoculum density and trace elements and its effect on biomass and lipid accumulation

In this study, four different inoculum volumes were evaluated and Table 22 represents the biomass, lipid accumulation at 120h. From the experimental results, inoculum concentration 5% v/v seems to be suitable for an initial glycerol concentration of 10%(w/v) and C/N ratio 100. A lipid concentration of 6.28g/L was achieved when the inoculum concentration was maintained at 5%v/v. On the other hand, the biomass concentrations at all these conditions were comparable and there were no significant increase or decrease. The yield of lipid from the amount of substrate utilized at different inoculum volume is represented in Table 22 and it suggests that the Yp/s was decreasing when the inoculum concentration was increased. The higher Yp/s

(0.192g/g) is observed in the case of 5%v/v inoculum with 6.28g/L lipid accumulation. The glycerol assimilation increased when the inoculum size was increased and the substrate to lipid conversion dropped significantly. Based on the Yp/s and the lipid concentration it is concluded that the inoculum concentration 5%v/v could be the optimum.

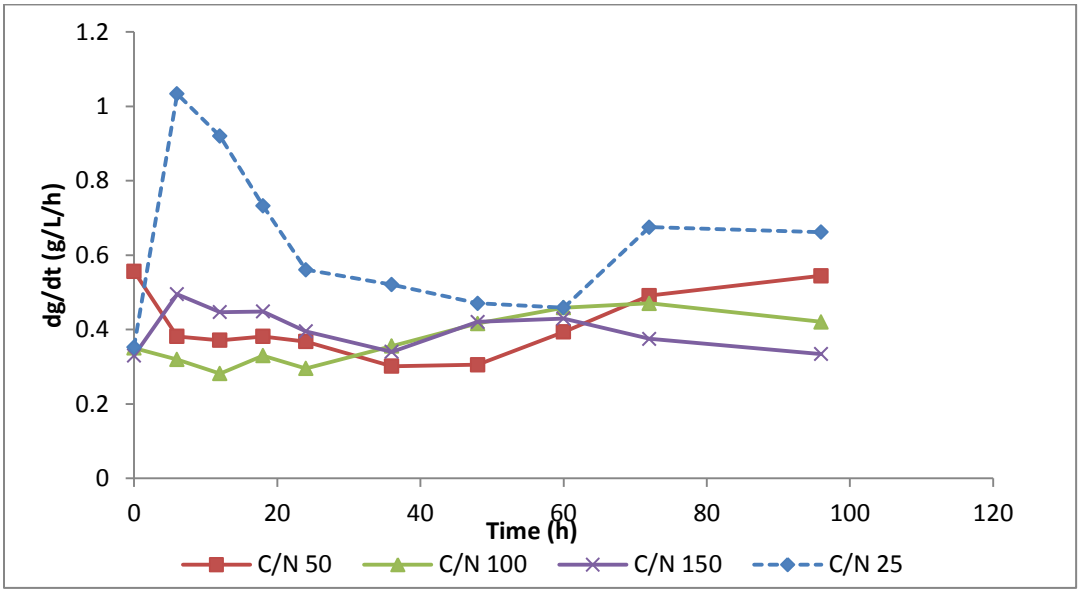

Figure 18 Variation in substrate utilization (dg/dt

Table 22 Effect of inoculum volume on biomass and lipid production

Trace elements (ml/L)	Biomass g/L	Lipid g/L	% Lipids	$Y_{P/S}$	$Y_{X/S}$
1	18.5	**5.5**	**30.0**	0.13	0.46
2.5	19.2	**5.4**	**28.2**	0.13	0.46
5	19.1	**4.2**	**22.2**	0.10	0.48
10	19.1	**4.8**	**25.1**	0.10	0.43

The effect of trace elements on lipid and biomass accumulation is presented in Table 23. As expected the trace elements improve the biomass accumulation when the trace element concentration is increased from 1 to 10ml/L. Decreasing the trace elements shows a positive effect on lipid accumulation. Whereas the glycerol utilization shows that the assimilated glycerol was mostly channelized to biomass accumulation this indicated that the enzymes related to the

lipid accumulation were under limited activity when the microelements are provided in sufficient amount. But this could be inverted as the fermentation time progresses due to the microbial consumption of trace elements. The present study illustrates that when the fermentation was carried out without any addition of trace elements the biomass and lipid accumulation was 14.6g/L and 6.6g/L (Figure 12A), respectively. In the case of trace elements supplemented experiments lipid accumulation dropped from 5.5g/L (trace elements 1mL/L) to 4.8g/L (trace elements 10 mL/L) and the biomass accumulation was increased to 18 to 19 g/L from 14.6g/L. From the above observations and from the literature, it is clear that the composition of the crude glycerol mainly contains the metal ions in the acceptable amount due to the addition of chemical catalyst during trans-esterification. Meanwhile, the literature survey showed that the addition of trace elements resulted in higher biomass conversion rather than lipid production (27). In the present study, the lipid concentration was also found higher in the case of non-trace element supplemented media.

Table 23 Effect of trace elements concentrations on biomass and lipid production

Concentration of Inoculum (% v/v)	Biomass g/L	Lipid g/L	% Lipid	$Y_{P/S}$	$Y_{X/S}$
2.5	11.6	4.3	37.2	0.16	0.44
5	14.1	6.2	44.6	0.19	0.43
7.5	14.5	5.4	37.6	0.14	0.39
10	13.4	5.5	41.3	0.12	0.29

3.5 Composition of TAG

The composition of lipid considerably depends on the carbon source, the concentration of carbon and the C/N ratio. Several studies showing that the lipid profile was changed with the increase of fermentation time. Table 24 represents the lipid composition of *Y.lipolytica* SKY7 at different glycerol concentration and C/N ratios at 120h of fermentation. Oleic acid was found to be the

major component in all the cases. Though *Y.lipolytica* is reported to produce high percentage of linoleic acid this isolate seems to produce other unsaturated fatty acids such as *Myristic* (C14:0), *Palmitic* (C16:0), *Stearic* (C18:0), *Oleic cis-9* (C18:1n9-cis), *Linoleic* (C18:2n6c) and *Arachidonic* (C20:4n6) acid in high quantity. Other than the listed components, this organism produces C15:0, C15:1, *Palmitoleate* (C16:1n7), *Elaidic* (C18:1n9t), *Linolenate* (C18:3n3), *Arachidic* (C20:0), *Eicosenoate* (C20:1n9), *Lignoceric* (C24:0) in minor quantities (Data not shown).

Table 24 Composition of lipids (%w/w)

Sample details	Myristic (C14:0)	Palmitic (C16:0)	Stearic (C18:0)	Oleic cis-9 (C18:1n9-cis)	Linoleic (C18:2n6c)	Arachidonic (C20:4n6)	Total	Others
Glycerol effect								
34.4g/L	4.08	13.45	11.44	47.59	8.38	1.93	86.88	13.12
52.3g/L	3.43	16.57	15.60	47.96	5.87	0.00	89.44	10.56
112.5g/L	3.69	17.51	11.04	36.92	7.43	0.00	76.59	23.41
168.2g/L	3.54	16.16	14.80	42.21	7.68	0.00	84.39	15.61
C/N ratio								
25	6.37	17.69	15.93	38.32	5.13	4.48	87.92	12.08
50	3.99	16.84	13.35	41.49	5.46	0.00	81.12	18.88
100	4.85	18.22	13.58	42.58	6.27	3.71	89.20	10.80
150	4.64	17.76	12.04	44.12	6.65	0.00	85.21	14.79

4 Summary and conclusion

The culture parameters such as glycerol concentration C/N ratio, inoculum concentration, and trace elements are evaluated in flask level. The results show that the glycerol concentration C/N

ratio and inoculum concentration have more influence in the case of lipid and biomass accumulation. According to the study, the preferable conditions for *Y.lipolytica* SKY7 are 100g/L (10%w/v) glycerol, C/N ratio of 100 with inoculum concentration of 5%v/v, which shows better biomass and lipid production. The supplementation of trace elements increase the biomass concentration, they fail to trigger the lipogenesis and which could be due to the nutrient availability that promotes the cell division and reduces the lipid production. Apart from nitrogen iron, phosphate and magnesium limitations are reported for lipid accumulation. Moreover, the supply of iron enhances the citric acid production in *Y.lipolytica*. As reported in the present study and by several authors, the inoculum age plays a key role in lipid accumulation. From the observations, the exponential phase of *Y.lipolytica* SKY7 falls within 18h and in the present study, the inoculum age was 36h. By reducing the inoculum age, the fermentation can be efficiently altered by shifting the late lipid production and this will eliminate the initial lag period (in this case 0-6h growth was insignificant).

Acknowledgement

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References

1. Easterling, E. R., French, W. T., Hernandez, R. and Licha, M. (2009) The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of Rhodotorula glutinis. Bioresour Technol*,* **100,** 356-361.
2. Poli, J. S., da Silva, M. A., Siqueira, E. P., Pasa, V. M., Rosa, C. A. and Valente, P. (2014) Microbial lipid produced by Yarrowia lipolytica QU21 using industrial waste: a potential feedstock for biodiesel production. Bioresour Technol*,* **161,** 320-326.
3. Papanikolaou, S., Chevalot, I., Komaitis, M., Marc, I. and 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.

4. Papanikolaou, S., Chevalot, I., Galiotou-Panayotou, M., Komaitis, M., Marc, I. and Aggelis, G. (2007) Industrial derivative of tallow: a promising renewable substrate for microbial lipid, single-cell protein and lipase production by Yarrowia lipolytica. Electronic Journal of Biotechnology, **10,** 0-0.
5. EIA. (2015) Monthly Biodiesel Production Report. U.S Energy information administration.
6. Anand, P. and Saxena, R. K. (2012) A comparative study of solvent-assisted pretreatment of biodiesel derived crude glycerol on growth and 1,3-propanediol production from Citrobacter freundii. New Biotechnology, **29,** 199-205.
7. CRFA. (2014) From biofuels to bioeconomy. Canadian renewable fuel Association.
8. Landress, L. (2014) Glycerine Prices, markets & analysis. ICIS.
9. Avila-Neto, P. M., da Silva, G. P., Lima, C. J. B., De Paula, F. C. and Contiero, J. (2014) Evaluation and optimization of growth and citric acid production by yarrowia lipolytica nrrl y-1095 using glycerol as carbon source as an alternative to use biodiesel byproduct. Journal of Experimental Biology and Agricultural Sciences, **2,** 25-31.
10. Morgunov, I., Kamzolova, S., Perevoznikova, O., Shishkanova, N. and Finogenova, T. (2004) Pyruvic acid production by a thiamine auxotroph of Yarrowia lipolytica. Proc. Biochem., **39,** 1469–1474.
11. Mantzouridou, F., Naziri, E. and Tsimidou, M. Z. (2008) Industrial glycerol as a supplementary carbon source in the production of B-carotene by Blakeslea trispora. J. Agric. Food Chem, **56,** 2668.
12. André, A., Diamantopoulou, P., Philippoussis, A., Sarris, D., Komaitis, M. and Papanikolaou, S. (2010) Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid. Industrial Crops and Products, **31,** 407-416.
13. Rymowicz, W., Rywinska, A. and Marcinkiewicz, M. (2009) High-yield production of erythritol from raw glycerol in fed-batch cultures of Yarrowia lipolytica. Biotechnol Lett, **31,** 377-380.
14. Chen, Y. H. and Walker, T. H. (2011) Biomass and lipid production of heterotrophic microalgae Chlorella protothecoides by using biodiesel-derived crude glycerol. Biotechnol Lett, **33,** 1973-1983.

15. Ageitos, J. M., Vallejo, J. A., Veiga-Crespo, P. and Villa, T. G. (2011) Oily yeasts as oleaginous cell factories. Appl Microbiol Biotechnol*,* **90,** 1219-1227.

16. Li, Q., Du, W. and Liu, D. (2008) Perspectives of microbial oils for biodiesel production. Appl Microbiol Biotechnol*,* **80,** 749-756.

17. Maddalena Rossi, A. A., Stefano Raimondi and Alan Leonardi. Getting Lipids for Biodiesel Production from Oleaginous Fungi.

18. Beopoulos, A., Chardot, T. and Nicaud, J. M. (2009) Yarrowia lipolytica: A model and a tool to understand the mechanisms implicated in lipid accumulation. Biochimie*,* **91,** 692-696.

19. Mironczuk, A. M., Rakicka, M., Biegalska, A., Rymowicz, W. and Dobrowolski, A. (2015) A two-stage fermentation process of erythritol production by yeast Y. lipolytica from molasses and glycerol. Bioresour Technol*,* **198,** 445-455.

20. Ratledge, C. and Wynn, J. P. (2002) The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms. Advances In Applied Microbiology*,* **51**.

21. Bondioli, P. and Della Bella, L. (2005) An alternative spectrophotometric method for the determination of free glycerol in biodiesel. European Journal of Lipid Science and Technology*,* **107,** 153-157.

22. Folch, J., M.Lees and G.H.Slane-Stanley. (1957) Simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem*,* **226,** 497–509.

23. Chatzifragkou, A. and Papanikolaou, S. (2012) Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes. Appl Microbiol Biotechnol*,* **95,** 13-27.

24. Machado, J., F. R. S., , Michelon, M., Burkert, J. F. M., Dalcanton, F., Burkert, C. A. V. and Furlong, E. B. (2015) Biomass production by Yarrowia lipolytica as a source of lipids: bench scale cultivation on raw glycerol-based medium. International Food Research Journal*,* **22,** 1253-1260.

25. Sestric, R., Munch, G., Cicek, N., Sparling, R. and Levin, D. B. (2014) Growth and neutral lipid synthesis by Yarrowia lipolytica on various carbon substrates under nutrient-sufficient and nutrient-limited conditions. Bioresour Technol*,* **164,** 41-46.

26. Dobrowolski, A., Mitula, P., Rymowicz, W. and 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.

27. Nambou, K., Zhao, C., Wei, L., Chen, J., Imanaka, T. and Hua, Q. (2014) Designing of a "cheap to run" fermentation platform for an enhanced production of single cell oil from Yarrowia lipolytica DSM3286 as a potential feedstock for biodiesel. Bioresour Technol*,* **173,** 324-333.

Chapter IV- Partie II

Research Article-3

Harnessing the effect of pH on lipid production in batch cultures of *Yarrowia lipolytica* – SKY7

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RÉSUMÉ

L'objectif visé par cette recherche était d'étudier la cinétique de la production de lipides par *Y. lipolytic* SKY7 dans un milieu de culture de glycérol brut supplémenté, avec et sans le control de pH. La production lipidique et d'acide citrique ont été améliorés dans les conditions de control de pH. Il n'y avait aucune différence significative dans la concentration de la biomasse produite avec ou sans le contrôle de pH. Dans les expériences où le pH a été contrôlé, la concentration de biomasse et de lipides a atteint 18 g/l et 7.78g/l (45,5% en poids/poids), respectivement, avec un rendement lipidique (Yp/s) de 0,179 g/g pendant 60 heures de fermentation. La production de lipides a été directement corrélée à la croissance et le processus a été associé à la cette dernière. Après 60h de la fermentation, la dégradation des lipides a été observée dans le réacteur dont le pH est contrôlé, Alors que le réacteur dont le pH n'est pas contrôlé, il est arrivé au même résultat en 84h En dehors des lipides, l'acide citrique a été généré en tant que produit extracellulaire majeur dans les deux fermentations, avec une concentration beaucoup plus faible avec le pH non contrôlé. Sur la base des résultats de l'expérience, il est évident qu'en contrôlant le pH, la production de lipides augmentera de 15% par rapport une fermentation avec un pH non contrôlé.

Mots clés : *Y.lipolytica*, le glycérol brut, Biodiesel, Lipide microbien, le rapport C/N, l'effet du pH

Abstract

The objective of this research was to investigate the kinetics of lipid production by *Y. lipolytic* SKY7 in the crude glycerol supplemented media with and without the control of pH. Lipid and citric acid production were improved with the pH control condition. There was no significant difference observed in the biomass concentration produced with or without the pH control. In the pH controlled experiments, the biomass and lipid concentration reached 18 g/L and 7.78g/L, (45.5%w/w), respectively, with lipid yield (Yp/s) of 0.179 g/g at 60h of fermentation. The lipid production was directly correlated with growth and the process was defined as growth associated. After 60h of fermentation, the lipid degradation was noticed in the pH controlled reactor whereas it occurred after 84h in the pH uncontrolled reactor. Apart from lipid, citric acid was produced as the major extracellular product in both fermentations but the much lower concentration in uncontrolled pH. Base on the experimental results it is evident that controlling the pH will enhance the lipid production by 15% compared to pH uncontrolled fermentation.

Keywords. Y.lipolytica, Crude glycerol, Biodiesel, Microbial Lipid, C/N ratio, effect of pH

1. Introduction

Ever growing energy demand and rapid industrialization leads to the generation of a huge amount of waste materials in various fields throughout the world. This necessitates the search for alternative waste disposal methods. One of the most important ways of waste treatment and disposal is the use of their carbon and other nutrients as a substrate to grow various microbes to generate value added products. In the present scenario, energy demand (in terms of oil, electricity, heat, etc.) has become vital for human activity. A rapid depletion of natural resources such as oil leads to the search for alternatives [1, 2]. In recent years, the revolution in green energy development technologies resulted in the advancement of research for the production of biofuels (biodiesel, bioethanol, biohydrogen, etc.) from renewable alternative sources [3]. The production of each ton of biodiesel generates 100kg of glycerol as a by-product. On the other hand, glycerol is generated along with wastewater from ethanol and soap producing industries [4]. Thus, the growth of biorefineries may lead to the generation of a large quantity of biological transformable waste materials. Among these wastes, glycerol has gained much attention of the research community due to a rapid growth of the biodiesel industry. Moreover surplus availability of glycerol, which has substantially decreased the market price of glycerol (0.04 $ - 0.33$/kg of crude glycerol) [1, 5].

Microbes have the advantage to store different energy reserve materials such as lipids, polymers, in specialized storage structures. In the case of oleaginous microbes, the excess carbon is converted to lipid and is stored in specialized organelles called lipid bodies, which are embedded in phospholipid monolayer. The lipids are mainly accumulated as triacylglycerides (TAG) or steryl esters (SE) [6]. In microbes, TAG mainly consists C:16 and C:18 or longer chain length fatty acid molecules and in composition they are similar to vegetable oils [7, 8]. This property of microbial oils makes it a suitable source for biodiesel production. In spite of ample amount of research reports available for microbial oil production using different substrates, the fermentation process challenges with reference to physicochemical aspects are yet to be fulfilled.

Several oleaginous microorganisms have been identified and reported to accumulate lipids more than 70% of the cell dry weight (CDW). For example, *Lipomyces starkeyi* 64.9% [9], *Rodhoturella gluttinis* was reported to accumulate lipid up to 72%, of cell dry weight when glucose was used as a carbon source [8], *Trichosporon porosum* 34.1% [10], 65% by

Cryptococcus curvatus on cheese whey[11], 49% in crude glycerol [12], 72.5% by *Mortirella isabellina* on commercial glucose medium [13] and so on. Various isolates of *Y.lipolytica* were reported to accumulate 18.9% w/w lipid on crude glycerol, 43% w/w on biodiesel derived glycerol, 40% w/w industrial lipid plus glycerol, 30.1% w/w industrial waste, 31% on industrial byproducts as a carbon source, and 58% in whey medium . When molecular tools based on *Y.lipolytica* were developed, the understanding about the lipid accumulation process was well addressed, which enhanced the knowledge about the biochemistry of lipid synthesis [8, 14]. Compared to the other lipid accumulating strains, *Y.lipolytica* has gained more attention due to the presence of multigene families to assimilate wide array of carbon sources such as hydrophobic as well as hydrophilic substrates. The ability of *Y.lipolytica* to produce different value added metabolites such as storage lipids, aroma compounds (γ- Deca lactone), single cell protein [15] and sugar alcohols [16] has made it very popular. Apart from the above-mentioned properties, *Y.lipolytica* has high tolerance towards glycerol which is reported to be the best substrate for lipid, and citric acid production by this organism [17, 18]. Therefore, the present study was planned to investigate the ability of *Y. lipolytica* strain SKY7 (isolated from nitrogen deficient soil samples collected from the region of Quebec, Canada) to produce lipid. To enhance the biomass and lipid, physicochemical parameters such as pH, DO (Dissolved Oxygen), mixing rate, were important. However, most of the existing literature on lipid production deals with the medium chemical composition and failed to explain the physical parameters (pH) associated with biomass and lipid production. The influence of pH on lipid production has a high importance since the lipid accumulation is favored by a slightly acidic pH (5 to 6.5). Similarly, most of the fungal strains grow well in an optimum pH range of 6.0 and 7.0 [19]. In unbuffered media, the pH falls rapidly during the growth of microbes resulting in growth retardation. In fact, the growth retardation is a result of poor nutrient assimilation by the microbes due to the change in pH, which alters the cell membrane permeability [20]. Moreover, there are studies showing a significant reduction (above 40%) lipid content at low pH. In the present study, we investigated the influence of pH control during fermentation on biomass and lipid production since the pH affects the biomass production and thereby reduces the overall lipid yield. Moreover, if fermentation could be conducted without pH control with less effect on lipid production, it could lead to a decrease in the cost of operation. The lipid production was studied in two identical 15 l fermenters to evaluate the influence of pH on biomass and lipid production with and without

control of pH. The data obtained were analyzed to study the kinetics of biomass and lipid production with and without pH control.

2. Materials and methods

2.1 Organism and pre-inoculum preparation

Yarrowia lipolytica isolate SKY7 (gene bank accession KF908256.1) was used in the present study. The culture was maintained in YPD (Yeast extract, Peptone and Dextrose) agar slants and revived at 30 days interval. Pre-inoculum was prepared in the crude glycerol. The crude glycerol sample was obtained from a biodiesel producing industry in Quéebec, Canada. Enriched media with the following composition was used; crude glycerol 20g/L, 1g/L $(NH_4)_2SO_4$, 1g/L Na_2HPO_4, 2.7g/L KH_2PO_4, 0.5g/L $MgSO_4.7H_2O$ and 0.3g/L yeast extract. The medium was sterilized in an autoclave at 121^oC for 15 minutes and a loop full of pure culture was aseptically inoculated. The flasks were incubated at 28^0C in an agitator operated at 180 rpm (revolution per minute) for 36h.

2.2 Fermentation conditions

To evaluate the influence of pH on biomass and lipid accumulation, fermentation was simultaneously performed (to ensure the experimental variations, the experiments were conducted twice and the results were presented with standard deviations) in two identical 15L bioreactors with a working volume of 10L (Biogenie, Quebec, Canada) equipped with programmable logic control (PLC) system, which includes dissolved oxygen probe (DO), antifoam, mixing, aeration, temperature and pH control system. The above parameters were maintained using the program ifix 3.5, Intellution, USA. The pH meter (Mettler Toledo, USA) was calibrated using buffers pH 4 and 7.0 (VWR, Canada). The OD probe was calibrated to zero using sodium sulfate and to 100% by purging the air in distilled water. All fermentation experiments were conducted with 100g/L crude glycerol and C/N ratio of 101. The other medium components are 1g/L $(NH_4)_2SO_4$, 1g/L Na_2HPO_4, 2.7g/L KH_2PO_4, 0.5g/L $MgSO_4.7H_2O$ and 1.5g/L yeast extract. The medium was sterilized at 121^0C for 30 minutes. After sterilization, the medium was cooled to 28^0C and aseptically inoculated with 5%v/v 36h grown pure culture

of *Y. lipolytica* SKY7. The samples were collected aseptically at each 6h interval. The samples were analyzed for biomass, lipid, citric acid, and residual glycerol.

3. Analytical methods

3.1 Quantification of biomass, lipid, and residual glycerol.

Fermented broth (25 ml) was used for biomass and lipid quantification. The collected samples were centrifuged at 4000rpm for 10 minutes and the supernatant was collected in a clean tube. The biomass was washed twice with distilled water and the cell pellets were collected by centrifugation. Each sample (pellet) for biomass quantification was transferred to a clean pre-weighed aluminum pan and it was kept at 105^0C until it reached a constant weight. A chloroform and methanol based conventional method of lipid extraction were employed for lipid quantification [21]. Wet sample was mixed with 15 ml of 2:1 chloroform: methanol mixture and zirconium beads (0.7mm) were added to the mixture and the cells were disrupted by bead beater for 3 minutes (BioSpec Products, Bartlesville, OK, USA). The samples after bead beating were filtered through Whatman filter paper with a pore size of 0.45µm. The solids, thus obtained were re-suspended in 1:1 chloroform-methanol mixture and the procedure was repeated. The resulting filtrates were pooled in a pre-weighed glass tube and the solvent was evaporated under low nitrogen pressure. The residual samples were further dried at 60^0C in a hot air oven until the sample reached a constant weight. The weight thus recorded represented as the mass of lipids in that sample.

Glycerol in the centrifuged supernatant was estimated based on a chemical method. Pure glycerol (Sigma-Aldrich, Canada) was used as the calibration standard [22]. Total dissolved nitrogen was analyzed by the Shimadzu VCPH automated analyzer. Citric acid estimation was carried out according to the method described by Marier et al, 1958 [23]. Appropriately diluted samples were mixed with pyridine and acetic anhydride. The reaction mixture was incubated in a water bath preset at 60^0C. After 5 minutes of incubation, the reaction mixture was cooled to room temperature and the OD (optical density) was measured at 510nm (Varian Cary 50, USA). Citric acid (Fisher, Canada) was used as the calibration standard.

3.2 Determination of yield

The lipid and biomass production rate were determined by the following equations. The specific growth rate (μ) was calculated according to Equation 1.

$$\mu = \frac{1}{X} \frac{dX}{dt} \quad \text{(Equn 1)}$$

Where X is the biomass concentration (g/L) at time t (h).

The biomass yield *(Yx/s g/g)* was calculated according to Equation 2, where X is the biomass concentration (g/L) and S is the substrate consumed to generate the biomass at time t (h).

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad \text{(Equn 2)}$$

The lipid yield *($Y_{P/S}$ g/g)* was calculated based on Equation 3, where P is the lipid concentration (g/L) and S is the substrate consumed to generate the biomass at time t (h)

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad \text{(Equn 3)}$$

The lipid content *($Y_{P/X}$ g/g)* was calculated from Equation 4

$$Y_{P/X} = \frac{\Delta P}{\Delta X} \quad \text{(Equn 4)}$$

The specific growth rate (μ), specific lipid production rate (U), lipid productivity, were calculated based on the model described by Giridhar et al.,2000 [24]

3.3 Characterization of lipid

Lipid sample of 25 mg was trans-esterified using acidified. Decahexanoic acid was used as the internal standard. The trans-esterified lipid fraction was extracted using hexane and the samples were further characterized by GC (Agilent 7890B) equipped with flame ionization detector (FID). Column length was 60m (Agilent J&W), the carrier gas was helium at a flow rate of 1.18 ml/min with the oven temperature 230^0C. Transesterified sample of 1μl was injected with an automated sample injector and the sample analysis was performed with Agilent GC chem station software. A 37 component FAME mixture from Supelco was used as the calibration standard at different concentrations.

4. Results and discussion

4.1 Effect of control of pH on biomass and lipid production

In the present study the crude glycerol solution used consists of glycerol concentration 78% (w/v), methanol (1.28% w/v), soap (2.4 %w/v), catalyst 0.12% (w/v) and water content of 2.48% (w/v). To calculate the C/N ratio the carbon present in glycerol was taken into account whereas the carbon in methanol and soap was not included since the amount was negligible.

Microbial assimilation of carbon source depends on the pH of the medium. The pH influences the surface properties of the cell membrane and thereby affects the carbon assimilation process. Several studies showed that a pH range of 6 to 6.5 is suitable for lipid production [25]. In the present investigation, initial pH of both fermenters was adjusted to 6.5. In one reactor, the pH was not controlled and in the other pH was controlled by adding 4N NaOH. Due to a rapid decline in pH in the uncontrolled reactor, the cells transformed into pseudohyphae. In the case of uncontrolled fermentation, the pH decreased from 6.5 to 3.98 at the end of fermentation (Figure 19) and this is due to the secretion of organic acids (mainly citric acid) into the medium. The production of citric acid by oleaginous microorganisms also has been reported by several authors [25, 26]. The pH control resulted in 15% (2g/L) higher lipid concentration than the uncontrolled experiments (Figure 20). From 60 h onward, the pH of the uncontrolled fermentation started to increase. After 108h of fermentation, in the pH uncontrolled reactor, the cells started to aggregate and pH of the medium increased to 4.3. This increase in pH could be attributed to maintaining the ionic balance in cellular metabolism; microbes tend to pump protons to intracellular space, which may lead to a slight increase in pH [27, 28].

The variation of concentration of biomass, lipid, and glycerol (g/L) with respect to fermentation time is presented in Figure 20. A short lag phase at the beginning of the fermentation was observed in both the pH controlled and the uncontrolled reactors. During the lag period (0-6h), a biomass increase of 1.25g/L and 0.24g/L was observed in the pH uncontrolled and the controlled reactors, respectively. This short lag period was brought by the sudden change in the medium composition, i.e. from a low concentration of glycerol (20g/L) during inoculum production to high concentration of glycerol (100g/L) in the fermenter medium. A substantial growth was achieved from 6 to 18h of fermentation in both reactors. The increase in biomass concentration was 9.3g/L in the pH controlled and 10.14g/L in the pH uncontrolled reactors during this time.

The biomass growth was decreased from 18h to 40h. After 40h, the increase in biomass was almost two times higher in the pH controlled reactor (3.52g/L pH controlled, 1.62g/L pH uncontrolled). At 60h the biomass concentration in the pH controlled and the pH uncontrolled reactor was 18 and 19.54g/L, respectively. The biomass concentration was almost stable from 80h onwards in both reactors. Growth reduction in both reactors could be because of the depletion of nutrients. On the other hand, a decrease in growth in the pH uncontrolled reactor was additionally contributed by low pH (4 to 4.5) in the medium. At the end of fermentation (120h), the biomass concentration was approximately the same in both reactors (20.58g/L pH controlled and 20.48g/L pH uncontrolled).

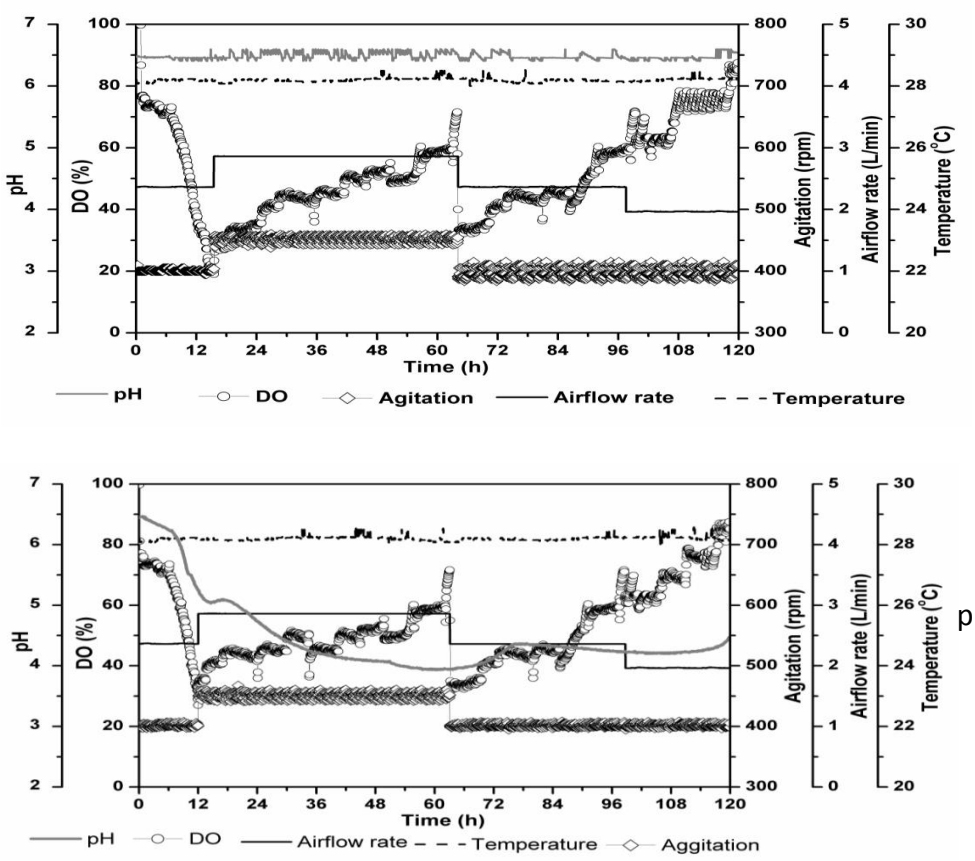

Figure 19 Fermentation profile of pH controlled and un-controlled fermentation

A monotonous decrease of glycerol concentration was observed in both reactors until 80h of fermentation (Figure 20). After 80h, glycerol consumption and increase in biomass and lipid concentration were very low. At this point, the consumed glycerol in the case of the pH controlled reactor was mostly converted into citric acid (Table 25) or other fermentation co-

products and in the case of the pH uncontrolled reactor, it may be used mainly for cell maintenance and some other co-product formation (polyalcohols, glycogens, etc) [29].

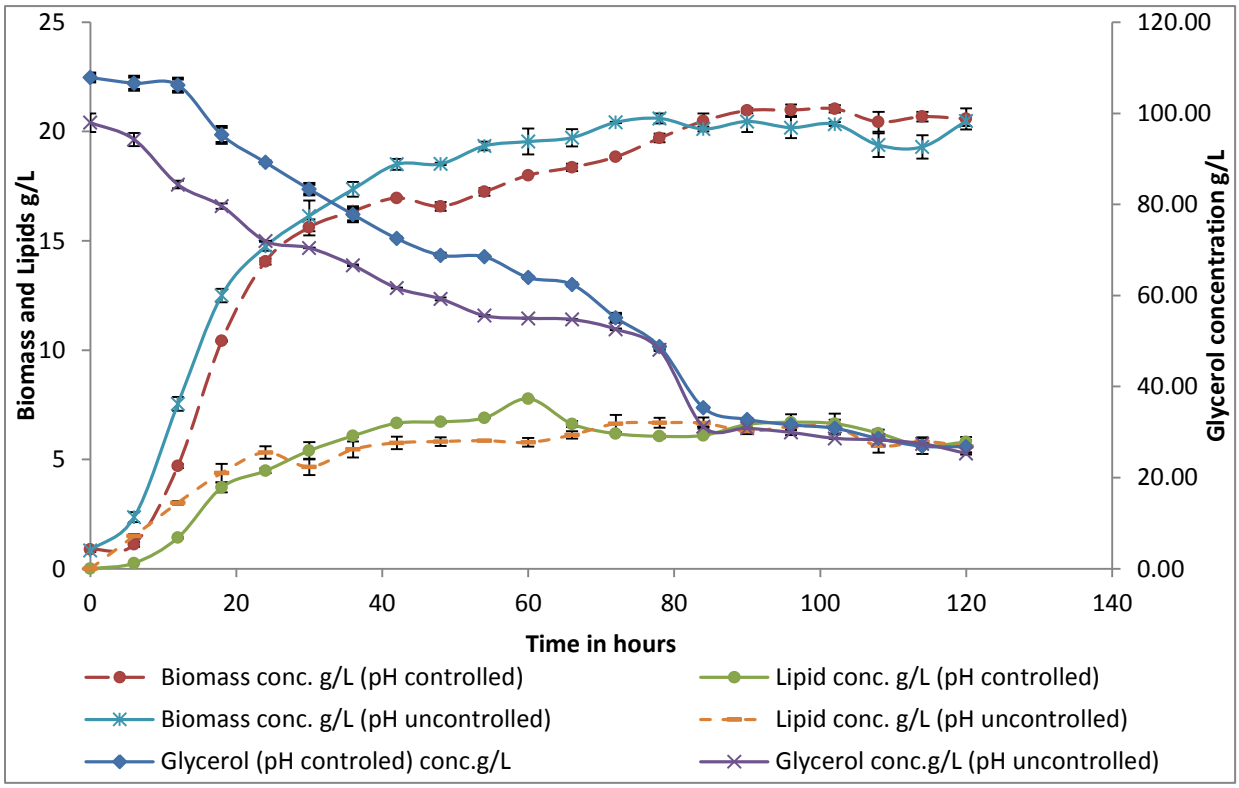

Figure 20 Variation of biomass, glycerol and lipid concentration during fermentation with and without pH control

The lipid concentration (Figure 20) was found to increase until 60h in case of the pH controlled and until 78h in the case of the pH uncontrolled reactor. However, significantly low increase in lipid concentration (0.9g/L) was observed in the pH uncontrolled fermentation from 60h to 78h. From 6-18h (the growth phase) of fermentation the lipid produced in the pH controlled and the uncontrolled reactors was 3.46g/L and 2.8 g/L, respectively. The concentration of lipid at 48h in the controlled and the uncontrolled reactor was 2.94g/L and 1.38g/L, respectively. From 48 to 60h, 1.12g/L lipid was produced in the pH controlled reactor, whereas no substantial lipid production (0.2g/L) was noticed in the uncontrolled reactor. In the case of pH controlled reactor, the lipid started to decrease after 60h of fermentation. From 60h to 120h of fermentation 1.98g/L of lipid was degraded, whereas in the pH uncontrolled reactor the lipid degradation was detected from 78h. The maximum lipid accumulation of 7.78g/L in the pH controlled reactor was observed at 60h. The biomass concentration at 60h was 18g/L with the lipid content of

45.44%w/w. The maximum lipid concentration 6.68 g/L with biomass concentration 20.6 g/L and lipid content of 33.8% (w/w) was observed at 78h in the pH uncontrolled fermentation. A slightly low lipid concentration can be correlated with the pH of the medium. Since the pH of the fermentation medium affects the cell membrane property and it has been reported that growth rate was higher when the pH was above 4.5 [30].

Y.lipolytica is a well-known producer of industrial citric acid. The concentration of citric acid was similar in both reactors at 24h of fermentation. In the case of a pH controlled reactor, the citric acid concentration was noticed to substantially increase from 24h and reached 5.71g/L at 60h and 11.1g/L at 120h (Table 25). In the case of pH uncontrolled fermentation, rapid decline in pH inhibits the citric acid production. The maximum concentration of citric acid obtained was 1.2g/L at 72h.On the other hand, the citric acid production (citrate concentration in the cytoplasm) is one of the important factors that control the lipid accumulation. A certain concentration of citrate in the cytoplasm is required to activate the intracellular lipid synthesizing enzymes. Citric acid is the substrate for ATP citrate lyase, which is involved in lipid production metabolism. Citric acid activates the ACC (Acetyl Co-A carboxylase), which is involved in the conversion of acetyl Co-A to malonyl Co-A (a step in lipid synthesis) [31]. A constant supply of citrate will generate adequate amounts of acetyl-CoA in the cytoplasm by the enzyme ACL (ATP citrate lyase). Narlin et al. (1983) showed that 2.0mM citrate concentration resulted in a steady rate of ACC activity [32]. Further, the relationship between the cellular concentration of citrate and carboxylase activity was confirmed by Moss & Lane [33]. Therefore, the supply of acetyl-CoA required for lipid synthesis depends on the cellular ACL and the ACC catalytic activity and these enzymes are guarded by cellular citrate concentration. In the case of pH uncontrolled fermentor, a low citrate concentration in the medium (0.55g/L at 24h and 1.25g/L at 66h, (Table 25) reflects that the intracellular citric acid concentration in the cells would be low. The low citric acid concentration reduced the lipid production, which resulted in a little lower lipid concentration in the pH uncontrolled fermentation. Thus, pH control favors the citric acid production that resulted in slightly higher biomass lipid content and higher medium lipid concentration in a shorter time than the pH uncontrolled fermentation.

Table 25 Variation of citric acid concentration during the pH controlled and the pH uncontrolled fermentation

	Citric acid (g/L)	
Time	pH controlled	pH un-controlled
0	0.4 ± 0.05	0.4± 0.02
24	0.51± 0.01	0.55± 0.001
48	2.35 ± 0.06	0.65± 0.004
72	5.71± 0.17	1.25± 0.02
96	7.92± 0.62	1.14± 0.006
120	11.10± 0.08	1.05± 0.1

From the above observations, it is clear that the fermentation can be stopped at 60h. At this instant, 53.9 % glycerol (added initially) was assimilated (23.2%w/w was converted to lipid-free biomass, 17.7%w/w to lipid and 13%w/w to citric acid) under the pH controlled condition. In the case of the pH uncontrolled fermentation 48.38% glycerol was assimilated (32.0%w/w was converted to lipid-free biomass, 13.4%w/w to lipid and 2.9%w/w to citric acid). The above comparison shows that 8.7% and 4.3% more glycerol was used for biomass production and lipid production, respectively, in the pH controlled reactor than the uncontrolled pH. Thus, controlling the pH during fermentation can lead to a little higher lipid production.

4.2 Variation of yield factors, specific growth rate, and specific lipid production rate during pH controlled and uncontrolled fermentation.

The values of biomass and lipid yields with respect to glycerol consumed in the case of pH controlled and uncontrolled fermentation are presented in Figures 21 A&B. In both cases, $Y_{X/S}$ (biomass produced/g of glycerol consumed) increased with the fermentation time until 24h. The value of maximum biomass yield ($Y_{X/S}$) of 0.538g/g (Yx/s 0.354g/g of lipid-free biomass) for pH controlled and 0.505g/g ($Y_{X/S}$ 0.339g/g of lipid-free biomass) for pH uncontrolled fermentation was attained at 24h (Figure 21A & B). Thereafter, the $Y_{X/S}$ value decreased with fermentation time in both cases.

The lipid yield ($Y_{P/S}$, g lipid produced/ g of glycerol consumed) increased in the beginning of the fermentation (0h to 18h) and then decreased until the end of fermentation (Figures 21 A& B). In

the case of the pH-controlled fermentation, the $Y_{P/S}$ value reached 0.2g/g at 18h whereas it was 0.168g/g in the pH uncontrolled reactor. The high value of lipid yield ($Y_{P/S}$) in the beginning of the fermentation could be attributed to lipid production as a part of cell wall synthesis. From 24h to 60h, the $Y_{P/S}$ decreased (from 0.18 to 0.17g/g) very slowly in the pH controlled reactor. In the case of the pH uncontrolled fermentation, $Y_{P/S}$ also decreased slowly, but for a longer period (24 to 78h, $Y_{P/S}$ 0.149 to 0.133g/g). Almost constant $Y_{P/S}$ value or a slow decrease up to 60h of fermentation leads to the conclusion that the fermentation should be terminated at 60h. After 60h of fermentation, the $Y_{P/S}$ value declined in both cases. The decline in $Y_{P/S}$ value could be explained by the lipid-free biomass concentration synthesized from 24 to 78h. The $Y_{X/S}$ value (with respect to lipid-free biomass) was noticed to be higher than the $Y_{P/S}$ value from 24 to 78h ($Y_{P/S}$ 0.13g/g and $Y_{X/S}$ lipid free biomass 0.3g/g). This indicates that most of the assimilated glycerol were directed towards biomass synthesis. Further, lipid free biomass yield reached maximum (0.354g/g) at 24h in the pH controlled reactor followed by a decrease and reached 0.205g/g at 60h. In the pH uncontrolled fermentation, the lipid-free biomass yield reached maximum 0.34g/g at 30h and then decreased slightly 0.3g/g at 60h.This high lipid free biomass production results in low lipid accumulation.

A different trend of lipid yield with respect to biomass ($Y_{P/X}$, g lipid produced/ g of biomass) in the pH uncontrolled reactor was perceived (Figure 21B). The lipid content of the biomass declined from 0.451g/g to 0.305g/g within the first 30h of fermentation and then remained almost constant (from 36h onward) until the end of fermentation. In the case of the pH-controlled fermentation, after a short decline from 12 to 24h of fermentation, an increasing trend in the $Y_{P/X}$ value was observed for 24 h (0.34g/g) to 60h (0.454g/g), which reflected high biomass lipid content at 60h (45.4%w/w). After 60h, a sharp decline in $Y_{P/X}$ value was evident (Figure 21 A), which indicates the lipid degradation in the pH-controlled fermentation. The lipid degradation could be attributed to β- oxidation of the produced lipid by the organism. In the case of the pH uncontrolled fermentation, the lipid degradation was noticed after 78h (about 1g/L lipid degraded from 78 to 120h).

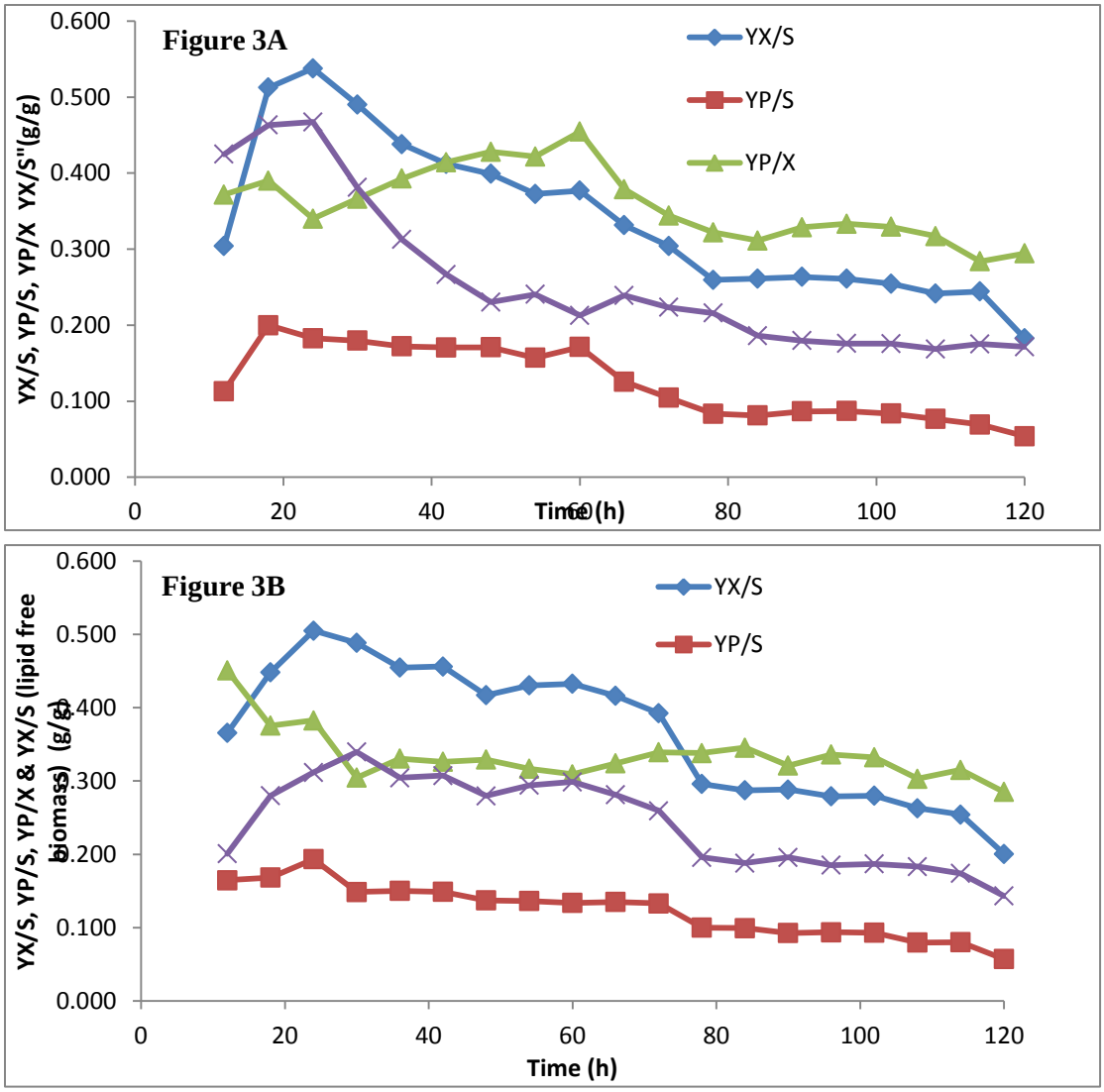

Figure 21 Variation of biomass (Yx/s), lipid free biomass (Yfx/s) and lipid yield (Yps) in the pH controlled (3A) and pH uncontrolled (3B) fermentation

The specific growth rates (μ) in both reactors reached maximum at 12h of fermentation (Figure 22 A & B). A Higher value of μ was observed for the pH uncontrolled reactor ($0.182h^{-1}$) than the pH controlled reactor ($0.139\ h^{-1}$). This shows that from six to 12h high growth occurred with or without control of pH. After 12h of fermentation, the specific growth rate value declined until the end of fermentation in both reactors. The decrease in specific growth rate could be due to the nutrient limitation in the medium. The specific lipid production rate (U) also followed a similar trend. The value of U for both fermentations was found to decrease with fermentation time (Figure 22 A & B) and reached near zero at around 60h. In the case of the pH uncontrolled fermentation, specific lipid production rate (U) was very low from 24h to 42h. In the pH-

controlled fermentation, the U was higher at 12h (0.061 h^{-1}). The U value was found to be 0.031 h^{-1} at 12h for the pH uncontrolled reactor, which is almost 50% lower than the pH-controlled fermentation.

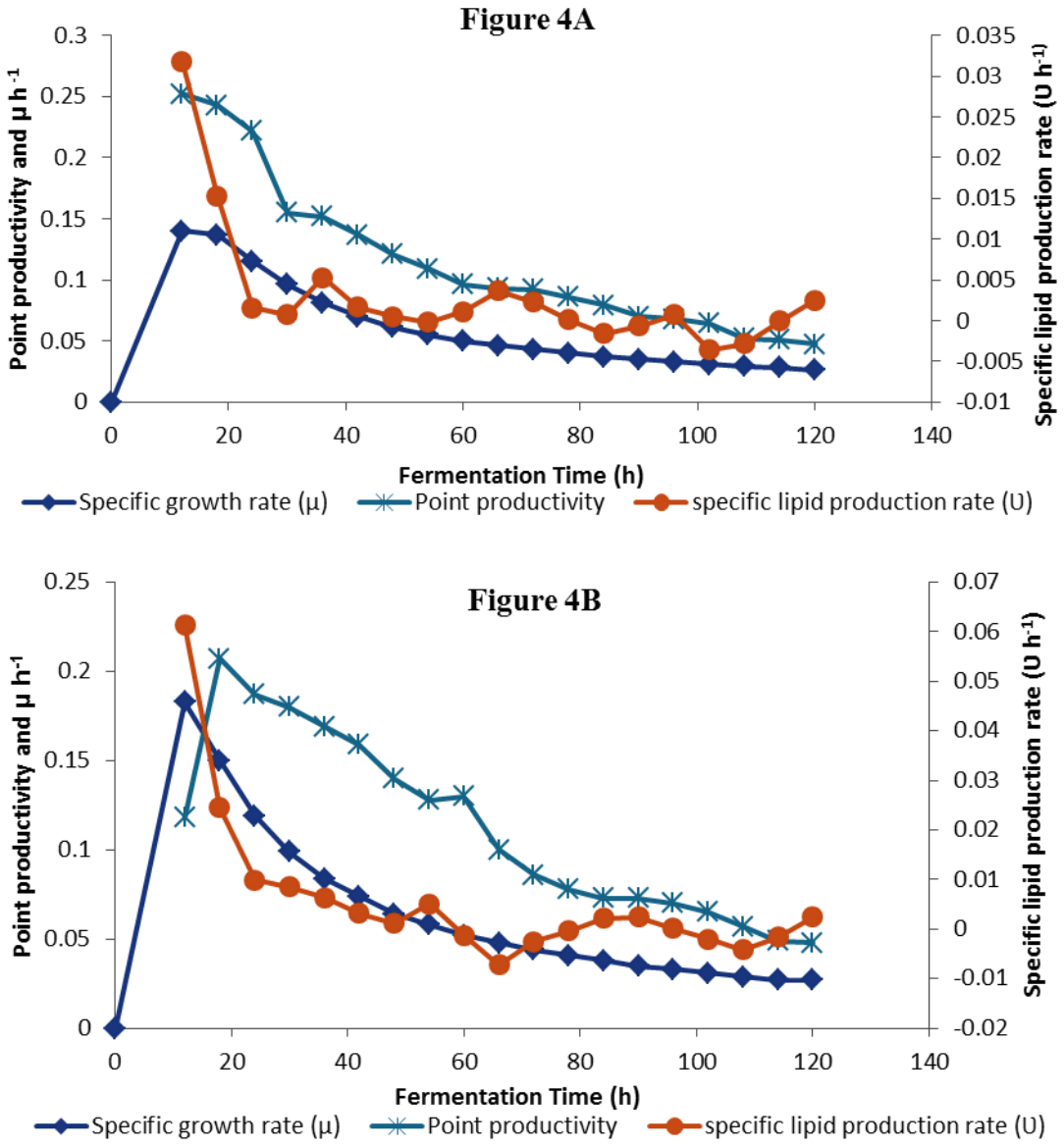

Figure 22 Variation of Specific growth rate (μ), specific lipid production rate (U) and point productivity in the pH uncontrolled (4A) and pH uncontrolled (4B) fermentation

On the contrary, lipid production in both reactors occurred throughout the fermentation but decreased with fermentation time. The lipid productivity in case of the pH-controlled fermentation increased from 0.118g/L/h to 0.207g/L/h from 12 to 18h followed by a slow decline

and reached 0.12 g/L/h at 60h (Figure 22A). The maximum lipid production rate of 0.252g/L/h was found at 12h in the pH uncontrolled reactor, which declined rapidly and reached to 0.096g/L/h at 60h (Figure 22B). The rapid decrease in lipid productivity with respect to the fermentation time in both cases could be attributed to a decrease in the malic enzyme activity [34]. It was reported that the malic enzyme (intracellular) activity decreases exponentially when nitrogen in the medium is depleted (discussed later). At the end of fermentation (120h), with or without pH control, the lipid productivity was noted to be the same (0.048 and 0.047g/L/h). These results clearly established that after 60h the lipid produced is not significant.

3.3 Relation between specific growth rate and specific lipid production rate

The specific growth rates and specific lipid production rates were computed by differentiating the concentration (biomass and lipid) versus time data and the results are presented in Figure 23, which revealed that the lipid production was proportional to growth. In general, the lipid accumulation has been defined as non-growth associated [19]. The maximum specific growth rate was attained between 6h to 12h (Figure 22A & B) in both cases and at this time a substantial amount of lipid was also synthesized (1.42 g/L with and 3.02 g/L without pH control at 12h). Thus, the lipid synthesis even occurred during the early stage of fermentation.

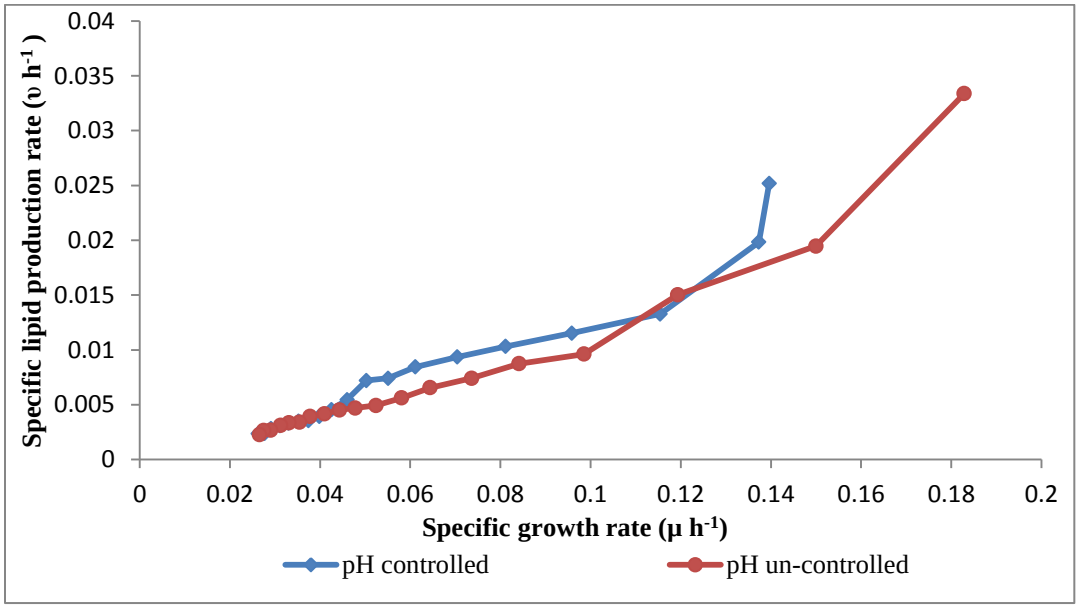

Figure 23 Specific lipid production rate of pH controlled and uncontrolled fermentation

These observations suggest that controlling the pH at an optimal level slightly improved the lipid productivity. In the case of the pH uncontrolled fermentation, lipid concentration 6.12g/L, and

biomass concentration 19.54g/L was attained at 66h and there was no significant increase afterward (Figure 20). On the other hand, 7.78g/L of lipid with 18g/L biomass was attained at 60h of fermentation in the pH controlled reactor. Meanwhile, in experiments with controlled pH, *Yp/s* (0.171 at 60h) stayed significantly high until 60h compared to the pH uncontrolled experiment ($Y_{P/S}$; 0.135 at 60h). Further, lipid productivity and specific lipid production tend to near zero in both cases. This suggests that the fermentation should be terminated before 60h

3.4 Evolution of C/N ratio on pH controlled and un-controlled conditions

The change in carbon flux alters the lipid accumulation in *Y.lipolytica* [8]. It is advocated that a low concentration of carbon in the medium leads to β- oxidation of stored lipids for re-utilization by the organism. However, in the presence of an abundance of carbon with high C/N molar ratio in the medium, *Y. lipolytica* generates low biomass and high lipid concentration. Further, a very high C/N ratio represses the metabolism due to nitrogen deficiency, which reduces the lipid accumulation and favors the secondary metabolite formation. In the present study initial C/N ratio was 113 for the pH controlled and 115 for the pH uncontrolled fermentation (Figure 24). The C/N ratio increased with the fermentation time. The nitrogen content of the fermentation broth reached 0.098 and 0.089g/L in 24h in the pH controlled and the uncontrolled fermentation, respectively, and after that remained approximately unchanged during the rest of the fermentation period (Figure 24). The glycerol was continuously assimilated by the organism and thus decreasing the carbon concentration in the medium, which resulted in a decrease of the C/N ratio below 300 in both fermentations. At this moment, the lipid production rate and an increase in biomass concentration were very low; therefore, it seems that most of the assimilated glycerol were diverted for cell maintenance purpose. Under the nitrogen limited condition, the glycerol consumed could also be diverted to the synthesis of secondary metabolites such as organic acids (citric acid, isocitric acid) or polyalcohols (erythritol, mannitol etc.). Citric acid production was enhanced when the nitrogen content was low or unavailable in the media (from 24h) in the pH controlled reactor (Table 25). Synthesis of citric acid should have concomitantly increased the lipid production, however, due to unavailability of nitrogen in the medium, the rate of the lipid production drastically decreased.

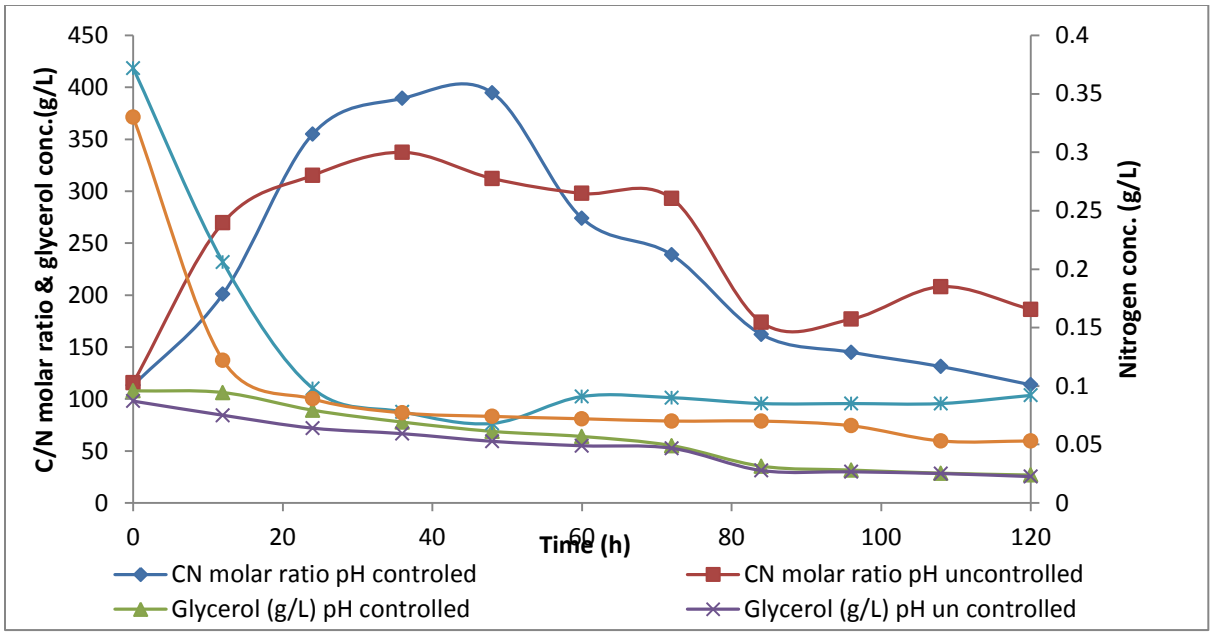

Figure 24 C/N evolution upon time in pH controlled and uncontrolled experiments

This can be further explained by correlating the specific lipid production rate with the C/N ratio. The specific lipid production rates of the pH controlled and the uncontrolled fermentation was noted to decline with increasing C/N ratio and reached very low at 24h in both cases (Figure 22A & B) when the nitrogen content of the medium was very low (0.098 and 0.089g/L). It is reported that when the organism enters the lipid synthesis mode, it needs a certain amount of nitrogen (as ammonium ion), which could serve as the co-factor for several enzymes in lipid biosynthesis [8, 25]. Thus, the present observation and the previously reported studies clearly suggest that it is necessary to maintain a low concentration of usable nitrogen in the medium after the growth, which will help to increase the lipid content of the cells.

3.5 Influence of pH control on lipid composition

The composition of lipid considerably depends on the carbon source, the concentration of carbon and the C/N ratio of the medium. There are several studies, which showed that the lipid profile changed with fermentation time [35]. Table 26 presents the lipid composition of *Y.lipolytica* (SKY7) for the pH controlled and the uncontrolled experiments. Oleic acid was found to be the major component in all cases. *Y.lipolytica* SKY7 isolate produced the following fatty acids in high quantity: *Myristic* (C14:0), *Palmitic* (C16:0), *Stearic* (C18:0), *Oleic cis-9* (C18:1n9-cis), *Linoleic* (C18:2n6c) and *Arachidonic* (C20:4n6). Other than the above listed triacylglycerols

(TAG), this isolate also produced C15:0, C15:1, *Palmitoleate* (C16:1n7), *Elaidic* (C18:1n9t), *Linolenate* (C18:3n3), *Arachidic* (C20:0), *Eicosenoate* (C20:1n9), *Lignoceric* (C24:0) in minor quantities (Data not shown). Linoleic acid concentration decreased from 27.76% (24h) to 15.25% w/w (120h) in the case of pH-controlled fermentation. In the case of the pH uncontrolled reactor, linoleic acid was decreased from 31.76% (24h) to 12.88% (120h). Myristic acid concentration decreased from 24h to 48 h and after that remained almost constant. The other TAGs were noted to increase with fermentation time.

Table 26 Lipid profile of *Y.lipolytica* in different culture conditions (digits represent % of the total lipid)

Sample details	*Myristic (C14:0)*	*Palmitic (C16:0)*	*Stearic (C18:0)*	*Oleic cis-9 (C18:1n9-cis)*	*Linoleic (C18:2n6c)*	*Total*	*Others*
pH Controlled							
24h	8.04	12.88	8.28	39.56	27.76	96.52	3.48
48h	0.76	12.44	8.01	38.44	27.02	86.68	13.32
72h	0.62	14.45	9.68	43.18	17.43	85.37	14.63
96h	0.80	15.38	9.10	43.95	18.65	87.87	12.13
108h	0.54	15.51	8.92	44.51	16.54	86.03	13.97
120h	0.53	16.24	9.37	44.53	15.25	85.93	14.07
pH Uncontrolled 24h	9.17	14.11	4.85	27.28	31.76	87.17	12.83
48h	0.48	15.67	9.06	45.13	18.27	88.59	11.41
72h	0.69	15.11	9.02	46.47	16.01	87.31	12.69
96h	0.56	15.95	9.56	47.47	14.67	88.21	11.79
108h	0.37	15.72	10.64	48.84	12.66	88.23	11.77
120h	0.42	16.11	9.98	49.47	12.88	88.85	11.15

4. Summary and conclusion

The results revealed that controlling pH at 6.5 produced 7.78g/L and uncontrolled 6.58g/L (60h) of lipid. In both cases, early lipid production was observed and this could be helpful to reduce the fermentation time. The specific lipid production was proportional to the specific growth rate and most of the lipid was produced during 60 h fermentation. Due to the low nitrogen concentration in the medium, the growth ended before 18h in both fermentations. In conclusion controlling pH lead to increase in lipid production significantly and further favors citric acid production in late fermentation stage.

Acknowledgement

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References

[1] M. Ayoub, A.Z. Abdullah, Critical review on the current scenario and significance of crude glycerol resulting from biodiesel industry towards more sustainable renewable energy industry, Renewable and Sustainable Energy Reviews, 16 (2012) 2671-2686.
[2] C.J. Campbell, The Rimini Protocol an oil depletion protocol: Heading off economic chaos and political conflict during the second half of the age of oil, Energy Policy, 34 (2006) 1319-1325.
[3] M.L. Savaliya, B.D. Dhorajiya, B.Z. Dholakiya, Recent advancement in production of liquid biofuels from renewable resources: a review, Research on Chemical Intermediates, 41 (2013) 475-509.
[4] R. Ciriminna, C.D. Pina, M. Rossi, M. Pagliaro, Understanding the glycerol market, European Journal of Lipid Science and Technology, 116 (2014) 1432-1439.
[5] X.F.a.R. Burton, Recent Development of Biodiesel Feedstocks and the Applications of Glycerol: A Review, The Open Fuels & Energy Science Journal, 2 (2009) 100-109.
[6] A. Beopoulos, Z. Mrozova, F. Thevenieau, M.T. Le Dall, I. Hapala, S. Papanikolaou, T. Chardot, J.M. Nicaud, Control of lipid accumulation in the yeast Yarrowia lipolytica, Applied and environmental microbiology, 74 (2008) 7779-7789.
[7] S. Papanikolaou, G. Aggelis, Selective uptake of fatty acids by the yeastYarrowia lipolytica, European Journal of Lipid Science and Technology, 105 (2003) 651-655.
[8] A. Beopoulos, J. Cescut, R. Haddouche, J.L. Uribelarrea, C. Molina-Jouve, J.M. Nicaud, Yarrowia lipolytica as a model for bio-oil production, Progress in lipid research, 48 (2009) 375-387.

[9] J. Lin, H. Shen, H. Tan, X. Zhao, S. Wu, C. Hu, Z.K. Zhao, Lipid production by Lipomyces starkeyi cells in glucose solution without auxiliary nutrients, Journal of biotechnology, 152 (2011) 184-188.
[10] S. Ines, H. Silla, G. Steffen, R. Thomas, O. Katrin, S. Christoph, N. Anke, Characterization of newly isolated oleaginous yeasts - Cryptococcus podzolicus, Trichosporon porosum and Pichia segobiensis, AMB Express, 4 (2014).
[11] Y.H. Seo, I. Lee, S.H. Jeon, J.-I. Han, Efficient conversion from cheese whey to lipid using Cryptococcus curvatus, Biochemical Engineering Journal, 90 (2014) 149-153.
[12] Y. Cui, J.W. Blackburn, Y. Liang, Fermentation optimization for the production of lipid by Cryptococcus curvatus: Use of response surface methodology, Biomass and Bioenergy, 47 (2012) 410-417.
[13] A. Chatzifragkou, S. Fakas, M. Galiotou-Panayotou, M. Komaitis, G. Aggelis, S. Papanikolaou, Commercial sugars as substrates for lipid accumulation in Cunninghamella echinulata and Mortierella isabellina fungi, European Journal of Lipid Science and Technology, 112 (2010) 1048-1057.
[14] A. Beopoulos, T. Chardot, J.M. Nicaud, Yarrowia lipolytica: A model and a tool to understand the mechanisms implicated in lipid accumulation, Biochimie, 91 (2009) 692-696.
[15] Y. Gao, D. Li, Y. Liu, Production of single cell protein from soy molasses using Candida tropicalis, Annals of Microbiology, 62 (2011) 1165-1172.
[16] Mhairi Workman, Philippe Holt, J. Thykaer, Comparing cellular performance of Yarrowia lipolytica during growth on glucose and glycerol in submerged cultivations, AMB Express, 3 (2013) 1-9.
[17] A. Rywińska, P. Juszczyk, M. Wojtatowicz, M. Robak, Z. Lazar, L. Tomaszewska, W. Rymowicz, Glycerol as a promising substrate for Yarrowia lipolytica biotechnological applications, Biomass and Bioenergy, 48 (2013) 148-166.
[18] S. Papanikolaou, L. Muniglia, I. Chevalot, G. Aggelis, I. Marc, Yarrowia lipolytica as a potential producer of citric acid from raw glycerol, Journal of applied microbiology, 92 (2002) 737-744.
[19] C. Ratledge, J.P. Wynn, The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms, Advances In Applied Microbiology, 51 (2002).
[20] L.R. Jarboe, L.A. Royce, P. Liu, Understanding biocatalyst inhibition by carboxylic acids, Frontiers in microbiology, 4 (2013) 272.
[21] J. Folch, M.Lees, G.H.Slane-Stanley, Simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem, 226 (1957) 497–509.
[22] P. Bondioli, B. Della, Laura, An alternative spectrophotometric method for the determination of free glycerol in biodiesel, European Journal of Lipid Science and Technology, 107 (2005) 153-157.
[23] J.R. Marier, M. Boulet, Direct determination of citric acid in milk with an improved pyridine-acetic anhydride method, Journal of Dairy sciences, 41 (1958) 1683-1692.
[24] R. Giridhar, A.K. Srivastava, Model based constant feed fed-batch L-sorbose production process for improvement in L-sorbose productivity., Chemical and biochemical engineering quarterly 14 (2000) 133-140.
[25] S. Papanikolaou, I. Chevalot, M. Komaitis, I. Marc, G. Aggelis, Single cell oil production by Yarrowia lipolytica growing on an industrial derivative of animal fat in batch cultures, Applied microbiology and biotechnology, 58 (2002) 308-312.

[26] C. Ratledge, Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production, Biochimie, 86 (2004) 807-815.
[27] V. Cristina, A. Paulo, C. Miguel, C. isabel, The H1-ATPase in the Plasma Membrane of Saccharomyces cerevisiae Is Activated during Growth Latency in Octanoic Acid-Supplemented Medium Accompanying the Decrease in Intracellular pH and Cell Viability, Applied and environmental microbiology, 64 (1998) 779-783.
[28] A. Ullah, R. Orij, S. Brul, G.J. Smits, Quantitative analysis of the modes of growth inhibition by weak organic acids in Saccharomyces cerevisiae, Applied and environmental microbiology, 78 (2012) 8377-8387.
[29] L. Tomaszewska, M. Rakicka, W. Rymowicz, A. Rywinska, A comparative study on glycerol metabolism to erythritol and citric acid in Yarrowia lipolytica yeast cells, FEMS yeast research, 14 (2014) 966-976.
[30] J. Van Leeuwen, M.L. Rasmussen, S. Sankaran, C.R. Koza, D.T. Erickson, D. Mitra, B. Jin, Fungal Treatment of Crop Processing Wastewaters with Value-Added Co-Products, Sustainable Bioenergy and Bioproducts,Green Energy and Technology, 8 (2012) 13-44.
[31] A.D. Landman, K. Dakshinamurti, Acetyl-Coenzyme A Carboxylase role of the prosthetic group in enzyme polymerization, Biochemical journal, 145 (1975) 545-548.
[32] B. Narlin, S. Beaty, M.D. Lane, Kinetics of Activation of Acetyl-coA Carboxylase by Citrate relationship to the rate of polymerization of the enzyme, The Journal of biological chemistry, 258 (1983) 13043-13050.
[33] J. Moss, M.D. Lane, Acetyl Coenzyme A Carboxylase, The Journal of biological chemistry, 247 (1972) 4952-4959.
[34] J.P. Wynn, Aidil bin Abdul Hamidt, C. Ratledge, The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi, Microbiology, 145 (1999) 1911-1917.
[35] S. Papanikolaou, I. Chevalot, M. Komaitis, Geor, Kinetic profile of the cellular lipid composition in an oleaginous Yarrowia lipolytica capable of producing a cocoa-butter substitute from industrial fats, Antonie van Leeuwenhoek, 80 (2001) 215-224.

Chapter-IV-Part-III

Research Article 4

Kinetics of lipid production at lab scale fermenters by a new isolate of *Yarrowia lipolytica SKY7*

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RÉSUMÉ

Yarrowia lipolytica est un organisme industriel important en raison de ses multiples applications dans différentes industries. Sur la base de notre étude précédente, la concentration de glycérol, le rapport C/N et la concentration d'inoculum ont été choisis pour améliorer la biomasse et la production de lipides. Sur la base de l'analyse statistique, la concentration du modèle d'inoculum du glycérol, et le rapport C / N avec l'inoculum ont été jugées importantes dans ce cas de production de lipides. La production maximale de lipides a été plus élevée dans le glycérol de 82,5 g/L, C/N 75 et un volume d'inoculum de 6,25% (condition de culture prédite). Les conditions de culture optimisées ont été testées dans un réacteur à petite échelle de 15L. La concentration de biomasse et la teneur en lipides obtenu sont à 29,5 g/l et 50% (p/p), respectivement. Les coefficients de rendement ont été calculées et aperçu à 0.332g/g (g biomasse / g de glycérol) de biomasse et de 0.179g/g (g lipide /g glycérol consommé) de lipide. Les taux observés de production de lipides ont montré une production à partir de 30h de la fermentation. Du glycérol total consommé, 41,1% de glycérol ont été converti en biomasse, en lipides et en acide citrique.

Mots-clés: lipides microbiens, *Yarrowia lipolytica*, Fermentation, optimisation du RSM, la biomasse

Abstract

The objective of this work was to study the kinetics of lipid production at lab scale fermenters by a new isolate of *Yarrowia lipolytica* SKY7. The model terms glycerol concentration inoculum and C/N ratio with inoculum were found to be significant for lipid production. Lipid production was found to be higher in glycerol 82.5g/L, C/N ratio 75 and inoculum volume 6.25%. Optimized culture conditions were tested at 15L bench scale reactor. The biomass concentration and lipid content obtained was 29.5g/L and 50% (w/w), respectively. The yield coefficients were calculated and found to be 0.332g/g (g biomass/g of glycerol) of biomass and 0.179g/g (g lipid /g glycerol consumed) for lipid. Observed rates of lipid production show lipid production from 30h of fermentation. Out of the total glycerol consumed, 41.1% glycerol was converted into biomass, lipid, and citric acid.

Keywords: Microbial lipids, *Yarrowia lipolytica*, Fermentation, RSM optimization, Biomass

1. Introduction

Bio-diesel is the clean, biodegradable, renewable, and non-toxic fuel produced from triacylglycerides (TAG) of bacteria, yeast, fungi, algae, plants and animals (Li et al., 2008; Okamura et al., 2015; Poli et al., 2014; Ruan et al., 2014). Biodiesel is considered as a green fuel since it does not release excess CO_2 and sulfur (Gustone, 2009). At present, identification and effective utilization of low-cost raw materials such as starch, crude glycerol, lignocellulosics, volatile fatty acids and other waste materials for biofuel product are a matter of active research (Li et al., 2015; Liu et al., 2015; Vajpeyi & Chandran, 2015; Xue et al., 2010). Currently, biodiesel is produced from vegetable oil, animal fat and used cooking oil; however, this practice is expensive and created food versus fuel competition (Demirbas, 2005; Gui et al., 2008). The lack of availability in sufficient quantity and high sulfur content and other impurities makes cooking oil as unsuitable raw material for the biodiesel application. Even though extensive research has been conducted to find a better raw material for microbial lipid (or oil) production; crude glycerol gained special importance among the researchers because it is available as a by-product at the biodiesel production site at low cost (Yazdani & Gonzalez, 2007). The other major industries that produce glycerol as a by-product are petrochemical industry and soap and saponification industry (Christophe et al., 2012). However, the microbial conversion of crude glycerol into lipids is not economical due to the following reasons. Primarily the capacity of glycerol tolerance and optimum concentration of glycerol required for lipid production varies based on the type of microbial strain. Tolerance of different inhibitors presents in the crude glycerol and adaptation to batch to batch variations of glycerol and inhibitors. Secondly the influence of raw material, that is the chemical composition of the crude glycerol, which varies based on the catalyst used in the transesterification process. This includes the glycerol content of crude glycerol solution (ranges from 36-96%w/w) and other major by-products such as methanol, soap, catalyst salt, non-glycerol organic matters, and water (Ayoub & Abdullah, 2012; Chatzifragkou & Papanikolaou, 2012).

The increased interest in microbial oil production initiated the bio-diesel industry to enter into a new generation of fuel production. Substantial research has been conducted and varying lipid accumulation capacities of different microbial strains ranging from 20 to 70% w/w have been reported. Among the microbes bacteria, yeast and fungi are suitable for utilizing crude glycerol

as the sole carbon source to produce lipid (André et al., 2010; Okamura et al., 2015; Poli et al., 2014).The main advantage of microbial oil production over oil crops is the fast growth, high lipid content and non-seasonal (Dias et al., 2015). However, a complete bioprocess for lipid production from microbes at low cost has not been developed.

Apart from high lipid accumulating capacity of a microbial strain, the second most important factor is the macro and micronutrients required for cell growth. The biochemical pathways of lipid production clearly dictate that the citrate is the precursor for the triacylglycerols (TAG) biosynthesis (Ratledge & Wynn, 2002). The limitation of nitrogen availability in the media triggers the pathway towards the lipid biosynthesis. Therefore, a limited supply of nutrients is one of the major concerns in the microbial lipid synthesis. Considering the fact of high lipid production in a feasible way requires primarily a well-optimized process that could be flexible enough to adapt to the variations in medium composition and produces the end product without any huge variation. Yet, in this case, more than one culture parameter determine the biomass and lipid production. Due to the complex nature of the process, a conventional way of optimizing the process makes the research laborious and time-consuming. Statistical tools are (RSM-Response Surface Methodology) well used in optimizing the culture growth media and to study the interaction between the various growth parameters.

In this study, a Box-Behnken design was used to understand the significance of principle parameters such as glycerol concentration, C/N molar ratio and inoculum volume for biomass production and lipid synthesis. In the case of *Y.lipolytica*, lipid accumulation was reported mostly with high glycerol concentration (100g/L) with high initial C/N ratio. However, in most of the cases, the glycerol was not completely utilized even after 5 days (120h) of fermentation (Kuttiraja et al., 2015). This could lead to a high waste of glycerol. At the same time, a high concentration of glycerol can influence the growth and finally reduce the yield of lipid production(Tchakouteu et al., 2015). Since the strain, *Y.lipolytica* SKY7 is a new isolate it is highly important to determine the optimum culture parameters to obtain a high biomass and lipid to develop an economically feasible process. Due to the above reason, a statistical method was introduced to know the significance of the important variables and their influence on biomass and lipid production. Meanwhile, the flask scale studies (statistical optimization) has limitation to draw a complete conclusion of process kinetics. To evaluate the RSM optimized culture

parameters a bench scale fermentation was also conducted to assess the optimal kinetic parameters of lipid production in crude glycerol as a growth medium.

2. Materials and methods

2.1.1. Culture and fermentation conditions

The pure culture of *Y.lipolytica* SKY7 was maintained at YPD slants (Yeast extract 10g/L, Peptone 10g/L dextrose 20g/L and agar 20g/L). The inoculum was prepared in YPCG (yeast extract 10g/L, peptone 10g/L, crude glycerol 20g/L) media. A loop full of the pure colony was aseptically transferred to the medium and it was incubated at 28^0C in an agitating shaker at 180 rpm for 24h. Well grown inoculum was transferred to a sterile centrifuge tube (500mL) and centrifuged at 6000 rpm for 10 minutes and the supernatant was discarded under sterile condition and then the cells were re-suspended in 250mL of sterile distilled water. This suspension was used as the inoculum for the study.

The crude glycerol used in the present study was kindly provided by a biodiesel producing industry in Quebec region, Canada. In all the cases the crude glycerol was used without any treatment. The glycerol solution used in this study contains 78%w/w glycerol, methanol 1.28% w/w, soap 2.4%w/w, water 2.48% w/w and catalysts 0.12%w/w.

2.1.2. Optimization of culture parameters (Box-Behnken design)

The glycerol concentration was varied from 75 to 100g/L. The glycerol stock solution was diluted according to the required glycerol concentration in the medium. The C/N molar ratio was calculated based on the carbon available in glycerol. Though several other components in the glycerol solution such as methanol, free fatty acids (FFA) and soap can contribute carbon, in the present investigation only glycerol was taken into account because the other component were comparatively low in concentration. In all experiments, 1g/L yeast extract was added to meet the trace elements requirement. $(NH_4)_2\ SO_4$ was used as the nitrogen source. The required C/N molar ratio was adjusted by varying the $(NH_4)_2\ SO_4$ concentration. Based on the above information a full-fledged factorial design was made with the design expert software (Design Expert 8- USA) and the process was optimized to obtain maximum biomass and lipid concentration. The range of process parameters was selected based on previous lab experiments. The C/N molar ratio was selected in the range of 75 to 150, glycerol concentration from 75g/L to 100g/L, inoculum

volume 2.5 to 10% v/v. The initial pH was adjusted to 6.5 and the other media components were same as described in previous sections.

2.1.3. Validation of design

The validation of the parameters values obtained was conducted by comparing the values given by the software and the correlation of the predicted and experimental values were performed.

2.2. Fermenter Studies

The validated experimental conditions were used further to verify the process in 15L bioreactors with working volume 10L (Biogenie, Quebec, Canada) equipped with programmable logic control (PLC) system, which included a dissolved oxygen (DO) probe, antifoam, mixing, aeration, temperature, and pH. The above parameters were maintained using the program ifix 3.5, Intellution, USA. The pH meter (Mettler Toledo, USA) was calibrated using buffers pH 4 and 7.0 (VWR, Canada). The DO probe was calibrated to zero using sodium sulfate and to 100% by purging air in distilled water. The fermentation media was fortified with 85.5g/L crude glycerol and the C/N molar ratio was maintained at 75. Well, grown (24h) inoculum was transferred aseptically. The fermentation was conducted up to 72h and the samples were collected at every 6h intervals and analyzed for biomass concentration, biomass lipid content, and residual glycerol. Citric acid was measured at every 24h intervals.

2.3. Analytical methods

Fermented broth (25 mL) was used for biomass and lipid quantification. The collected samples were centrifuged at 4000 rpm for 10 minutes and the supernatant was collected in a clean tube. The biomass was washed twice with distilled water and the cell pellets were collected by centrifugation. The samples for biomass quantification were transferred to a clean pre-weighed aluminum pan and it was kept at 105^0C until it reached a constant weight. A chloroform-methanol based conventional method of lipid extraction was employed for lipid quantification (Folch et al., 1957). Wet sample was mixed with 15 mL mixture of 2:1 chloroform: methanol and zirconium beads (0.7mm) were added to the mixture and the cells were disrupted by a bead beater for 3 minutes (BioSpec Products, Bartlesville, OK, USA). The samples after bead beating were filtered through Whatman filter paper with a pore size of 0.45µm. The solids thus obtained were re-suspended in 1:1 chloroform-methanol mixture and the procedure was repeated. The resulting filtrates were pooled in a pre-weighed glass tube and the solvent was evaporated under

low nitrogen pressure. The residual samples were further dried at 60^0C in a hot air oven until the sample reached a constant weight. The weight thus recorded represented an amount of lipids in that sample.

Glycerol in the centrifuged supernatant was estimated based on the chemical method. Pure glycerol (Sigma-Aldrich, Canada) was used as the calibration standard (Bondioli & Della, 2005). Citric acid estimation was carried out according to the method described by (Marier & Boulet, 1958). Appropriately diluted samples were mixed with pyridine and acetic anhydride. The reaction mixture was incubated in a water bath preset at 60^0C. After five minutes of incubation, the reaction mixture was cooled to room temperature and the OD was recorded at 510nm (Varian Cary 500, USA). Citric acid (Fisher, Canada) was used as the calibration standard.

2.4. Determination of yield constants

The rate of lipid and biomass production was determined by the following equations. The specific growth rate (μ) was calculated according to the following equation

$$\mu = \frac{1}{x} \frac{dX}{dt} \quad \text{(Equn 1)}$$

Where X is the biomass concentration (g/L) at time t (h)

The biomass yield *(Yx/s, g/g)* was calculated according to Equation (2), where $\Delta X$the biomass (g/L) is produced and ΔS is the substrate consumed to generate the biomass at time t (h^{-1}).

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad \text{(Equn 2)}$$

The lipid yield *(Yp/s, g/g)* was calculated based on Equation 3, where ΔP is the lipid produced (g/L^{-1}) and ΔS is the substrate consumed to generate the biomass at time t (h^{-1})

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad \text{(Equn 3)}$$

The lipid content *(Yp/x, g/g)* was calculated from Equation (4)

$$Y_{P/X} = \frac{\Delta P}{\Delta X} \quad \text{(Equn 4)}$$

The specific lipid production rate (Ʊ) was calculated based on the Equation (5), were Ʊ is specific lipid production rate.

$$Ʊ = \frac{1}{p}\frac{dP}{dt} = Y_{P/X}\,\mu \text{ (Equn 5)}$$

3. Results and discussion

3.1. Optimization of culture parameters (Box-Behnken design)

RSM (Response Surface Methodology) study was designed based on the previous lab data and the results obtained from 15L fermenter under controlled conditions. *Y.lipolytica* SKY7 was found to tolerate a glycerol concentration of 150g/L. meanwhile, the current literature also supports the fact that *Y.lipolytica* can grow and produce a high concentration of lipid when initial glycerol concentrations were high. ((Dobrowolski et al., 2016; Kuttiraja et al., 2016). The time of incubation was reduced to 72h since the lipid production was noted to substantially decrease 227after 60h. The lipogenic phase in *Y.lipolytica* was reported from 30 to 60h. The inoculum age was 24h because the exponential growth was observed from 12 to 24h in previous reactor studies. Nitrogen source (YE) was replaced with $(NH_4)_2SO_4$ because YE furnishes un-utilizable nitrogen, which cannot be assimilated (Pawlak & Bizukojć, 2012). This can be further explained by the fact of yeast assailable nitrogen (YAN) (Bely et al., 1990), that is when the nitrogen concentration goes below the assimilable limit; it will not be utilized by the microbes. From the current results and previous experiments, a nitrogen concentration below 70mg/L was not utilized by *Y.lipolytica* SKY7. After a certain time, when all assimilable nitrogen of YE is exhausted, glycerol will be used for lipid production. This will result in a decrease of C/N ratio towards the end of fermentation as observed in previous work i.e. after 66h of fermentation the C/N ratio was decreased from 400 to 250 (after 24h of fermentation the C/N ratio was decreased from 310 to 136 in the present study). In order to know the impact of yeast extract on lipid accumulation the organic nitrogen source was replaced by inorganic nitrogen ($(NH_4)_2SO_4$). As expected the biomass concentration decreased from 15g/L to 10g/L at 72h in shake flask experiments (Table 27).This phenomenon was also observed in several other studies the inorganic nitrogen favors mostly product formation (lipid in the present case) rather than the biomass, whereas the organic nitrogen favors the biomass and product (lipid) accumulation

(Kalam et al., 2014). For this reason in the present study, both organic (yeast extract) and inorganic nitrogen ($(NH_4)_2SO_4$) sources were used.

3.2. Interaction of model terms in Biomass production.

Glycerol concentration, C/N ratio, and inoculum concentration were optimized to produce maximum biomass concentration. The actual biomass and lipid produced for each combination of experiments are presented in Table 25. Higher biomass was produced for 75g/L glycerol irrespective of C/N molar ratio and inoculum volume. The average biomass concentration at 75g/L glycerol was 10g/L and at 87.5g/L glycerol the biomass concentration varied from 9 to 9.8 g/L. A lower biomass concentration (7.2 to 9.1g/L) was observed at 100g/L glycerol concentration. This indicates that a low glycerol concentration favors the biomass production, whereas the interaction between (B&C) C/N molar ratio and inoculum volumes are ineffective in the range studied. To confirm the above claims, interactions between the culture parameters were analyzed with quadratic model and the results are discussed in the following sections.

Table 27 Box Behnken design of experiments

Run order	Glycerol conc (g/L)	C/N molar ratio	Inoculum volume (v/v)	Biomass (g/L)	Lipid (g/L)	% of lipid (w/w)
1	100	150	6.25	7.5	3.8	50.7
2	87.5	112.5	6.25	9	4.0	45.3
3	100	75	6.25	8.7	3.6	41.2
4	75	75	6.25	10.4	3.4	32.8
5	87.5	112.5	6.25	9.4	4.0	43.1
6	87.5	112.5	6.25	9.4	4.1	43.8
7	100	112.5	10	9.1	4.4	48.2
8	87.5	112.5	6.25	9.8	3.9	40.5
9	87.5	150	2.5	9.3	4.1	44.0
10	75	112.5	10	10.4	3.0	29.1

11	87.5	75	10	9.9	4.2	42.5
12	87.5	112.5	6.25	9.2	4.1	44.3
13	75	112.5	2.5	10.3	2.4	23.6
14	75	150	6.25	10.0	3.4	33.9
15	87.5	150	10	9.3	3.4	36.3
16	87.5	75	2.5	9	3.2	35.5
17	100	112.5	2.5	7.2	2.6	37.0

3.3. Interaction of glycerol concentration, C/N molar ratio and inoculum volume on biomass production

At low glycerol concentration and low C/N molar ratio a high biomass concentration was observed compared to other combinations (Table 27). The biomass concentration decreased with the increase in glycerol concentration or C/N molar ratio. The lowest biomass concentration (7.2g/L) was observed at glycerol concentration 100g/L and C/N ratio 150. The highest biomass in this experiment was (10.44g/L) obtained with glycerol concentration 75 g/L and C/N molar ratio 75. Under the studied inoculum volumes, the highest biomass was obtained in 10%v/v inoculum supplemented experiment. However, a moderate biomass (9 to 10g/L) was accumulated in experiments with 6.2% v/v inoculum, which revealed a close significance with 10% v/v inoculum supplemented experiments. A trend of decrease in biomass concentration was noticed with a decrease in inoculum volume and increase in glycerol concentration. Meanwhile, low C/N ratio with high inoculum volume showed high biomass production. However, the study shows that there was no significant variation in biomass concentration when C/N ratio and inoculum concentration was changed. Thus, the biomass concentration strongly depends on the glycerol concentration, C/N molar ratio, and inoculum concentration. Based on the data obtained the Fischer's test for the analysis of variance (ANOVA) was performed. The final equation for optimizing the biomass concentration thus obtained is given below

$$Y= 9.38-1.08A-0.22B+0.37C-0.19AB+0.46AC-0.24BC-0.16A^2-0.023B^2+0.067C^2 \text{ – (Equn 6)}$$

Where A, B, and C are the coded values for glycerol concentration, C/N molar ratio, and inoculum volume, respectively. Based on the ANOVA test, the model terms glycerol concentration (A), inoculum volume (C) and coded term (AC), glycerol concentration and inoculum volume are found to be significant with a p-value of 0.0001 and 0.01. Further, the results showed that the selected model terms are significant with a p-value of 0.0014. However, as mentioned above the model terms AB and BC were not significant. This could be explained by taking into account the nitrogen added to the medium. The actual nitrogen concentration added to the medium ranged from 0.2g/L to 0.4g/L, which is low compared to the concentration added in common fermentation medium (0.36 to 0.6g/L). Hence, the biomass concentration observed was also low.

3.4. Glycerol concentration, C/N molar ratio, and inoculum volume interaction with lipid accumulation

High lipid content was attained when the glycerol concentration was 100g/L with a C/N ratio of 150 and inoculum volume of 6.5 % (v/v). This shows that an increase in C/N molar ratio will result in a high content of lipid in the biomass; however, high C/N ratio reduces the biomass production. The low biomass production on high C/N ratio could be the result of low nitrogen content in the media. Low biomass lipid content was noted with 75g/L glycerol concentration, C/N ratio 112.5 with an inoculum volume of 2.5% v/v. In this case high C/N ratio and low inoculum volume resulted in only biomass accumulation instead of lipid. When glycerol concentration was 87.5g/L with C/N ratio 112.5 and 6.25%v/v inoculum an average lipid content of 40.5 to 45.3% w/w was attained. Thus, in order to achieve a high biomass lipid content, glycerol concentration and C/N ratio needs to be high (Table 27).

A significant increase in lipid concentration was noted when the inoculum and glycerol concentration increased. An average lipid concentration was obtained with a glycerol concentration of 87g/L with 6.2%v/v inoculum. Under above conditions, the increase in lipid concentration was about 0.4g/L. This indicates that when the glycerol concentration is 87.5g/L an inoculum volume of 6.2% would be ideal for lipid accumulation. Further, the interaction of glycerol concentration and C/N molar ratio with lipid accumulation was evaluated. As perceived in the case of lipid production, C/N ratio was noted to be insignificant in lipid accumulation. It is evident that when the C/N ratio is low the glycerol concentration needs to be optimum in order to

accumulate high lipid content. In this case, at 87.5g/L of glycerol concentration, the lipid accumulation was similar with respect to all C/N ratios. However, when the glycerol concentration was high (100g/L) the lipid accumulation decreased at low C/N ratio (75). It is evident that C/N ratio of 75 with 87.5g/L glycerol could be optimum for batch cultures. Moreover, the change in C/N ratio during the course of batch fermentation may lead to a change in lipid accumulation. Here the only factor that affects the lipid accumulation is the glycerol (carbon) concentration since lipid synthesis requires excess carbon with low nitrogen concentration. The interaction of C/N molar ratio and inoculum volume with lipid concentration is evaluated. A high inoculum with low C/N molar ratio was noted to be better for lipid accumulation. This can be explained by the assimilation of nitrogen by the yeast cells when a high concentration of inoculum is used in the fermenter. A rapid depletion of nitrogen in the fermentation medium coerces the cell biomass to route the available glycerol towards lipid synthesis. Here in the present case, this condition was observed when 6.25%v/v inoculum was used. The Fischer's test for the analysis of variance (ANOVA) was performed based on the data obtained. Thus, the final equation obtained for improving the biomass lipid content was given below.

$$Y= 4.07+0.27A+0.038B+0.33C+0.065AB+ 0.29AC-0.44BC-0.55A^2+0.050B^2-0.38C^2$$

Where A, B, and C are the coded values for glycerol concentration, C/N molar ratio, and inoculum volume, respectively. From the ANOVA test, it is clear that the model selected for the study was significant (p-0.0165). The model variables glycerol concentration (p-0.0373 0) and inoculum volume (p-0.0172) were the significant parameters with respect to lipid synthesis. The model term C/N molar ratio was non-significant with a p- value of 0.734. In the coded terms BC, $A^{2,}$ and C^2 were found to be significant.

3.5. Validation of the model

Based on the analysis, 10 different solutions were predicted by the software with reference to accumulate high biomass concentration with high lipid content. Based on the high (0.95%) desirability values 4 experimental conditions were chosen to conduct the experiments. The obtained results are presented in Table 28. A correlation analysis of predicted versus experimental data was conducted and it shows a correlation coefficient value of 0.85 for biomass and 0.92 for lipid. This indicates that the predicted experimental conditions are suitable to

produce a high concentration of biomass and lipid. Based on the validated data glycerol concentration of 85g/L, C/N ratio 75 and inoculum volume of 6.25%v/v were chosen to further verify the conclusion at 15L bench scale fermenter, as described below.

Table 28 Validation of predicted values

Column1	Glycerol concentration g/L	C/N molar ratio	Biomass predicted (g/L)	Biomass obtained (g/L)	Lipid Predicted (g/L)	Lipid Obtained(g/L)
1	85.5	75	10.3	9.2	4.4	5.1
2	85.9	75	10.3	9.5	4.4	4.124
3	85.9	75	10.3	9.2	4.4	4.524
4	85.8	75	10.3	9.3	4.3	3.268

3.6. Fermenter studies

The fermentation media was modified according to the optimal media composition obtained through RSM studies. In order to maintain the steady growth, the initial aeration was maintained at 0.35vvm (volume/volume/minute) and pH of fermentation was controlled by adding 4N NaOH solution. The mixing was varied according to the DO (dissolved oxygen) requirement and the temperature was maintained at 28^0C. The DO value in the fermenter decreased to 30% saturation within 12h of fermentation, which indicated an active microbial growth. When growth decreased, the DO started to increase. When DO reached 60% of saturation, the mixing was reduced to 400 rpm and then the aeration was reduced to 0.325vvm in order to maintain the DO at about 30% of saturation.

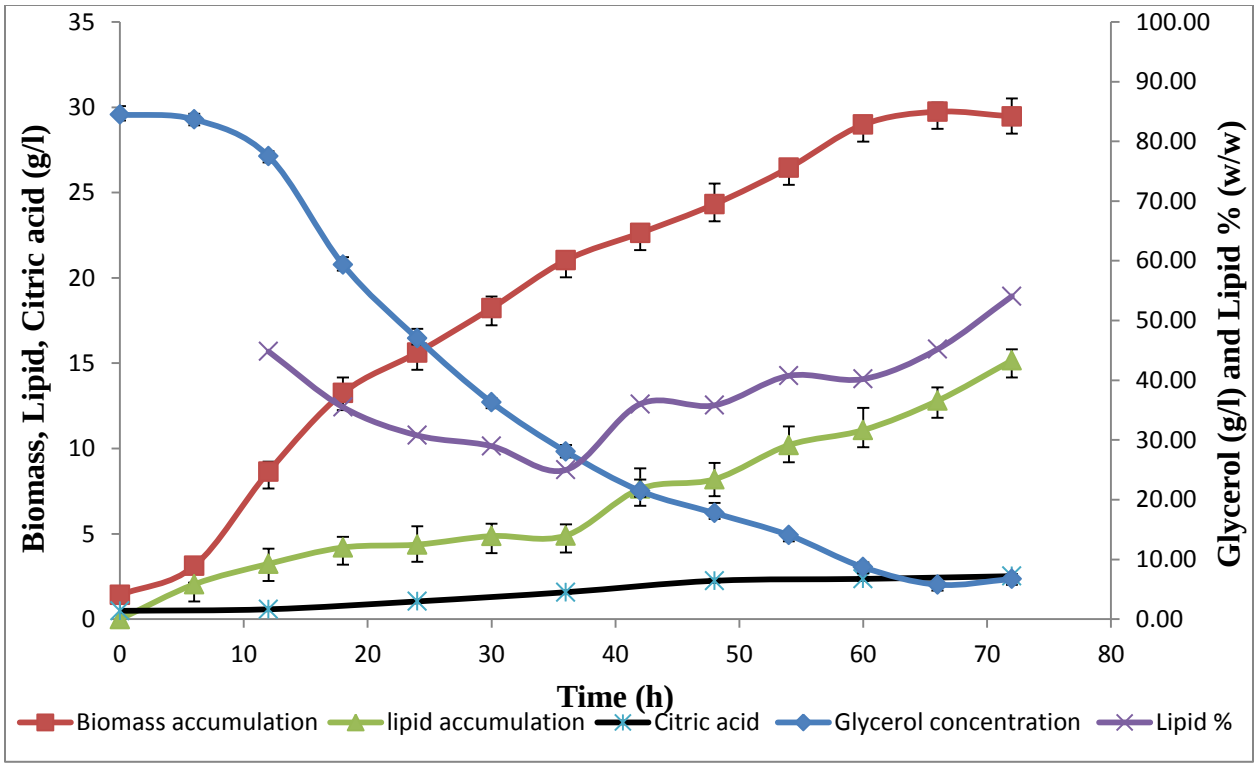

Figure 25 Variation of Biomass, glycerol, citric acid and lipid concentration during fermentation in 15L fermenter

The biomass, lipid, citric acid production and glycerol consumption during the fermentation process is described in Figure 25. The biomass concentration reached 29.5g/L at 72h of fermentation with a lipid concentration of 15.1g/L. The final lipid content in the biomass was 54.1% (w/w) at 72h of fermentation. From 0 to 6 h only 0.82g/L of glycerol was consumed by the cells and the biomass produced was 1.7g/L. However, the biomass produced during this time period was higher than the carbon assimilated in the form of glycerol. (Carbon content of glycerol= 0.39g/g, 1gm of yeast cell constitutes 0.4-0.5g/g carbon (Teixeira & Vicente, 2013; Wagner et al., 2012), therefore 1.7 g of cells requires 0.81g of carbon). However, only 0.82g of glycerol was consumed by the cells during the first 6h of fermentation, which can only provide 0.32g of carbon. Therefore, the rest of the carbon required for biomass production must be supplied by other carbonations components (yeast extract, soap, methanol) available in the fermentation medium (Holwerda et al., 2012). After a short lag period of 0-6h, 5.53g/L biomass was produced during 6 -12 h of fermentation and then the production of biomass was decreased due to the nutrient depletion.

At initial stage (12h) of fermentation, the lipid production was observed higher. When fermentation proceeds the lipid production drops and the biomass production is noticed to increase until 30h of fermentation. The lipid produced during this time could be contributed by cell wall lipids and a small quantity of TAGs. From 36h, the lipid production was observed to be stable and the lipid concentration at 36h of fermentation was 4.9g/L with the biomass lipid content 24.9%w/w. The maximum lipid concentration reached was 15.1g/L at 72h.

The citric acid concentration reached 2.52g/L at 72h. In previous studies under pH control condition citric acid concentration up to 11.32, g/L was observed using same strain (Kuttiraja et al., 2016 under review). A low citric production in the present case may be due to the fact that the synthesized citric acid is used by the strain and converted to lipids efficiently under optimized culture conditions; it has already been reported that the citric acid produced can be converted to lipid (Ratledge & Wynn, 2002). Meanwhile, the citric acid production also varies based on carbon concentration (high initial sugar concentration 10 to 14%(Xu et al., 1989), fermentation time (4-8days)(Maddox et al., 1985) and the amount of available nitrogen.

The biomass yield (Yx/s) shows a slow increase from 24 to 72h (Figure 26), which is due to the cumulative increase of biomass and lipid content of the biomass. An average biomass production of 0.321g/g was observed from 12 to 72h. To assess the actual biomass produced, the yield of lipid-free biomass was calculated (Figure 26), which decreased with the fermentation time. The above observation helps conclude that most of the assimilated carbon is converted to lipid synthesis rather than biomass production. The lipid yield (Yp/s, g lipid produced/g glycerol consumed) initially decreased from 0.129g/g to 0.078g/g during 12 to 36h. From 36h onwards more substrate was transformed into lipid and, therefore, Yp/s increased until 72h (0.179g/g). The trend of Yp/s and Yp/x were noted to decrease initially and then increase with time of fermentation. The decrease in yield coefficients (Yp/s and Yp/x) until 36h was due to the diversion of most of the glycerol carbon for biomass production. After 36 h the depletion in nitrogen content in the medium limited the biomass production and diverted the carbon available for lipid production, which increased the lipid yield coefficients. The lipid free biomass yield was decreased from 30 to 72h, which confirms the substrate conversion to lipids.

Based on the fermentation profile the glycerol concentration was 5.78g/L at 66h. Further increase in fermentation time may lead depletion of glycerol to a low critical level, which may

result in lipid degradation. This could be further explained by assessing the variation in specific lipid production rate. The specific lipid production rate was noted to decrease from 0.007 h^{-1} to 0.006 h^{-1} at 60 to 72h (Figure 27).

Based on glycerol conversion to biomass, lipid, and citric acid, a simple mass balance was made. It was found that 41.1% of the total assimilated glycerol, 18.38% was converted into biomass, 19.49% into lipids and 3.23% citric acid at the end of fermentation. The rest of the glycerol consumed could be mainly converted to other metabolic byproducts such as CO_2 or other organic acids, sugar alcohols, and carbohydrates and for maintenance metabolism (Tomaszewska et al., 2014). When it is compared with the un-optimized fermentation condition (same strain) lipid production was improved 2% and citric acid production was reduced to10%.

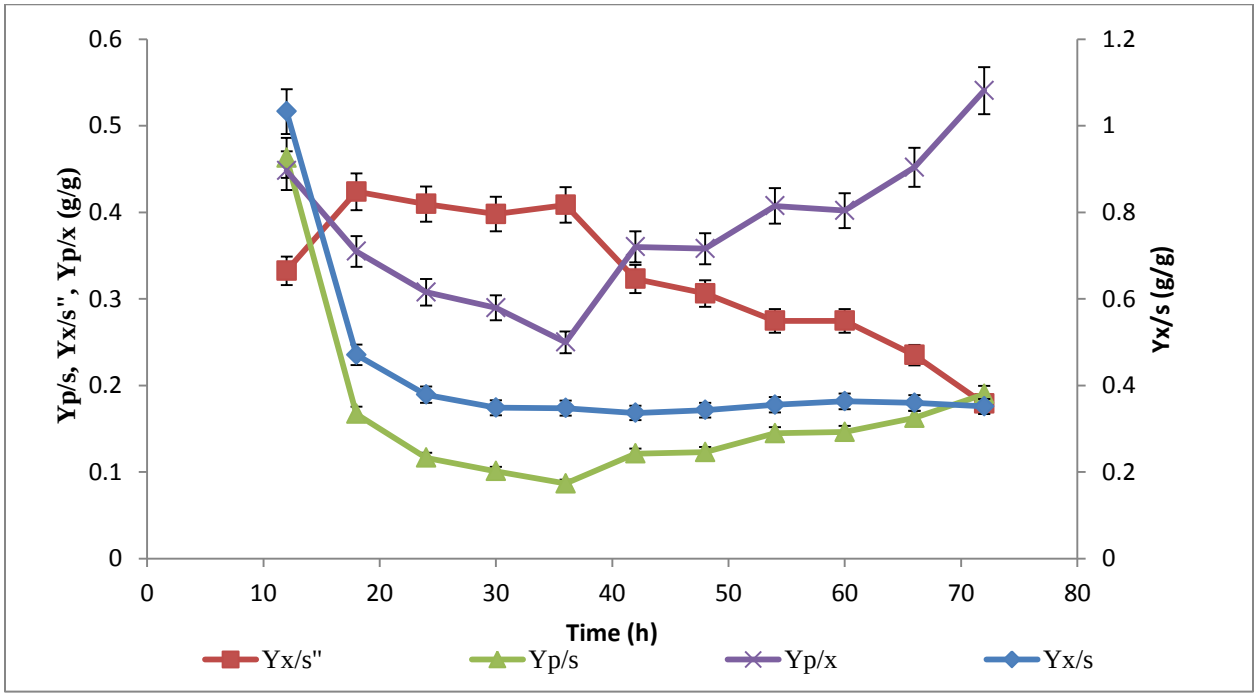

Figure 26 Variation of the yield of biomass (Yx/s), lipid free biomass (Yfx/s), the yield of lipid (Yp/s) and yield of lipid content per gram of dry biomass (Yp/x) with fermentation time. The yield was calculated based on the average biomass and lipid obtained on duplicates

The maximum specific growth rate 0.15h^{-1} was attained at 12h (Figure 27). From 12h onwards the specific growth rate declined and reached 0.042h^{-1} at the end of fermentation (72h). The specific lipid production rate attained maximum value at 12h of fermentation (Figure 27). A decreasing trend of specific lipid production was found from 12h to 30h, which correlated with the decreasing trend of yield coefficients (Yp/x and Yp/s) (Figure 26). After 36h, the specific

lipid production was approximately constant until the end of fermentation. As described above, the specific lipid production was proportional to specific growth rate at the initial stage of fermentation. However, it decreased with the declining specific growth rate at mid-point (12 to 30h) of fermentation.

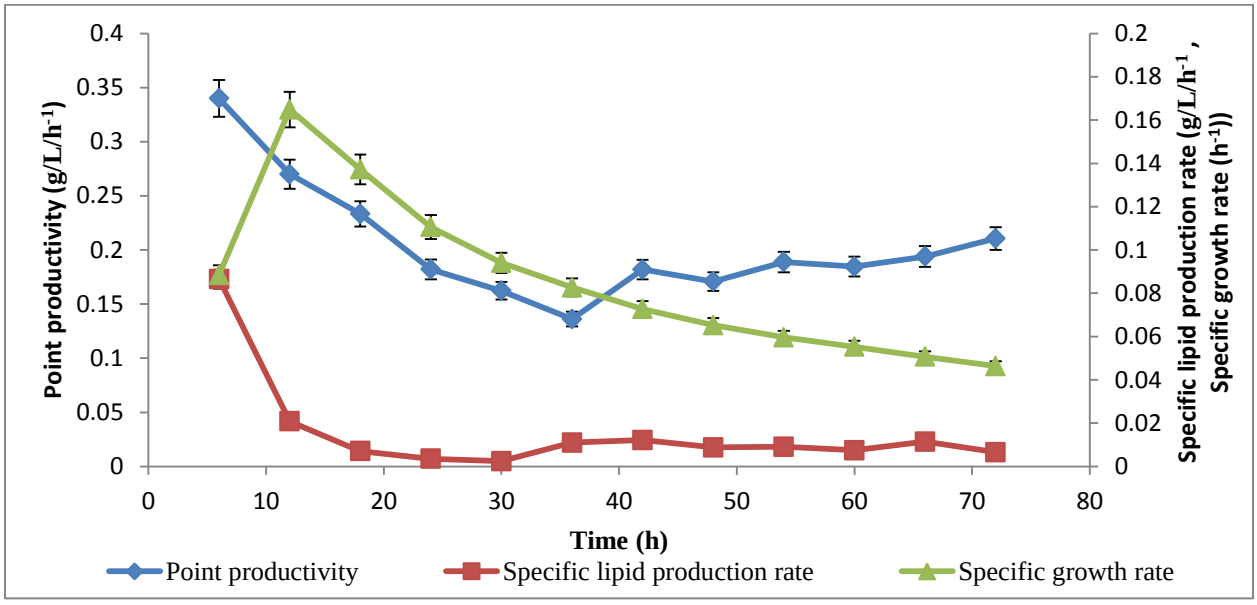

Figure 27 Variation of specific growth rate and specific lipid production rate and lipid productivity with fermentation time

The μ value reached $0.1h^{-1}$ at 30h where the U starts to rise again and this was denoted as the lipogenic phase by different authors (Ratledge & Wynn, 2002). The lipid accumulated in cells during 36 to 72h was 10.29g/L and 4.87g/L of lipid was accumulated at the early stage (6 to 30h) of fermentation. To confirm the above statement the (lipid) point productivity was calculated by differentiating the lipid concentration data and presented in Figure 27. The point productivity was noted to decline from 6h to 36h of fermentation. Thus, it is assumed that most of the glycerol consumed during this time period were routed for biomass synthesis. From 36h onward the lipid productivity increased and attained 0.21g/L/h at 72h. This shows that the TAG was mainly synthesized by the cells during 36 to 72h fermentation (as it also clear from yield coefficients)

4. Conclusion

The current study shows a glycerol concentration of 85g/L, C/N ratio75, with an inoculum volume of 6.25%v/v was found to be ideal for biomass and lipid production. The maximum

biomass and lipid concentration were attained at 72h with a biomass and lipid yield of 0.332g/g and 0.179g/g, respectively. At the initial stage of fermentation, the organism found to assimilate carbon from methanol, yeast extract, and free fatty acids. During growth at optimal culture conditions, the citric acid production was noticed to decrease significantly compared to non-optimized conditions. The specific growth rate was noted to be declined from 12h onwards and the specific lipid production was found to increase from 30h.

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References

André, A., Diamantopoulou, P., Philippoussis, A., Sarris, D., Komaitis, M., Papanikolaou, S. 2010. Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid. *Industrial Crops and Products*, **31**(2), 407-416.

Ayoub, M., Abdullah, A.Z. 2012. Critical review on the current scenario and significance of crude glycerol resulting from biodiesel industry towards more sustainable renewable energy industry. *Renewable and Sustainable Energy Reviews*, **16**(5), 2671-2686.

Bely, M., Jean-Marie, S., Pierre, B. 1990. Automatic Detection of Assimilable Nitrogen Deficiencies during Alcoholic Fermentation in Oenological Conditions. *Journal of Fermentation and bioengineering*, **70**(4), 246-252.

Bondioli, P., Della, B., Laura. 2005. An alternative spectrophotometric method for the determination of free glycerol in biodiesel. *European Journal of Lipid Science and Technology*, **107**(3), 153-157.

Chatzifragkou, A., Papanikolaou, S. 2012. Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes. *Appl Microbiol Biotechnol*, **95**(1), 13-27.

Christophe, G., Kumar, V., Régis, N., Geneviève, G., Pierre, F., Ashok, P., Carlos, R., Soccol,, Christian, L. 2012. Recent Developments in Microbial Oils Production: a Possible Alternative to Vegetable Oils for Biodiesel Without Competition with Human Food? *Brazilian archives of biology and technology*, **55**(1), 29-46.

Demirbas, A. 2005. Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods. *Prog Energ Combust*, **31**, 466-487.

Dias, C., Sousa, S., Caldeira, J., Reis, A., Lopes da Silva, T. 2015. New dual-stage pH control fed-batch cultivation strategy for the improvement of lipids and carotenoids production by the red yeast Rhodosporidium toruloides NCYC 921. *Bioresour Technol*, **189**, 309-18.

Dobrowolski, A., Mitula, 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.

Folch, J., M.Lees, G.H.Slane-Stanley. 1957. Simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*, **226**, 497–509.

Gui, M., Lee, K., Bhatia, S. 2008. Feasibility of edible oil vs. non-edible oil vs. waste edible oil as biodiesel feedstock. *Energy*, **33**, 1646-1653.

Gustone, F. 2009. Lipids as source of food and fuel—will there be enough? Dissertation, 2nd International Congress on Biodiesel. *The Science and The Technologies Munich, Germany*.

Holwerda, E.K., Hirst, K.D., Lynd, L.R. 2012. A defined growth medium with very low background carbon for culturing Clostridium thermocellum. *J Ind Microbiol Biotechnol*, **39**(6), 943-7.

Kalam, A., Abul, Abu, Y., Atia, F., Md, M., Hasan, Md, R., Karim, Asraful, J. 2014. Production of Microbial Lipids from Rice Straw Hydrolysates by Lipomyces starkeyi for Biodiesel Synthesis. *Journal of Microbial & Biochemical Technology*, **S8**(01).

Kuttiraja, M., Douha, A., Valero, J.R., Tyagi, R.D. 2016. Elucidating the Effect of Glycerol Concentration and C/N Ratio on Lipid Production Using Yarrowia lipolytica SKY7. *Appl Biochem Biotechnol*.

Kuttiraja, M., Krishna, S., Dhouha, A., Tyagi, R.D. 2015. A substrate-based approach for the selection of oil-bearing heterotrophs from nitrogen-deficient soil for lipid production. *Appl Biochem Biotechnol*, **175**(4), 1926-37.

Li, J., Liu, R., Chang, G., Li, X., Chang, M., Liu, Y., Jin, Q., Wang, X. 2015. A strategy for the highly efficient production of docosahexanoic acid by Aurantiochytrium limacinum SR21 using glucose and glycerol as the mixed carbon sources. *Bioresour Technol*, **177**, 51-7.

Li, Q., Du, W., Liu, D. 2008. Perspectives of microbial oils for biodiesel production. *Appl Microbiol Biotechnol*, **80**, 749-756.

Liu, Y., Wang, Y., Liu, H., Zhang, J. 2015. Enhanced lipid production with undetoxified corncob hydrolysate by Rhodotorula glutinis using a high cell density culture strategy. *Bioresour Technol*, **180**, 32-9.

Maddox, I.S., Spencer, K., Greenwood, J.M., Dawson, M.W., Brooks, J.D. 1985. Production of citric acid from sugars present in wood hemicellulose using Aspergillus niger and Saccharomycopsis lipolytica. *Biotechnol.lett*, **7**, 815-818.

Marier, J.R., Boulet, M. 1958. Direct determination of citric acid in milk with an improved pyridine-acetic anhydride method. *Journal of Dairy sciences*, **41**(12), 1683-1692.

Okamura, Y., Nakai, S., Ohkawachi, M., Suemitsu, M., Takahashi, H., Aki, T., Matsumura, Y., Tajima, T., Nakashimada, Y., Matsumoto, M. 2015. Isolation and characterization of bacterium producing lipid from short-chain fatty acids. *Bioresour Technol*, **201**, 215-221.

Pawlak, M., Bizukojć, M. 2012. Kinetic modelling of lovastatin biosynthesis by aspergillus terreus cultivated on lactose and glycerol as carbon sources. *Chemical and Process Engineering*, **33**(4), 651-665.

Poli, J.S., da Silva, M.A., Siqueira, E.P., Pasa, V.M., Rosa, C.A., Valente, P. 2014. Microbial lipid produced by Yarrowia lipolytica QU21 using industrial waste: a potential feedstock for biodiesel production. *Bioresour Technol*, **161**, 320-6.

Ratledge, C., Wynn, J.P. 2002. The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms. *Advances In Applied Microbiology*, **51**.

Ruan, Z., Zanotti, M., Archer, S., Liao, W., Liu, Y. 2014. Oleaginous fungal lipid fermentation on combined acid- and alkali-pretreated corn stover hydrolysate for advanced biofuel production. *Bioresour Technol*, **163**, 12-7.

Tchakouteu, S.S., Kalantzi, O., Gardeli, C., Koutinas, A.A., Aggelis, G., Papanikolaou, S. 2015. Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency. *J Appl Microbiol*, **118**(4), 911-27.

Teixeira, J.A., Vicente, A.A. 2013. Engineering aspects of food biotechnology. 436-437.

Tomaszewska, L., Rakicka, M., Rymowicz, W., Rywinska, A. 2014. A comparative study on glycerol metabolism to erythritol and citric acid in Yarrowia lipolytica yeast cells. *FEMS Yeast Res*, **14**(6), 966-76.

Vajpeyi, S., Chandran, K. 2015. Microbial conversion of synthetic and food waste-derived volatile fatty acids to lipids. *Bioresour Technol*, **188**, 49-55.

Wagner, A.O., Hohlbrugger, P., Lins, P., Illmer, P. 2012. Effects of different nitrogen sources on the biogas production - a lab-scale investigation. *Microbiol Res*, **167**(10), 630-6.

Xu, D., Madrid, C.P., Rohr, M., Kubicek, C.P. 1989. The influence of type and concentration of the carbon source on production of citric acid by Aspergillus niger. *Applied microbiology and biotechnology*, **30**, 553-558.

Xue, F., Gao, B., Zhu, Y., Zhang, X., Feng, W., Tan, T. 2010. Pilot-scale production of microbial lipid using starch wastewater as raw material. *Bioresour Technol*, **101**(15), 6092-5.

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

Chapter-V

Research Article-5

Replacement of commercial nutrients sources by silo leachate for biomass and lipid production using crude glycerol as a sole carbon source by *Y.lipolytica* SKY7

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Résumé

La cellule unique d'huile à partir des microbes hétérotrophes était dans la phase expérimentale depuis des décennies. Les principaux obstacles rencontrés dans la production d'huile microbienne étaient principalement le coût des matières premières et les nutriments. Plusieurs matières premières alternatives ont été introduites au cours des dernières années, cependant, les études de remplacement des sources de nutriments pour la production de l'OCS étaient limitées. Par conséquent, le but de cette étude était de remplacer la source d'azote commerciale coûteuse avec le liquide de Silo. Les résultats expérimentaux obtenus avec le liquide de silos seul montrent une application potentielle de production dans le SCO. Cependant, lorsque le liquide de silos a été supplémenté comme source d'azote pour la production de biomasse lipidique et de lipides, il a présenté une concentration de biomasse et de lipides de 8.72g/L et de 3,6 g/L, respectivement. Lorsque la condition similaire a été reproduit dans un réacteur à échelle de laboratoire de 15L à, cela a donné lieu à 25.82g/L de biomasse et 15.15g/L de lipides en 51h de fermentation. L'accumulation lipidique globale montre 63,85% p/p de la biomasse cellulaire sèche était des lipides avec un rendement de biomasse et de lipides de 84.39g/C mol et de 36.83g / C mol respectivement. L'addition du glycérol brut contenant une grande quantité de savon pourrait aboutir à la sécrétion de la lipase comme l'un des sous-produits. Sur la base des résultats expérimentaux le liquide de silos pourrait être susceptible de remplacer les sources d'azote et les substances nutritives commerciales.

1. Summary of work

The single cell oil (SCO) from heterotrophic microbes was in the experimental phase for decades. The main hurdles observed with microbial oil production were mainly the costs of feedstock and nutrients required for the fermentation. There are many alternative feedstocks were introduced in recent years, however, the studies replacements for nutrient sources for SCO production were limited. Therefore, the aim of this study was to replace the expensive commercial nitrogen source with silo leachate. The experimental results obtained with silo leachate alone shows a potential application in SCO production. However when the silo leachate was supplemented as a nitrogen source for lipid biomass and lipid production, it shows a biomass and lipid concentration of 8.7g/L and 3.6g/L respectively. When the similar condition was replicated in 15L bench scale reactor, it resulted in 25.8g/L biomass and 15.1g/L lipid within 51h of fermentation. The overall lipid accumulation shows 63.8%w/w of dry cell biomass was lipid with a biomass and lipid yield of 84.3g/C mole and 36.8g/C mole respectively. The addition of high soap containing crude glycerol could result in secretion of lipase as one of the co-products. Based on the experimental results the silo leachate could be a potential replacement for the commercial nitrogen and nutrient sources.

2. Introduction

Oil producing microbial cells is a natural workhorse as a future energy source and an excellent alternative for fossil fuel. The oil accumulated by the microbial source is known as single cell oil (SCO), which is accumulated intracellularly (Ratledge & Wynn, 2002a). The oil producing microbes are found in bacteria, yeast, fungi and algae. In recent days microbial oil production from renewable sources is given high importance due to the rapid depletion of global oil reserves, increasing oil price and growing environmental concerns. With respect to the above-mentioned facts, biofuels have recognized as one of the suitable alternatives for liquid fuel. Biofuels can be used in conventional motor engines without any modifications and in recent days many countries in the world proposed to blend biofuels such as bioethanol in petroleum and biodiesel in diesel fuels at 2-20% accordingly (Lane, 2013). Although biofuels are considered as potential alternatives, the feasibility of production depends on the efficiency of the process (Koutinas et al., 2014). In contrast, the production cost solely depends on the feedstocks (Demirbas, 2009). Even though, used cooking oil, animal fat, non-edible oils and to certain extent algal oil have been used for biodiesel application, to meet the global demand an alternate must be identified (Gnanaprakasam et al., 2013; Khan et al., 2014; Papanikolaou et al., 2002; Yaakob et al., 2013). This eventually opens the quest for the search for new alternative sources for microbial oil production.

The quest for alternative low-cost feedstocks for oil production was extended to lignocellulosic biomass, municipal solid and liquid waste, waste from food materials, slaughter housed waste, industrial tallow, kitchen waste, forestry solid waste, biodiesel derived glycerol and waste water (Dobrowolski et al., 2016; Karmee et al., 2015; Muller et al., 2014; Papanikolaou et al., 2002; Tchakouteu et al., 2015). In general, the waste originated from food materials harbors 5-30% of lipids, and the extraction of lipid from food waste is the only concern associated with it (Pleissner et al., 2014). Meantime, the other waste materials require being converted into biofuels by chemical or biological means. Biological conversions of wastes into valuable products are green technologies compared to the chemical methods. In the case of biological conversion of waste into usable oil requires potential microbial candidates. In nature, many microbes were identified to convert the waste carbon into single cell oil (SCO). Among the low-cost carbon for bioconversion into various value added products, glycerol has become one of the most prominent in recent days. The recent boom in biodiesel industry has swung the crude

glycerol price to as low as 5cents to 15cents/Lb. The current world biodiesel production reached over 35billion liters (OECD-FAO, 2011). Considering the fact the 30billion liters of biodiesel can produce 3 billion liters of crude glycerol as a major byproduct. Furthermore, the composition of biodiesel driven glycerol makes it as unusable for food and pharma applications. At this point, the disposal of crude glycerol has become one of the challenging tasks for the biodiesel industries (Yazdani & Gonzalez, 2007). To the alternative, microbial conversion of glycerol into oil has become one of the suitable alternative ways to utilize the glycerol into oil. At the same time, the conversion of crude glycerol into oil helps the biodiesel industry to boost the overall biodiesel production with sustainable management of waste streams from the industry.

Developing a viable technology for SCO production is not only dependent upon cheap carbon source but also determined by the other important medium components (macro and micro nutrients). The growth medium for lipid production usually prepared with one or more nutrients deficiency. In general, nitrogen-deficient medium opts for lipid production (Lin et al., 2011; Ratledge & Wynn, 2002a). However for the biomass production apart from nitrogen, other organic and inorganic nutrients are vital. Meantime utilization of commercially available nitrogen (Yeast extract, Peptone, Ammonium salts) was impractical with respect to the process economy. To overcome the issues related to nutrient supplementation, alternative approaches such as substitution of industrial waste materials containing important nutrients were under investigation. There were fewer reports were available in the case of supplementation of industrial waste as a nutrient source for SCO production (Mondala et al., 2011).

In the present study silo leachate (the leachate from grain storage) is investigated as a complete nutrient source for SCO production. Silos seepage is considered as one of the common pollutants from the agricultural industry. The silos seepage is responsible for the corrosion of the silo tower or pollution related to the land and ground water along with unpleasant odor. The chemical composition of seepage shows a high BOD (12000- 90000bg/L) (Clarke & Stone, 2004). Since silo leachate is an organic pollutant the microbial load could be also high. The other components present in seepage are lactic acid, acetic acid, and butyric acid. Along with this, the silo seepage is known to contain 1.5 to 4.4g/L of total nitrogen(Clarke & Stone, 2004). Considering the fact of low nitrogen requirement for lipid production, silage could be utilized as the nitrogen and to a

certain level nutrient source for any fermentation. This could lead to the value addition of silo leachate as well as reduce the potential negative impact of seepage on the local environment.

3 Materials and methods

3.1 Culture and inoculum preparation

Y.lipolytica SKY7 and *Rhodococcus sps.* were used during an initial study. *Y.lipolytica* SKY7 was maintained on YPD agar plates (Yeast extract 10g/L, peptone 5g/L, dextrose 20g/L, and agar 20g/L). Pre-inoculum was prepared in YPD broth (Yeast extract 10g/L, peptone 5g/L, dextrose 20g/L). The pre-inoculum medium was sterilized by autoclaving at 121°C for 20 minutes and then a loop full of pure culture of *Y.lipolytica* was aseptically inoculated. The final inoculum was prepared in YPG medium (yeast extracts 10g/L, peptone 5g/L, and glycerol 20g/L). The glycerol content of the crude glycerol solution was 48%w/w, a soap content of 25.33%w/w. Methanol was the other major component with 11.7%w/w (Table 29). In order to make the final glycerol of 20g/L concentration in the inoculum, 41.6g/L of crude glycerol was added. Hence, the addition of 41.6 g/L crude glycerol solution resulted in supplementation of 10.5g/L soap and 4.86g/L methanol in the medium. The final inoculum medium was sterilized and aseptically inoculated with (5%v/v) 18h grown pre-inoculum. The inoculum was incubated for 18h in a shaking incubator at 180rpm and 28°C. The well-grown inoculum was used in all studies.

Table 29 Composition of Crude glycerol

Component	Concentration (g/L)	Concentration (%w/w)
Glycerol	480	48
Soap	253.3	25.33
Methanol	150ml/L	11.7
Catalyst	40	4
Water	11.3ml/L	11.3

Rhodococcus sps was isolated from the soil samples collected from Quebec province Canada and the identification were carried out with Biolog system (Biolog USA). *Rhodococcus* sps was maintained in nutrient agar plates (dextrose 20g/L, peptone 5g/L, beef extract 3g/L, agar 15g/L and pH 7). The pre-inoculum was prepared on a nutrient broth having the following composition; dextrose 20g/L, peptone 5g/L, beef extract 3g/L and pH 7. The medium was sterilized by autoclaving at 121°C for 15 minutes. The sterilized medium was inoculated with a pure culture of *Rhodococcus sps*. A well grown (18h) pre-inoculum was transferred aseptically into the final inoculum. The final inoculum was prepared in YPG medium with the following composition; yeast extracts 10g/L, peptone 5g/L, and glycerol 20g/L of crude glycerol.

3.2 Assessment of silo leachate as carbon and nutrient source for biomass and lipid production

The initial fermentation was carried out in silo leachate without any addition of additional carbon source and other nutrients. In other words, the silo leachate was used as a complete fermentation medium. The leachate contains high carbon (10 to 12g/L BOD) and 1.5 to 4g/L nitrogen and other nutrients as described in Table 30. The medium was sterilized by autoclaving the flask containing medium at 121^0C for 15minutes. The inoculum was prepared as mentioned in Section 1.1 using crude glycerol. Then the medium was inoculated with 5% v/v inoculum of *Y. lipolytica* and *Rhodococcus sps*. The fermentation was carried out at 28^0C with 180rpm for 72h.

3.3 Comparison of silo leachate as nitrogen source with commercial nitrogen sources supplemented to crude glycerol solution as substrate.

In order to evaluate the possibility of using silo leachate as a nutrient source for biomass and lipid production, the silo leachate waste as nitrogen source was compared with other commercial nitrogen sources $(NH_4)_2SO_4$ and yeast extract.

In the current study, the crude glycerol stock had high soap content. The original composition of crude glycerol is presented in Table 29. The fermentation medium contains 50g/L glycerol, soap + FFA 17.5g/L and methanol 5.85g/L with pH 6.5. The nitrogen source was varied according to the source of nitrogen as follows; Yeast extracts 2.25g/L, $(NH_4)_2SO_4$ 1.25g/L and silo leachate 100 to 200 ml/L. KH_2PO_4 2.7g/L, Na_2SO_4 1g/L, $MgSO_4$ 0.5g/L were also added in all cases. The medium in the flask was sterilized by autoclaving at 121^0C for 15 minutes. After cooling, the medium was inoculated with 5% v/v inoculum *of Y. lipolytica*. The fermentation in duplicate

shake flasks was conducted in a shaking incubator at 28^0C with 180 rpm for 72h. The samples were collected at every 12h.

3.4 Fermentation at 15L bench scale bioreactor

The optimal condition for lipid production was derived based on the flask results and further used to test in 15L bench scale fermentor. The fermentation medium was prepared by diluting the medium components at the following concentration. The initial glycerol concentration was adjusted at 50g/L with soap and FFA 17.5g/L and methanol 5.85g/, pH 6.5, Na_2HPO_4 1g/L; KH_2PO_4 2.7g/L, $MgSO_4.7H_2O$ 0.5g/L, and silo leachate 200mL/l as a nitrogen source. The initial C/N ratio was 145.2. The C/N ratio was based on the glycerol and soap content and the total nitrogen content in the fermentation medium, i.e. 44.39g glycerol (= 17.53g carbon), 17.53g soap (= 12.10g carbon) and 204mg/L nitrogen from silo leachate). The fermentation volume was maintained at 10L (Biogenie, Quebec, Canada) (9.5L medium+0.5L inoculum) and the fermentation was conducted in replicates. The fermenter was equipped with programmable logic control (PLC) system, which included a dissolved oxygen (DO) probe, antifoam, mixing, aeration, temperature, and pH. The above parameters were maintained using the program ifix 3.5, Intellution, USA. The pH probe (Mettler Toledo, USA) was calibrated using buffers pH 4 and 7.0 (VWR, Canada). The DO probe was calibrated to zero using sodium sulfate and to 100% by purging air in distilled water. A well grown (18h) pure inoculum (5% v/v) of *Y. lipolytica* was transferred aseptically to the fermentation medium. Samples were collected at each 3h intervals to determine the biomass, lipid, and residual glycerol concentrations. The pH was maintained at 6.5 and DO was maintained above 30% of saturation.

3.5 Analytical methods

Fermented broth (25 ml) was used for biomass and lipid quantification. The collected samples were centrifuged at 4000rpm for 10 minutes and the supernatant was collected in a clean tube. The biomass was washed twice with distilled water and the cell pellets were collected by centrifugation. Each sample (pellet) for biomass quantification was transferred to a clean pre-weighed aluminum pan and it was kept at 105^0C until it reached a constant weight. A chloroform and methanol based conventional method of lipid extraction were employed for lipid quantification (Folch et al., 1957). Wet biomass sample of known weight was mixed with 15 ml of 2:1 chloroform: methanol mixture and zirconium beads (0.7mm) were added to the mixture and the cells were disrupted by bead beater for 3 minutes (BioSpec Products, Bartlesville, OK,

USA). The samples after bead beating were filtered through Whatman filter paper with a pore size of 0.45µm. The solids, thus obtained were re-suspended in 1:1 chloroform-methanol mixture and the procedure was repeated. The resulting filtrates were pooled in a pre-weighed glass tube and the solvent was evaporated under low nitrogen pressure. The residual samples were further dried at 60^0C in a hot air oven until the sample reached a constant weight. The weight thus recorded represented as the mass of lipids in that sample.

Glycerol in the centrifuged supernatant was estimated based on chemical method. Pure glycerol (Sigma-Aldrich, Canada) was used as the calibration standard (Bondioli & Della, 2005). *Y.lipolytica* is known for lipase production in the presence of hydrophobic substrates such as FFA soap. Hence the culture broth was subjected to lipase assay. Lipase activity was measured spectrophotometrically using pNPP (Para-nitrophenol palmitate) as substrate and pNP (Para-nitrophenol) as standard (Sigma-Aldrich USA). The absorbance was measured at 415nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1Umol of pNP per minute under the assay condition (Prim et al., 2003).

Soap and FFA were measured according to the method described by Hu et al (Hu et al., 2012). 5g of sample mixed with a neutral solvent (Ethanol acetone at 1:1v/v at pH 7). The resulting mixture was heated to 50C and the sample was mixed with soap and FFA dissolves. Then the sample was titrated against 0.1N KOH solution. Phenolphthalein was used as an indicator and the end point was the appearance of pale pink color. The soap and the FFA were calculated as described below.

$$AV = \frac{mL\ of\ KOH * N * 56}{Weight of\ sample} \quad (\%of\ FFA = AV * 0.503)$$

Where N is normality of KOH, 56 is molecular weight of KOH 0.503 is conversion factor and AV is the acid value (the amount of KOH required to neutralize the sample)

Total carbon and nitrogen were analyzed with Shimadzu automated TOC analyzer (TOC-VCSH) as described by Avramidis et al (Avramidis et al., 2015).

3.6 Calculation of yield coefficients and specific growth

The specific growth rate was calculated according to the following equation

$$\mu = (\frac{1}{x})\frac{dx}{dt}\,(1)$$

Where μ is the specific growth rate, x is the concentration of biomass (g/L) and t is the fermentation time.

The yield of biomass and lipid was calculated according to the following equations (based on moles of carbon consumed to produce each gram of biomass and lipid)

$$\frac{Yx}{s} = \frac{g\ of\ biomass\ produced}{total\ moles\ of\ carbon\ consumed} = \frac{g\ of\ biomass\ produced}{C\ mole\ consumed\ from\ \ glycerol + C\ mole\ consumed\ from\ \ in\ soap}\,(2)$$

$$\frac{Yp}{s} = \frac{g\ of\ Lipid\ produced}{total\ moles\ of\ carbon\ consumed} = \frac{g\ of\ Lipid\ produced}{C\ mole\ consumed\ from\ \ glycerol + C\ mole\ consumed\ from\ \ in\ soap}\,(3)$$

The lipid (TAG) composition was determined by GC-FID (gas chromatograph coupled with Flame Ionization Detector). The column length was 60m, the carrier gas was helium at a flow rate of 1.18ml/min. the oven temperature was 230C. 1µl of transesterified sample was loaded with an automated sample injector and the sample analysis was performed with Agilent GC chem station software. A 37 component FAME mixture from Supalco was used as the reference standard.

4 Results and discussion

4.1 Assessment of silo leachate as carbon and nutrient source for biomass and lipid production

Prior to all the experiments, the carbon and nitrogen concentration of silo leachate were estimated. The results showed that silo leachate contains 12.55g/L carbon concentration and 2.04g/L nitrogen concentration. The initial pH of the leachate was 4.2 (Table 30). Prior to fermentation, when the pH of the medium was adjusted to 6.5 with 1N NaOH solution, the medium turned turbid. After sterilization of the silo leachate as fermentation medium, the suspended solids were precipitated. This is due to the aggregation of suspended solids during the sterilization at high temperature and pressure and change in the ionic strength due to the neutralization (4.5 to 7 pH).

Table 30 Comparison of nitrogen content of commercial nitrogen sources with silos leachate

	$(NH4)_2SO_4$	Yeast extract	Silos leachate(Clarke & Stone, 2004)	Silos leachate(current study)
Nitrogen content	21%w/w (0.210g/g)	12%w/w (0.120g/g)	1.5 to4.4g/L	2.048g/L
Carbon content	0	45%w/w	10-12g/L (BOD)	12.55g/L
Lactic acid			4-6%	
Acetic acid			1-2%	
Butyric acid			>1%	
pH			3.5-5.5	4.5
Concentration of nitrogen sources added to the medium and corresponding C/N ratio				
	Ammon. sulfate 1.25g/L	Yeast extract (2.25g/L)		Silo leachate (100mL/ 200mL)
Nitrogen content	0.262g/L	0.270g/L		0.102 / 0.204g/L
Carbon concentration	29.9g/L	28.98g/L		25.18 / 27.98g/L
C/N ratio	114.1	107.3		246/ 137.1

The C/N ratio was calculated based on the carbon available in glycerol and soap content added to the fermentation. Methanol was neglected because it was assumed that methanol will evaporate during medium sterilization

The results obtained for silo leachate fermentation are presented in Table 31. Due to the precipitation of suspended solids, the initial suspended solids concentration was 9.12g/L. In the case of *Y.lipolytica*, the biomass or suspended solids concentration increased to 25.1g/L at the end (72h) of fermentation. In the case of *Rhodococcus sps*, the suspended solid concentration reached only 13.04g/L at 72h of fermentation from an initial suspended solid concentration of 8.88g/L. Higher concentration of lipid was observed in the case of *Y lioplytica* (or YL) (Table 31). Only a slight increase in lipid concentration was obtained in *Rhodococcus* fermentation. In the case of *Y.lipolytica*, lipid concentration of 1.84g/L was attained at 72h, which represents 11.56%w/w of biomass or suspended solids. However, lipid was 0.72 g/L in the case of *Rhodococcus sps* and which contributes 5.52%w/w of total solids. The strain of *Y.lipolytica* is known to produce lipid content of biomass up to 60%w/w. The low lipid content (11.56%w/w) in the present case is due to the lack of carbon in the silos leachate.

Table 31 Comparison of biomass and lipid production by *Y.lipolytica* SKY7 and Rhodococcus sps in silos leachate as complete fermentation medium

Sl no	Age (h)	Suspended solids (g/L)	Lipid g/L	% of lipid(w/w)
Y.lipolytica	0	9.12	0	0
	24	12.8	1.4	11.5
	48	19.1	1.7	9.0
	72	25.1	1.8	7.3
Rhodococcus	0	8.8	0	0
	24	10.0	0.2	1.9
	48	11.2	0.48	4.2
	72	13.0	0.72	5.5

In general, the lipid accumulation was reported to occur in the presence of excess carbon with low nitrogen concentrations (Papanikolaou & Aggelis, 2011; Ratledge & Wynn, 2002b). Based on the results it is evident that supplementing additional carbon source or using silo leachate as nitrogen source could be a better way of biomass and lipid production.The current results proved that yeast *(Y.lipolytica)* is a better candidate for growing in silo leachate than bacterial species. In the case of *Rhodococcus sps*, low growth could be due to the presence of bactericide or fungicide in the leachate arising from their use during the grain storage, or the presence of organic acids in the leachate (Table 30), which are inhibitory to bacterial growth (Clarke & Stone, 2004). Moreover, *Y.lipolytica* is known to utilize different (hydrophobic and hydrophelic) carbon sources. Based on this observation further experiments were conducted with *Y.lipolytica SKY7*.

4.2 Comparison of silos leachate as nitrogen source with commercial nitrogen sources supplemented to crude glycerol solution used as the substrate.

Crude glycerol obtained from a biodiesel industry (Biocardal Quebec) and the composition is presented in Table 29.The high methanol concentration may be due to the poor transesterification or excess use of methanol during transesterification due to the presence of high FFA content in the oil. The glycerol concentration in biodiesel derived crude glycerol highly depends on the feed oil and the final purification process employed. The variation of glycerol concentration in crude glycerol was already reported by various authors (Hu et al., 2012; Schultz et al., 2014). The high

soap content of crude glycerol solution clearly indicates the presence of high FFA concentration in the feed oil used for transesterification. In the presence of water, FFA reacts with the catalyst (in the present case alkali) and produces soap.

The biomass concentration thus obtained is presented in Figure 28. The maximum biomass concentration obtained was 10.6g/L in yeast extract supplemented experiments followed by 9.6 g/L in the case of $(NH_4)_2SO_4$ and 8.7g/L for silos leachate 200 ml/L supplemented medium. The lowest biomass concentration of 5.2g/L was obtained in 100 ml/L silos leachate supplemented experiments. Based on the results, it is clear that growth on different nitrogen sources had a lesser effect on biomass production in *Y.lipolytica*. In the case of 100ml/L silos leachate supplementation, the low biomass concentration was due to the low nitrogen and other nutrient concentrations. The nitrogen added in case of 100ml/L silos leachate fortified flask was 102mg/L, which is lower compared to 200ml/L silos (204mg/L) leachate, $(NH_4)_2SO_4$ and Yeast extract (Table 30).

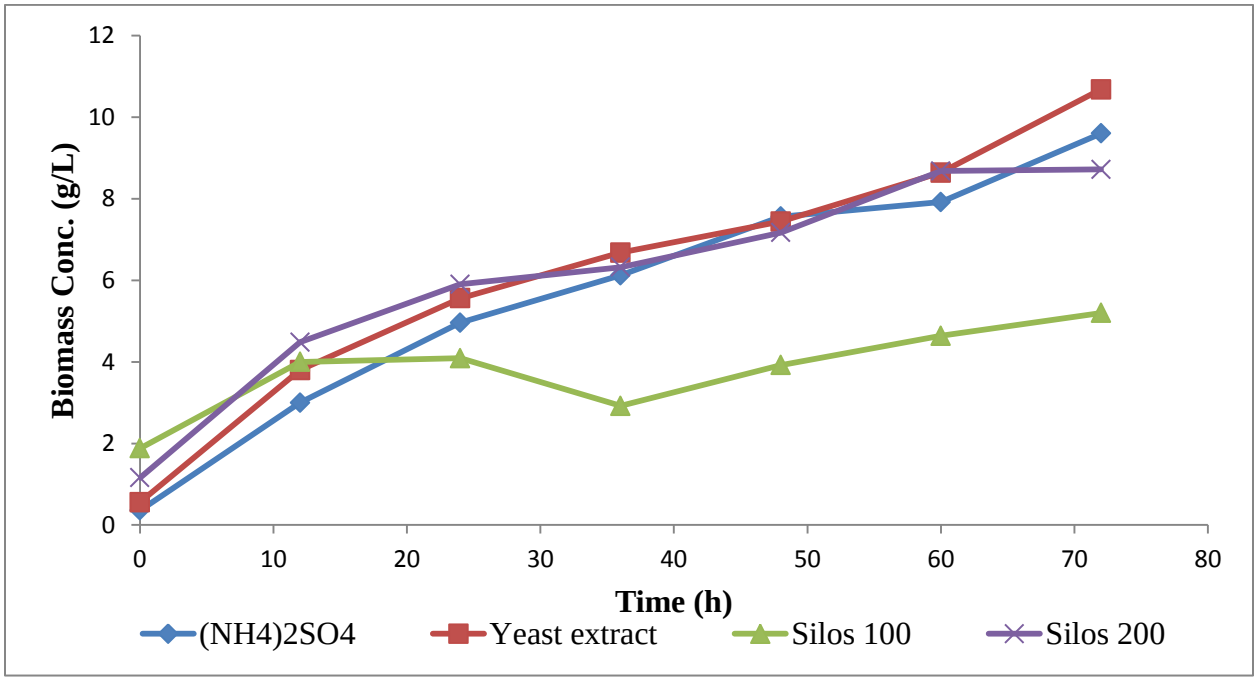

Figure 28 Biomass production of Y.lipolytica SKY7 on different nitrogen sources in crude glycerol medium

The lipid concentration obtained with different nitrogen sources is presented in Figure 29. The maximum lipid (4.04g/L) was produced in yeast extract supplemented experiments. When yeast extract was used the 4.04g/L of lipid was produced at 60h and represents 46.7%w/w of the total

dry biomass produced. Lipid concentration of 3.6g/L of was produced when the fermentation was supplemented with silos leachate at 200ml/L, which is 41.2% w/w of the total dry biomass. In the case of $(NH_4)_2SO_4$, 2.44g/L lipid was accumulated at 72h of fermentation, which is comparatively lesser than the previous two conditions. However, in the case of silos leachate concentration 100ml/L the lipid content was noted to be 2.16g/L, which is 55%w/w of the total dry biomass. At low nitrogen concentration (100ml/L silo leachate) a low cell proliferation resulted in high lipid accumulation. Moreover, the C/N ratio in the above experiments was noted to be (according to the glycerol and soap +FFA concentration and the nitrogen content of $(NH_4)_2SO_4$, Yeast extract and silos leachate) vary as follows: $(NH_4)_2SO_4$ - 114.1, Yeast extract - 107.3, silos 100ml/L -246 and 200ml/L -137.1. From the calculated C/N ratio it is evident that the C/N ratio in 100ml/L silos leachate supplemented experiments was higher than other experimental condition, which resulted in low biomass with high lipid content. It is well known that the high C/N ratio in the case of *Y.lipolytica* favors lipid accumulation rather than the biomass production. From Figure 29 it is evident that the concentration of lipid in all cases significantly increased from 12h to 36h of fermentation. From 36h onwards the increase in lipid concentration was comparatively low. In the case of yeast extract supplemented flask, the lipid concentration was dropped significantly from 60h. This may occur due to the lipid degradation. However, in other conditions there was no significant change was noticed.

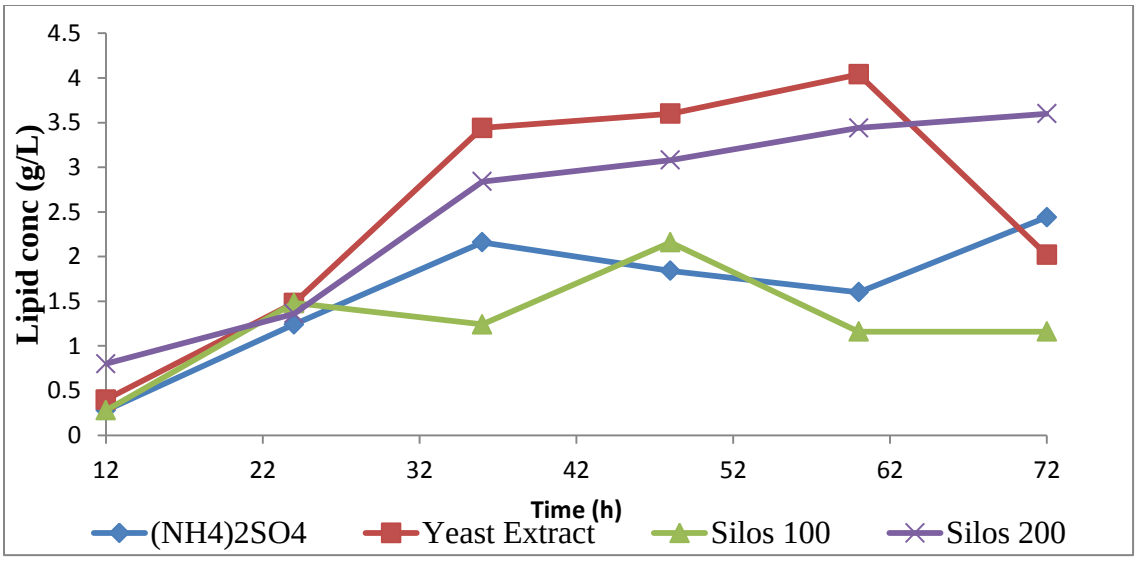

Figure 29 Lipid production of Y.lipolytica SKY7 on different nitrogen sources in crude glycerol medium

The glycerol concentration during different nitrogen supplemented sources fermentation was presented in Figure 30. In general, glycerol was not completely utilized, which is due to the deficiency of available nitrogen in the medium. Comparatively, high glycerol (29.01g/L) consumption was evident in yeast extract (highest nitrogen concentration or lowest C/N ratio) supplemented experiments. The availability of nitrogen and other nutrients were higher with comparatively low C/N ratio. In the case of $(NH_4)_2SO_4$, 19.96g/L glycerol was consumed. Compared to the yeast extract supplemented flask the biomass and lipid concentration obtained were low, thus the glycerol consumption also low. In the case of 200m/L silos leachate supplemented flask 16.55g/L glycerol was utilized and in 100ml/L silos leachate supplemented experiments it was 7.36g/L. From the experimental data, it is clear that the nitrogen concentration and C/N ratio has a high impact on glycerol consumption, thus, it affects the biomass and lipid production.

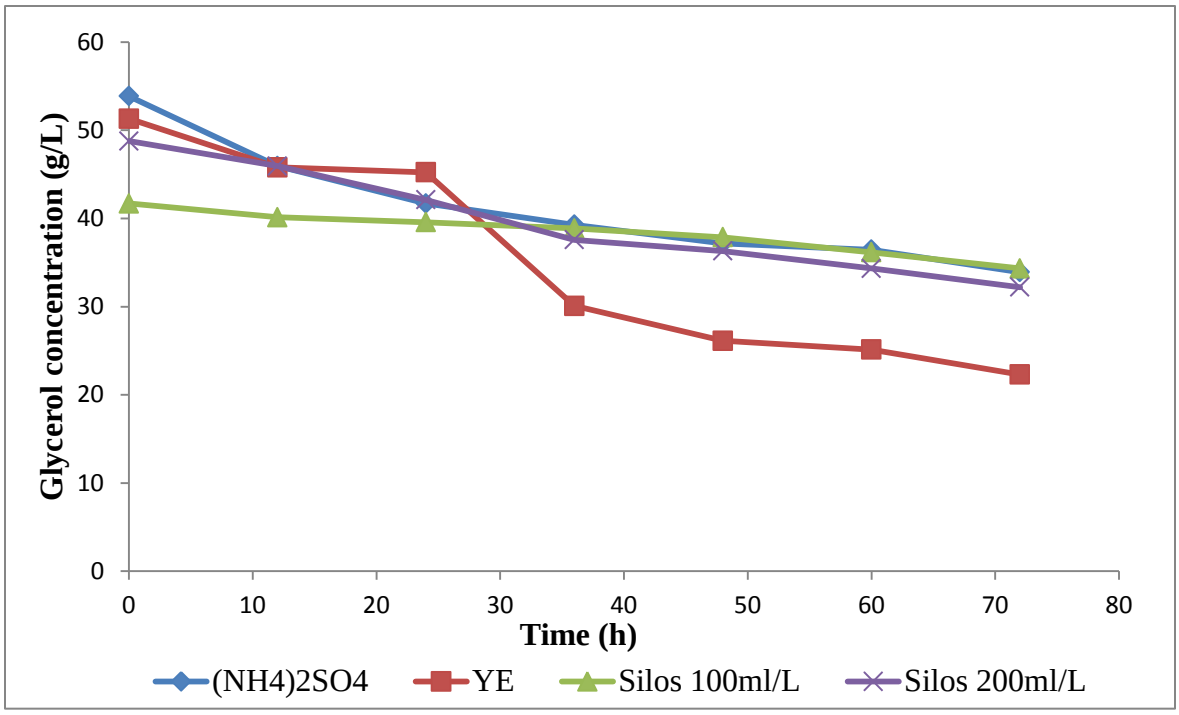

Figure 30 Glycerol consumption of Y.lipolytica on different nitrogen sources

Further to compare the obtained results the yield of biomass and lipid produced with respect to glycerol consumed were calculated and presented in Table 32. Since the soap concentration was not determined the yield of biomass and the lipid was calculated according to the glycerol consumed. In the case of biomass yield (Yx/s- yield of biomass/ gram of glycerol consumed. was higher in silos 100 with a Yx/s of 0.707g/g was achieved at 72h of fermentation. However, a low Yx/s value of 0.368g/g was observed in yeast extract supplemented experiments. When the medium was fortified with $(NH_4)_2SO_4$ the Yx/s value was 0.481g/g and it was 0.527g/g in the case of silos 200ml/L supplemented studies. The higher biomass yield compared to the $(NH_4)_2SO_4$ and yeast extract supplemented experiment suggests that the silos can be utilized as an alternative source of nitrogen for biomass production.

Table 32 Evaluations of different nitrogen source for biomass lipid productions and overall yield of biomass and lipid obtained at 72h of fermentation in crude glycerol supplemented medium.

Nitrogen **source**	**Glycerol consumed** (g/L)	**Biomass concentration** (g/L)	**Lipid concentration** (g/L)	**Yx/s (g/g)**	**Yp/s (g/g)**
Yeast extract	29.0	10.6	4.0	0.36	0.07
$(NH_4)_2SO_4$	19.9	9.6	2.4	0.48	0.12
Silos 100	7.3	5.2	1.1	0.70	0.15
Silos 200	16.5	8.7	3.6	0.52	0.21

The lipid yield (Yp/s yield of lipid/ gram of glycerol consumed) was noted to be higher in 200ml/l silos supplemented flask. A 0.217g/g lipid was obtained at the end of fermentation (72h). The low lipid yield was obtained in yeast extract supplemented experiments (0.07g/g). The low lipid yield could be due to the division of consumed substrate into other co-product such as citric acid and CO_2. The strain *Y.lipolytica* is well known for its citric acid production capabilities and it is accepted as one of the industrial scale citric acid producer (Makri et al., 2010). Based on the flask scale studied it has been concluded that a silos leachate concentration of 200ml/L could be favorable for high biomass and lipid production. However, it highly depends on the concentration of nitrogen and other nutrient availability in silos leachate.

4.3 Silos leachate supplemented fermentation for biomass and lipid production at bench scale reactor (15L).

Based on the flask experiments 200ml/L silos supplemented crude glycerol medium was further tested in 15L (duplicates) scale. The initial glycerol concentration was 44.39g/L and in addition to that 17.53g/L soap was also present in the medium. The glycerol concentration decreased faster than soap from the early stages of fermentation. At the end of fermentation (72h) 18.74g/L glycerol and 7.4g/L soap was left in the fermentation. The low glycerol and soap+FFA assimilation is due to the addition of extra carbon in silo leachate. It is known that silo leachate harbors 12g/L BOD, thus, *Y.lipolytica* may use the carbon from silo leachate and leaving unused glycerol and soap in the medium. In our previous studies, the glycerol and soap consumption was comparatively higher than the present case. At 60h glycerol was completely consumed in a non-

sterile fermentation and only 8g/L soap was left in the fermentation medium. The high glycerol concentration even at 72h of fermentation could result due to the addition of silos leachate as a nitrogen source. In addition to the nitrogen content of 2048 mg/L, the silo leachate contains 12550mg/L BOD (biological oxygen demand). Hence, the addition of 200ml/L silos leachate will contribute a certain amount of free carbon for the microbe (ie 200X10=2000ml/10L= 25100mg/10L). The main components reported in silos leachate were lactic acid 4-6%w/v, acetic acid 1-2%w/v and butyric acid less than 1%w/v and BOD 12-19g/L (Clarke & Stone, 2004). Hence, it is clear that some carbon from the silos leachate was used for biomass and lipid production by the microbe as is also clear from the experiments conducted in the silos leachate alone (Table 30).

The biomass production notably started after a lag of 6h (Figure 31). From 6h onwards exponential growth occurred. The specific growth rate was calculated and presented in Figure 32. A maximum specific growth rate of $0.151h^{-1}$ was attained at 9h of fermentation and up to 18 h it was approximately same. From 18h onwards, the specific growth rate declined rapidly and reached $0.0455h^{-1}$ at 72h of fermentation. The recent studies show that the stationary phase of *Y.lipolytica* commence between 17 to 21h when the medium was fortified with pure glycerol (Sestric et al., 2014) and these results were comparable with the current study. A sharp increase in biomass concentration from 6 to 18h period also supports high growth of *Y.lipolytica*. To further confirm the results of lipid concentration obtained during the fermentation was subtracted from the total biomass to know the actual biomass concentration (or lipid-free biomass). It clearly indicates that up to 18h the major increase was contributed from the biomass accumulation rather than lipid production. From 18h onwards, the lipid-free biomass stays approximately similar until 51h of fermentation. From there onwards the lipid-free biomass starts to climb to 17.28g/L. The increase in biomass concentration was probably due to the use of stored lipid as a carbon source; lipid concentration decreased from 51h. In the case of lipid production during the early 18h of fermentation, it is 5.32g/L. However, the lipid concentration was notably increased from 5.32g/L to 15.12g/L, which represents 63.85% w/w of the cell dry weight between 18 to 51h of fermentation. In the case of *Y.lipolytica*, this is the highest lipid concentration achieved within 51h of fermentation using crude glycerol as substrate and industrial waste water (silos leachate) as the nitrogen source. At the same time, a genetically modified *Y.lipolytica* was reported to produce higher lipid concentration (25.3g/L) than the wild

strains (Blazeck et al., 2014). The high lipid production during 18h to 51 h and a constant lipid free biomass concentration at this time period shows that the lipid production was highly favored in this period. At the same time, the initial increase in lipid concentration can be explained by the presence of a hydrophobic substrate in the fermentation medium. It has been known that when the fermentation was supplemented with hydrophobic substrates, the lipid biosynthesis occurs through ex de novo synthesis and thus the biomass and lipid accumulation occurs simultaneously (Papanikolaou & Aggelis, 2011). The decline of lipid concentration from 51h onwards could be due to the lipid degradation by the beta oxidation process. At 72h of fermentation, the lipid concentration was significantly reduced to 11.88g/L. The primary enzyme in beta oxidation is the intracellular lipase, which is reported to be present by many authors in *Y.lipolytica (Fickers et al., 2005).* In the present study, the extracellular lipase activity was measured to know the function of degardation of hydrophobic substrate. A maximum lipase activity of 16.6U/ml was obtained at 36h of fermentation (Figure 33). During the early stage of fermentation, the lipase activity was remarkably increased. From 36h onward the enzyme activity in the fermentation broth was notably decreased. Further, the soap degradation in the fermentation medium also follows a similar trend. From early stage to 30h of fermentation a high soap degradation was observed. However, when the fermentation progressed the lipase activity decreased and the soap consumption by *Y.lipolytica* also slows down significantly. From our earlier study, it is evident that with the current medium composition the lipase production occurs maximum within 36h of fermentation. Moreover, production of multiple products (lipid, citric acid, lipase activity) value added products from single fermentation may boost the overall economy of the process. Based on the fermentation profile (Figure 31) it is clear that the fermentation can be terminated at 48 or 51h of fermentation.

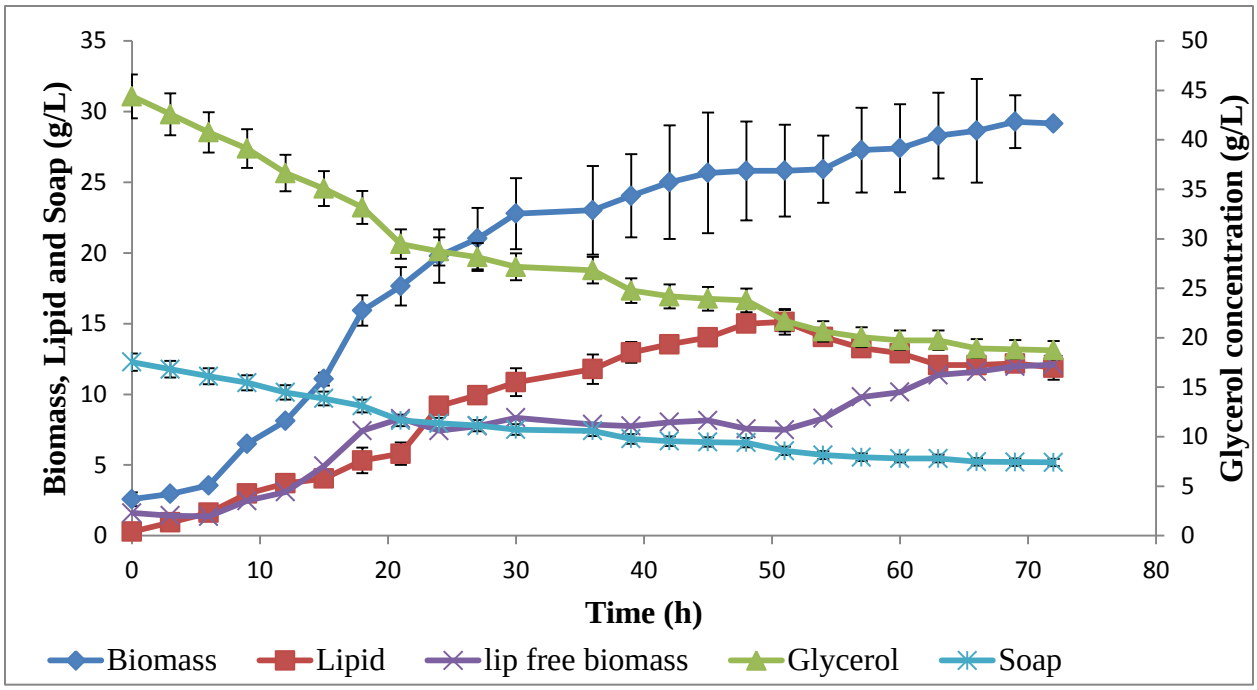

Figure 31 Time course analysis of Fermentation conducted in a 15L fermenter with 200ml/L silos leachate supplementation

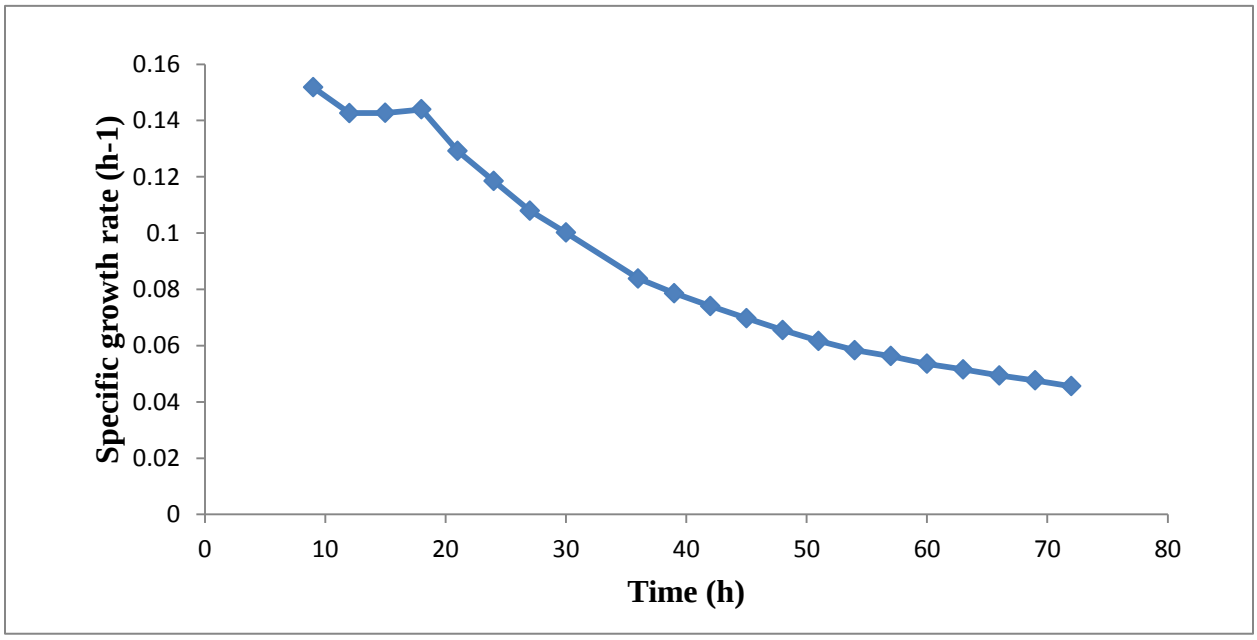

Figure 32 Variation of specific growth rate during fermentation

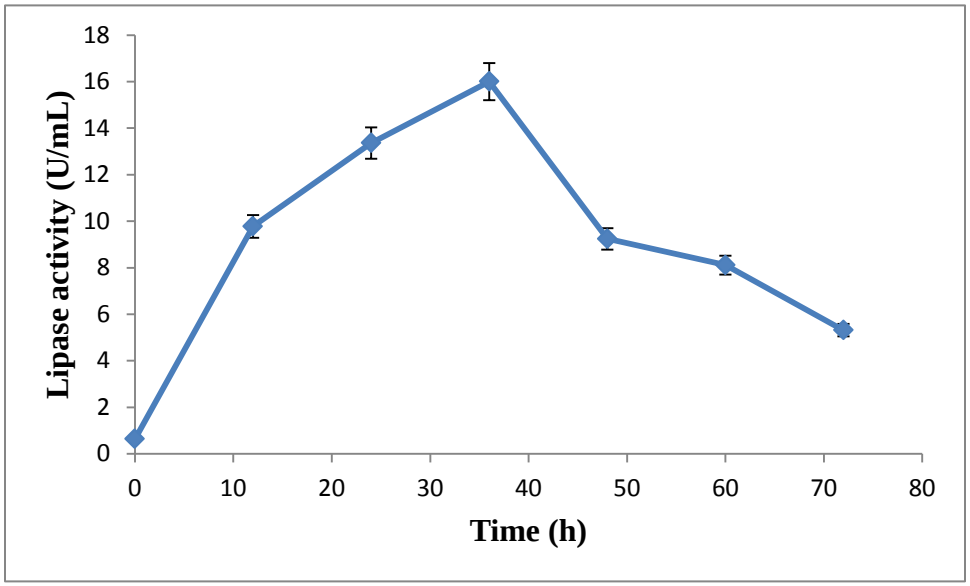

Figure 33 Variation of lipase activity with fermentation time

4.4 Time course analysis of yield of biomass and lipid

The yield coefficient of biomass, lipid, and lipid free biomass is presented in Figure 34. The cumulative biomass yield (Yx/s g biomass/C mole) shows a rapid increase from 6h of fermentation. It reached 60.4g/C mole at 9h and 96.6 g/C mole at 18h of fermentation. A decline in biomass yield was evident from 18h. When comparing the biomass yield and the lipid-free biomass yield it is clear that the biomass accumulation was prominent during 6 to 18 of fermentation. The maximum lipid-free biomass yield of 60.2g/C mole was achieved at 18h. The decline of yield in lipid-free biomass from 21h onward shows the decline of growth, which is also confirmed by a decrease in specific growth from 21h of fermentation. Further, to support the claim the nitrogen content of the fermentation broth was analyzed (Figure 35). The results show when the cells were in active growth phase the depletion of nitrogen from the medium was rapid. Nevertheless, the decrease in nitrogen concentration from the medium was significantly low after 24h of fermentation.

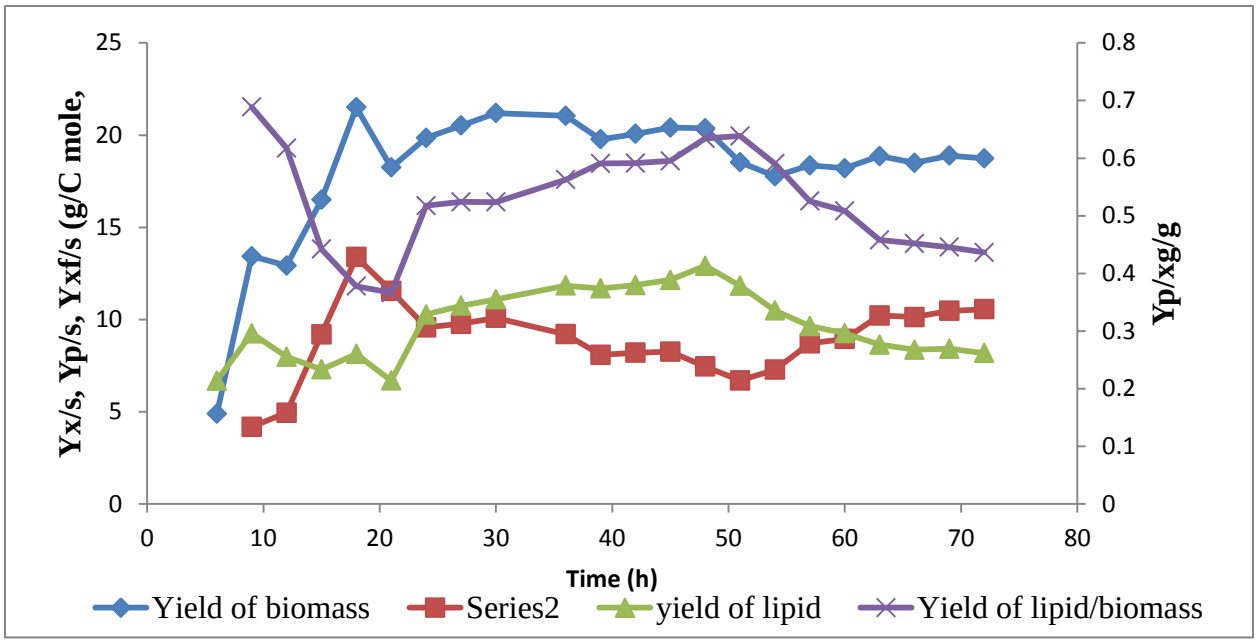

Figure 34 Variation of the yield of biomass, lipid free biomass, and lipid produced with fermentation time

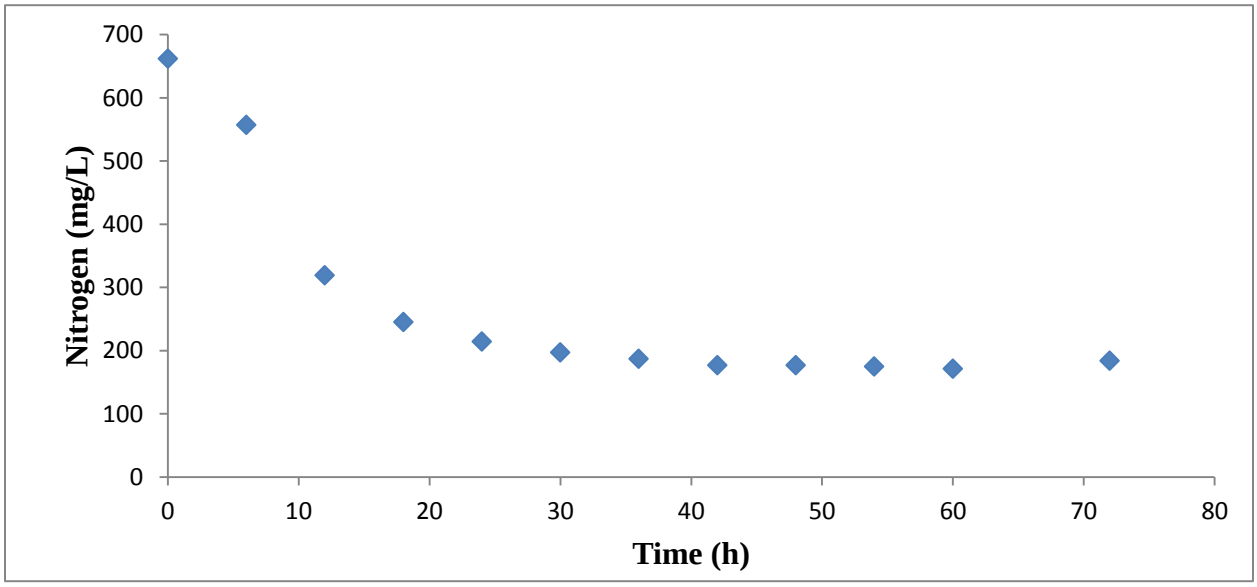

Figure 35 Variation in nitrogen concentration during fermentation

As reported by (Papanikolaou & Aggelis, 2011) when the fermentation was supplied with hydrophobic substrate the lipid accumulation also occurs concomitant to biomass production. From the results of Yp/s g lipid/C mole (lipid yield), it is evident that Yp/s increased from 30.07g/C mole at 6h to 41.6 g/C mole at 9h of fermentation. This could be due to the short lag phase 0 to 6h may cause a significant lipid accumulation in the cells. However, Yp/s was

significantly decreased until 18h (30 g/C mole) of fermentation and then from 24h onward the Yp/s started increasing until 51h. In other words, when Yx/s (lipid-free) value declines the Yp/s value increases. This was denoted by lipogenic phase by several authors (Beopoulos et al., 2009; Papanikolaou & Aggelis, 2011; Ratledge & Wynn, 2002b). The Yp/s declined from 51 h onward and which is further evident from the total lipid concentration at 51h. the decrease in the lipid concentration may occur due to the β-oxidation. A similar trend was observed in the case of Yp/x (yield of lipid per gram of biomass). A maximum Yp/x was obtained at 51h (0.638g/g).

The GC-FID data of FAME analysis showed a high concentration of oleic acid (37.98%). Followed by that linoleic acid with 28.59%. the other components were palmitic acid 12.53%, stearic acid 7.11% and arachidonic acid 1.72%. Other than the above-mentioned components trace amounts of Linolenate, Eicosenoate, and Lignoceric were present in the TAG. The main components contribute 87.94% of the total TAG produced and other components contribute 12.06% of the total TAG.

5 Conclusion and summary

The current study of silos leachate as a nitrogen source for biomass and lipid production of crude glycerol-based medium shows the potential application of silos iodate on biodiesel industry. When the fermentation medium was supplemented with silos leachate alone the strain *Y.lipolytica* showed a comparatively better growth over *Rhodococcus* and accumulated 16.04g/L biomass and 1.84g/L lipid. Meantime, the addition of crude glycerol with high soap content resulted in high biomass and lipid accumulation in a short period of time. Based on the results it has been concluded that the silos leachate could be one of the potential waste material that can replace the commercial nitrogen sources such as $(NH_4)_2SO_4$ and yeast extract without affecting the biomass and lipid accumulation. Meantime using silo leachate will reduce the risk of contamination of ground water by silo leachate. Moreover, replacing the commercial nutrients from SCO production will boost the economy of biodiesel production from heterotrophic microbes.

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References

Avramidis, P., Nikolaou, K., Bekiari, V. 2015. Total Organic Carbon and Total Nitrogen in Sediments and Soils: A Comparison of the Wet Oxidation – Titration Method with the Combustion-infrared Method. *Agriculture and Agricultural Science Procedia*, 4, 425-430.

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(6), 692-6.

Blazeck, J., Hill, A., Liu, L., Knight, R., Miller, J., Pan, A., Otoupal, P., Alper, H.S. 2014. Harnessing Yarrowia lipolytica lipogenesis to create a platform for lipid and biofuel production. *Nat Commun*, 5, 3131.

Bondioli, P., Della, B., Laura. 2005. An alternative spectrophotometric method for the determination of free glycerol in biodiesel. *European Journal of Lipid Science and Technology*, 107(3), 153-157.

Clarke, S., Stone, R. 2004. How to handle seepage from farm silos- Fact sheet. *Agricultural Engineering*.

Demirbas, A. 2009. Progress and recent trends in biodiesel fuels. *Energy Conversion and Management*, 50(1), 14-34.

Dobrowolski, A., Mitula, 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.

Fickers, P., Benetti, P.H., Wache, Y., Marty, A., Mauersberger, S., Smit, M.S., Nicaud, J.M. 2005. Hydrophobic substrate utilisation by the yeast Yarrowia lipolytica, and its potential applications. *FEMS Yeast Res*, 5, 527-543.

Folch, J., M.Lees, G.H.Slane-Stanley. 1957. Simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*, 226, 497–509.

Gnanaprakasam, A., Sivakumar, V.M., Surendhar, A., Thirumarimurugan, M., Kannadasan, T. 2013. Recent Strategy of Biodiesel Production from Waste Cooking Oil and Process Influencing Parameters: A Review. *Journal of Energy*, 2013, 1-10.

Hu, S., Luo, X., Wan, C., Li, Y. 2012. Characterization of crude glycerol from biodiesel plants. *J Agric Food Chem*, 60(23), 5915-21.

Karmee, S.K., Linardi, D., Lee, J., Lin, C.S. 2015. Conversion of lipid from food waste to biodiesel. *Waste Manag*, 41, 169-73.

Khan, T.M.Y., Atabani, A.E., Badruddin, I.A., Badarudin, A., Khayoon, M.S., Triwahyono, S. 2014. Recent scenario and technologies to utilize non-edible oils for biodiesel production. *Renewable and Sustainable Energy Reviews*, 37, 840-851.

Koutinas, A.A., Chatzifragkou, A., Kopsahelis, N., Papanikolaou, S., Kookos, I.K. 2014. Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. *Fuel*, 116.

Lane, J. 2013. Biofuels Mandates Around the World: 2014. *Biofuels Digest*.

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(4), 184-8.

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(7), 2351-8.

Mondala, A., Hernandez, R., french, T., McFarland, L., Domingo, J.w.S., Meckes, M., Ryu, H., Iker, B. 2011. Enhanced lipid and biodiesel production from glucose-fed activated sludge: Kinetics and microbial community analysis. *Environmental and energy engineering*, 58(4), 1279-1290.

Muller, E.E., Sheik, A.R., Wilmes, P. 2014. Lipid-based biofuel production from wastewater. *Curr Opin Biotechnol*, 30, 9-16.

OECD-FAO. 2011. OECD-FAO Agricultural Outlook 2011-2020.

Papanikolaou, S., Aggelis, G. 2011. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. *European Journal of Lipid Science and Technology*, 113(8), 1031-1051.

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(3), 308-12.

Pleissner, D., Kwan, T.H., Lin, C.S. 2014. Fungal hydrolysis in submerged fermentation for food waste treatment and fermentation feedstock preparation. *Bioresour Technol*, 158, 48-54.

Prim, N., Sánchez, M., Ruiz, C., Pastor, F.I.J., Diaz, P. 2003. Use of methylumbeliferyl-derivative substrates for lipase activity characterization. *J Mol Catal B Enzym*, 22, 339–346.

Ratledge, C., Wynn, J.P. 2002a. The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms. *Advances In Applied Microbiology*, 51.

Ratledge, C., Wynn, J.P. 2002b. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. in: *Advances in Applied Microbiology*.

Schultz, E.L., Souza, D.T.d., Damaso, M.C.T. 2014. The glycerol biorefinery: a purpose for Brazilian biodiesel production. *Chemical and biological Technologies in Agriculture*, 1(7).

Sestric, R., Munch, G., Cicek, N., Sparling, R., Levin, D.B. 2014. Growth and neutral lipid synthesis by Yarrowia lipolytica on various carbon substrates under nutrient-sufficient and nutrient-limited conditions. *Bioresour Technol*, 164, 41-6.

Tchakouteu, S.S., Kalantzi, O., Gardeli, C., Koutinas, A.A., Aggelis, G., Papanikolaou, S. 2015. Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency. *J Appl Microbiol*, 118(4), 911-27.

Yaakob, Z., Mohammad, M., Alherbawi, M., Alam, Z., Sopian, K. 2013. Overview of the production of biodiesel from Waste cooking oil. *Renewable and Sustainable Energy Reviews*, 18, 184-193.

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

Chapter-VI

Research Article-7

Studies on influence of methanol in biomass and lipid production and development of a non-sterile fermentation process for lipid production from crude glycerol obtained from biodiesel industry

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Résumé

L'augmentation de la demande de production du biodiesel a créé une nouvelle alternative et une source de carbone durable sous forme de glycérol pour la production d'huile microbienne. L'essor de l'industrie du biodiesel dans le monde entier a directement influencé le prix du glycérol global, ainsi le glycérol brut provenant de l'industrie du biodiesel est devenu l'une des alternatives prometteuses les moins chers pour la production du SCO. Cependant, le glycérol brut provenant de l'industrie du biodiesel héberge de multiples composants, tels que le méthanol, le savon, les acides gras libres, des catalyseurs et de l'eau. Une conversion efficace des multiples sources de carbone en une seule fermentation est un moyen très intéressant pour ajouter de la valeur aux sous-produits de l'industrie du biodiesel. Cependant la présence des composés mentionnés ci-dessus est connue pour son inhibition de la croissance des cellules. En Pour cette raison, les composés inhibiteurs tels que le méthanol et le savon ont été éliminés avant la fermentation et le procédé a été opéré dans un état de stérilité. Bien que des résultats prometteurs aient été atteints, le processus de stérilisation exige une grande énergie, ainsi l'industrialisation de l'OCS des hétérotrophes est toujours à l'échelle du laboratoire. En utilisant le méthanol comme agent de contrôle de la contamination est une nouvelle stratégie de la production de lipides microbiens depuis le glycérol brut contient une grande quantité de méthanol. Dans la présente enquête, une fermentation supplémentée et non stérile de3% v/v de méthanol a été tentée. Sur la base des résultats expérimentaux, il a été constaté que la supplémentation par méthanol pouvait contrôler la croissance des contaminants dans une grande mesure. Mais l'ajout d'UV traité de l'eau du robinet pour diluer les milieux de fermentation a prolongé de façon significative la croissance des contaminants. En utilisant cette méthode de fermentation non stérile une concentration de biomasse de 17,57 ± 1.7g/L et 8,8 ± 0,7 g production/l de lipides a été réalisée en moins de 72h de fermentation. Plus encore, la croissance de contaminants a été limitée jusqu'à 42h de fermentation, ce qui a fourni un suffisamment de temps pour la croissance des monocultures (*Y.lipolytica* SKY7). L'étude actuelle démontre la possibilité d'utiliser le méthanol, produit dérivé du glycérol brut et l'UV de l'eau traitée du robinet pourrait être utilisé pour la production future d'huile microbienne utilisant une monoculture sans stérilisation classique.

Summary of the work

Increasing demand for biodiesel production sets up a new alternative and sustainable carbon source in the form of glycerol for microbial oil production. The booming of biodiesel industry throughout the globe directly affected the global glycerol price thus the crude glycerol from biodiesel industry became one of the cheap promising alternatives for SCO production. However, the crude glycerol from biodiesel industry harbors multiple components such as methanol, soap, free fatty acids, catalyst, and water. An effective conversion of multiple carbon sources into a single fermentation is an interesting way to add value to the biodiesel industry by-product. Still, the presence of above-mentioned compounds is known to inhibit the cell growth. The inhibitory compounds such as methanol and soap were removed prior to fermentation and the process was operated in a sterile condition. Although promising results were attained, the sterilization process demands high energy thus industrialization of SCO from heterotrophs are at lab scale. Using methanol as a contamination control agent is a new strategy in microbial lipid production since the crude glycerol used to contain a considerable amount of methanol. In the present investigation, a 3%v/v methanol supplied non-sterile fermentation was attempted. Based on the experimental results it has been found that methanol supplementation could control the growth of contaminants to a large extent. But the addition of UV treated tap water for diluting the fermentation media significantly prolonged the growth of contaminants. Using this nonsterile fermentation method a biomass concentration of 17.57±1.7g/L and 8.8±0.7g/L lipid production was achieved within 72h of fermentation. Moreover, the growth of contaminants was restricted until 42h of fermentation, which provides enough space for the growth of monoculture *(Y.lipolytica SKY7*). The current study proves the possibility of using crude glycerol derived methanol and UV treated tap water could be used for future microbial oil production using monoculture without classical sterilization.

Introduction

Biofuels are the raw energy source (oil, firewood etc.) used by the ancient civilization. However, the discoveries of crude oil change the scenario and made the industrial revolution at middle age. Then onwards the crude oil based fuels were dominated in industrial and transportation sector as a major energy source (Machado et al., 2015). However, in recent decades, the availability of the fossil fuels started to decrease and the oil price increased (Tchakouteu et al., 2015). Furthermore, the over utilization of crude oil based fuels the globe ended up in serious environmental problems including global warming, changes in the ecological system, etc. Due to the ever increasing oil price and increasing concerns about the global warming, searches of alternative fuels were primary concerns in the world. At this critical scenario biofuel from sustainable and renewable sources are being investigated as a potential alternative for high pollutant fossil fuels (Da Silva et al., 2009).

Biofuels are generally produced from renewable resources such as agro-industrial by-products. Among them, oils from food industry gained more attention due to its high availability at low cost (Attia & Hassaneen, 2016). The conventional conversion of oils and fat into biodiesel from food industry involves the trans-esterification with short chain alcohols such as methanol or to certain extent ethanol (Leung et al., 2010). Conversion of oils and fat into biodiesel results in the formation of glycerol as a major byproduct (every 10kg of biodiesel production from oil results in 1kg of glycerol) (Meesters et al., 1996). The current increase in the biodiesel production resulted in a significant boost in the glycerol production globally. The disposal of glycerol obtained from biodiesel industry is a tedious process due to the presence of a large amount of impurities (Samul et al., 2014). At the same time refining the glycerol and utilizing it of pharma and food industry is not an economical process (Gerpen, 2005). Moreover, the impurities such as high salt content and methanol content are toxic to the environment and reduces the application of biodiesel derived glycerol in industrial applications(Tan et al., 2013). However, the crude glycerol is considered as one of the best carbon sources for microbes. Due to the boom in the biodiesel industry, an increased glycerol production can serve as an excellent renewable and sustainable resource for microbial oil production. Thus, glycerol can substitute the traditional carbohydrates in the industrial microbial production of value added components.

Meanwhile, the composition of the glycerol produced in transesterification reaction results in the high amount of methanol, soap, free fatty acids (FFA), inorganic salts, water, methyl esters, etc. (Mario Pagliaro, 2010). However, utilization of crude glycerol without purification in microbial fermentation process leads to a poor efficiency of metabolite formation due to inhibition of metabolic pathways of the microbes (Chatzifragkou & Papanikolaou, 2012). In general, impurities present in the crude glycerol interact with the microbial cell wall and either increase or decreases the permeability. However, the alteration in permeabilization strictly depends on the concentration of the impurities (Samul et al., 2014). Low concentration of methanol (1%v/v) has no significant effect on cell metabolism. At low concentration, methanol can be utilized as a carbon source for the cell growth (Woon-Yong et al., 2011). On the other hand, when the concentration increases methanol can become a potent toxicant to the cell. Initially, the externally supplied methanol is oxidized via alcohol oxidases (AOX EC 1.13.13) to formaldehyde and hydrogen peroxide. Then the catalase converts the H_2O_2 into water and oxygen. Further, the formaldehyde is oxidized into CO_2 by glutathathaone and NAD+ dependent formaldehyde dehydrogenase (FLD, EC, 1.2.1.1). Thus, the high concentration of methanol or deregulation of AOX or FLD fails, the assimilation of methanol halts, which finally results in high toxicity to the cells (Krainer et al., 2012). The second most important growth inhibitor is the high concentration of inorganic salts present is the crude glycerol. The monovalent salts reduce the van der Waals force in the lipid bilayers thereby reduces the transportation of nutrients through the cell membrane (Petrache et al., 2006). However, their low concentration (monovalent salts) favors the cell growth by acting as co-factors for intracellular enzymes. The third class of inhibitors is the free fatty acids and soap. The unsaturated fatty acids and soap influence the diffusion of nutrients through the membrane (Venkataramanan et al., 2012).

Based on the transesterification process and the efficiency of the transesterification reaction the final glycerol composition varies. The variation in methanol content from various biodiesel industry has been found between 12-18% w/w (Venkataramanan et al., 2012). Conversely, our lab studies showed that methanol concentration can vary from 1.5 to 35%v/v. Exceptionally, in nature, many microorganisms are equipped to convert these short chain alcohols into biomass and other value added products. A methanol utilizing *P pastoris* was reported to cultivate methanol based fermentation media (fed-batch mode) to produce single cell proteins. The above study found that the methanol concentration of 3.65g/L was found to inhibit cell growth (Krainer

et al., 2012). However, 1%v/v methanol was used as a carbon source for cultivating *chlorella sps* to produce SCO. Further, a methanol concentration above 3%v/v was reported to be inhibitory for cell growth (Woon-Yong et al., 2011). On the other hand, *Y.lipolytica* is known to utilize ethanol as carbon source up to 3% concentration (Barth & Künkel, 1979). Moreover, *Y.lipolytica* processes several NAD+ and NADP+ dependent alcohol dehydrogenases, which make it as a potential organism to utilize/tolerate short chain alcohols and grow without inhibition at preferable concentration levels (Coelho et al., 2010).

Until date, plenty of research has been undertaken for single cell oil (SCO) production from crude glycerol. However, an economically sustainable process for making SCO from renewable resources by microbial fermentation is still in experimental stage. Though the process economy depends on the cheap carbon sources; 60% of overall production cost is for substrate (Fei et al., 2011). The current availability of the low-cost substrate for biotransformation such as crude glycerol from biodiesel industry, lignocellulosic biomass, food and other industrial effluents could change the philosophy of SCO production. Apart from a carbon source, the operational cost of the fermentation has been another major factor, which influences the final SCO cost. In such a scenario sterilization of fermenters and the media is a tedious and high energy demanding process. Elimination of sterilization process from SCO production or sterilization of the fermentation vessels by other low-cost options may boost the overall process economics. In this concern, there are few strategies that were adopted such as low pH and low temperature based non-sterile fermentation process to generate lipids from renewable sources. However, the biomass and lipid content of *Y.lipolytica* achieved at 15^0C was 7.4 ±0.25g/L and 39%w/w, respectively (Taskin et al., 2015). Whereas, biomass concentration 7.4g/L and 40% w/w lipid content were recorded at low pH conditions (pH 5.5 and temperature 15^0C by *Metschnikowia pulcherrima (Santamauro et al., 2014).* Considering the fact that the low temperature and low pH may be helpful to maintain the fermentation contamination free but it affects both the biomass growth and lipid production. Therefore, an alternative way needs to be identified to grow the cells without or with low contamination in a feasible way to produce high biomass and lipid. A low-level contamination without major effect on lipid yield and productivity should be acceptable as the contaminant will not affect the quality of lipid and biodiesel. Based on the available literature it has been found that supplementation of methanol to the fermentation media in a controlled manner may eliminate or suppress the possible contaminants to a large extent.

With this knowledge, the current study aims to develop a nonsterile process for SCO production using *Y.lipolytica* and crude glycerol supplemented media. Moreover, the crude glycerol from biodiesel industry contains a minimum concentration of methanol. The development of such a bioprocess from crude glycerol may eliminate the distillation of crude glycerol after transesterification process, which can be used to control the contaminants.

1 Materials and methods

1.1 Organism and inoculum preparation

Y.lipolytica SKY7 was maintained on YPD agar plates (Yeast extract 10g/L peptone 5g/L dextrose 20g/L and agar 20g/L). Pre-inoculum was also prepared in YPD broth. The medium was sterilized by autoclaving at 121^0C for 20 minutes and then a loop full of a pure culture of *Y.lipolytica* was aseptically inoculated. The final inoculum was prepared in YPG medium (yeast extract 10g/L, peptone 5g/L, and glycerol 20g/L. In order to get a glycerol concentration of 20g/L, 66.5g/L crude glycerol was added to the media. Hence along with glycerol 17.9g/L soap and 6.7g/L methanol was also added additionally to the inoculum. An 18h grown pre-inoculum was aseptically transferred to final inoculum. The inoculum was incubated for 18h in a shaking incubator with 180rpm and 28^0C. The well-grown inoculum was used in all the studies.

1.2 Impact of methanol concentration on biomass and lipid production

The initial screening for methanol tolerance of *Y.lipolytica* SKY7 was conducted in a shake flask fermentation containing 500 ml media and 1 to 5% v/v methanol supplementation. The initial glycerol concentration was maintained at 40g/L. Due to the presence of soap and methanol in crude glycerol sample (Table 33), the addition of (133g/L) glycerol 35.9g/L soap and 13.51g/L methanol and was also added to the medium. The media was fortified with (g/L) Na_2HPO_4 1g; KH_2PO_4 2.7g, $MgSO_4.7H_2O$ 0.5g, $(NH_4)_2SO4$ 2g and yeast extract 1g. Initially, the 2L Erlenmeyer flasks were sterilized without the media and 475ml of non-sterile media supplemented with 1 to 5 %v/v methanol was prepared. A 5% v/v inoculum was aseptically transferred to the fermentation media. Samples were collected at each 12h interval and analyzed for residual glycerol content, biomass and lipid content. The fermentation broth was monitored under the microscope with proper dilutions to check for microbial contamination. In addition to that, the samples were plated on YPD agar plates to check the contamination and cell count

(CFU- colony forming units). All the fermentation media were prepared with normal tap water and the initial C/N molar ratio was maintained at 75.

Table 33 Composition of Glycerol

Component	Content (W/W)
Glycerol	30.0
Soap	27.4
Methanol	12.8
Catalyst	1.2
Water	28.6

1.3 Non-sterile methanol supplemented experiments on bench scale fermenters

Based on the methanol tolerance, methanol supplementation of 3%v/v in the medium was chosen to test in 15L bench scale fermentor. The fermentor was sterilized with water initially to make sure that the contamination may not occur from the fermentor. The fermentation media was prepared by diluting the media components with non-sterile tap water at the following concentration. The initial glycerol concentration was maintained at 40g/L (35.9g/L soap, 13.51g/L methanol and 1.59g/L catalyst), Na_2HPO_4 1g/L; KH_2PO_4 2.7g/L, $MgSO_4.7H_2O$ 0.5g/L, and yeast extract 3g/L. The initial C/N ratio was 113.3 (taking into account the carbon content in soap and glycerol). The working volume of the fermenter was 10L (Biogenie, Quebec, Canada) (9.5L media+0.5L inoculum) and the fermentation was conducted in replicates at 6.5pH. The fermenter was equipped with programmable logic control (PLC) system, which included a dissolved oxygen (DO) probe, antifoam, agitator, aeration, temperature, and pH control devices. The above parameters were maintained using the program ifix 3.5, Intellution, USA. The pH meter (Mettler Toledo, USA) was calibrated using buffers pH 4 and 7.0 (VWR, Canada). The DO probe was calibrated to zero using sodium sulfate solution and to 100% by purging air in distilled water. A well grown (18h) pure inoculum (5% v/v) was transferred aseptically to the fermentation media. The DO in the fermenter was maintained above 30% saturation. Samples were collected at 6h intervals to analyze the biomass, lipid, and residual glycerol and soap content. Further, the fermentation broth was aseptically plated on YPD agar plates to check the growth of *Y.lipolytica* and also to monitor the contamination. Moreover, the culture broth was

routinely examined using a microscope and the change in DO level also monitored to ensure the fermentation progress without contamination.

1.4 UV treated tap water as diluent for media preparation for methanol based non-sterile fermentation.

Initially, the tap water was treated with UV (UV-30W/model G30 T8 from Philips) source for 10 minutes and the UV treated water was used to prepare the media. UV treatment is one of the widely accepted techniques used in portable water treatment to eliminate the vegetative cells. Figure 36 represents the overall flow diagram of a non-sterile fermentation process based on UV and methanol treatment. A UV source was added before the water was used to dilute the culture media. Then the treated water was used for the fermentation. Further, the presence of methanol in the cultivation media was expected to control the growth of contaminants. Moreover, to minimize the lag in growth, the inoculum was acclimatized for methanol by adding 1.5%v/v methanol during inoculum preparation. The initial glycerol concentration was maintained at 40g/L and the crude glycerol was reported in Table 33 and contains 30%w/w glycerol and 27.4% w/w soap. In order to obtain a glycerol concentration of 40g/L in the medium, 133.3g/L of crude glycerol was used. Hence, the soap concentration in the fermentation media was 36.5g/L. In addition to that, a methanol concentration of 12.9mL/L was also added to control the contamination to make the final methanol concentration 30mL/L (addition of 133g crude glycerol will supply 17.1mL methanol). The media was fortified with (g/L) Na_2HPO_4 1g; KH_2PO_4 2.7g, $MgSO_4.7H_2O$ 0.5g, and yeast extract 1g and $(NH_4)_2SO_4$0.5g/L. The media 9.5L prepared in UV sterilized water and supplemented with 3 %v/v methanol was added to the fermenter. A well grown 5% v/v inoculum was transferred to the fermenter aseptically. The fermentation was conducted at pH 6.5; temperature 28^0C and DO were kept above 30% saturation. Samples were collected at the 6h interval and analyzed for residual glycerol, biomass, and lipid concentration. The fermentation broth was monitored under the microscope with proper dilutions to evaluate the sterility. In addition, the samples were plated on YPD agar plates to check the contamination

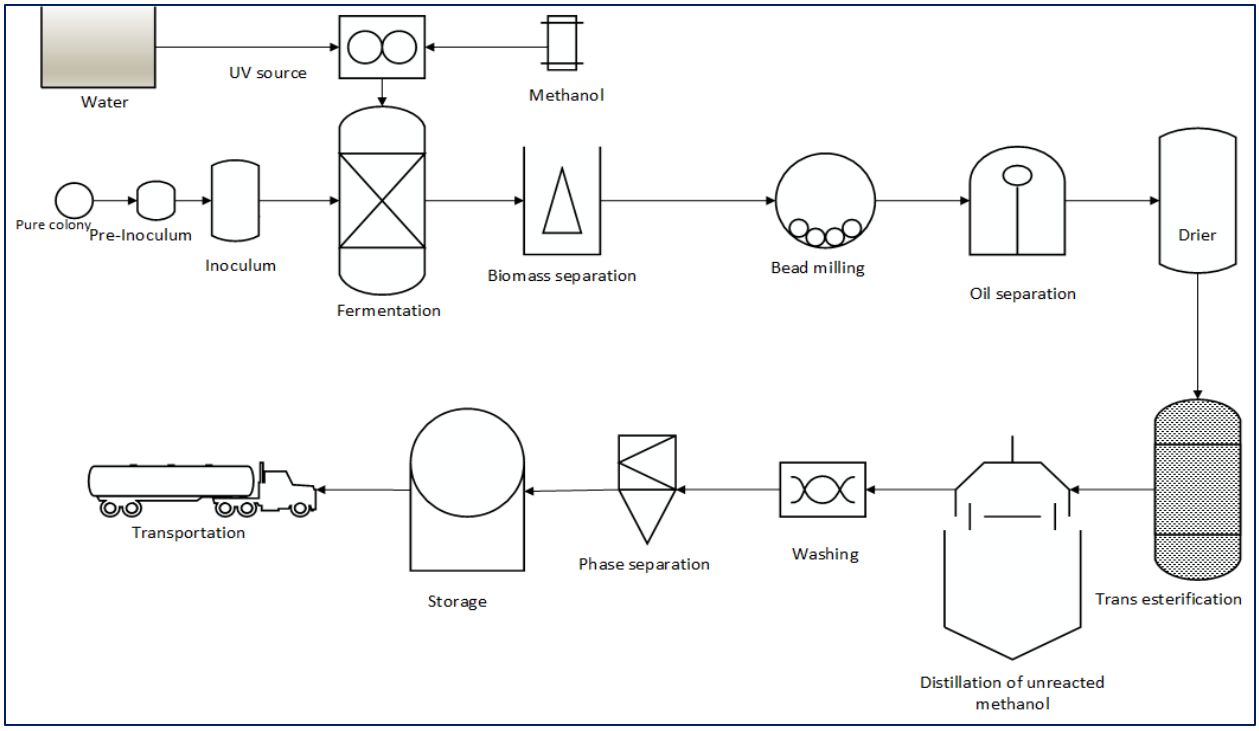

Figure 36 The flow diagram of lipid and biomass production on UV treated tap water with methanol supplementation

1.5 Analytical methods

The biomass content was measured using 25 ml of fermentation broth. The broth was centrifuged at 8000rpm for 10 minutes. The collected pellets were washed twice with hot water (60-65^{0}C). The supernatant was collected in a clean tube and the cell pellet was carefully transferred into a pre-weighed aluminum pan and heated at pre-set hot air oven at 105^{0}C for 12h or until it reached a constant weight. The lipid extraction was carried out with 20ml of fermentation broth. The cell pellets collected after centrifugation (8000rpm) was washed twice with hot water and 15ml of chloroform and methanol mixture (2:1) was added. To the mixture, an adequate amount of 0.7mm zirconium beads were added and the mixture was agitated on a wrist action shaker for 12h. After 12h, the samples were centrifuged at 3000rpm and the supernatant was collected into a clean preweighed glass tube. To the residual cell pellet 15ml of chloroform-methanol mixture (1:1ratio) was added again and the extraction was continued for 12h. The extracts were then filtered using Fisher brand filter paper (0.04µm). Then the samples were kept at 65^{0}C in a hot air oven to evaporate the chloroform and methanol. The final lipid content was measured by gravimetric method. The supernatant collected earlier was further used to analyze the residual

glycerol content described by (Bondioli & Della, 2005). Lipase activity in the supernatant was measured spectrophotometrically using pNPP as substrate and pNP as standard (Sigma-Aldrich USA). The absorbance was measured at 415nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1Umol of pNP per minute under the assay condition (Prim et al., 2003).

1.6 Calculation of yield coefficients and specific growth rate

The specific growth rate was calculated according to the following equation

$$\mu = (\frac{1}{x})\frac{dx}{dt} \quad (1)$$

Where μ is the specific growth rate, X is the concentration of biomass (g/L) and t is the fermentation time.

The yield of biomass and lipid was calculated according to the following equations (based on moles of carbon consumed to produce each gram of biomass and lipid).

$$Y\frac{x}{s} = \frac{g\ of\ biomass\ produced}{total\ moles\ of\ carbon\ consumed} = \frac{g\ of\ biomass\ produced}{C\ mole\ consumed\ from\ \ glycerol + C\ mole\ consumed\ from\ \ in\ soap} \quad (2)$$

$$Y\frac{x}{s} = \frac{g\ of\ Lipid\ produced}{total\ moles\ of\ carbon\ consumed} = \frac{g\ of\ Lipid\ produced}{C\ mole\ consumed\ from\ \ glycerol + C\ mole\ consumed\ from\ \ in\ soap} \quad (3)$$

2. Results and discussion

2.1. Impact of methanol concentration on biomass and lipid production

The initial methanol concentration was varied from 1% v/v to 5%v/v. A control experiment with sterile medium without methanol (control 1) and another control without methanol and non-sterile (control 2) were conducted to compare the results with non-sterile methanol supplemented experiments. The CFU data shows that when methanol concentration was increased from 1 to 5%v/v the total cell count at the end of fermentation decreased due to the inhibitory effect of methanol on *Y.lipolytica* SKY7 (Table 33). However, the formation of colonies at 5%v/v methanol indicated the cells survived at elevated methanol concentration. Furthermore, the

plating results revealed strong evidence that methanol concentration at or above 4%v/v completely restricts the growth of contaminants. However, in the case of 3%v/v methanol, the contaminants started to appear after 96h of fermentation. The occurrence of contaminants in experiments with methanol concentration less than 3%v/v suggests that the inhibition of methanol on contaminants (microbes) was not significant. At the same time, prolonged fermentation may lead to the reduction of methanol due to either by microbial utilization or evaporation loss, which favored the contaminants to appear at 96h onwards in 3%v/v methanol supplemented experiment. In another case without methanol and without sterilization (control 2), the contaminants were noticed at an early stage (12h) of the fermentation.

The effect of methanol concentration on biomass and lipid production is presented in Figure 37. Compared to the sterile fermentation, all other conditions resulted in low biomass concentrations. A decrease in biomass concentration was evident in methanol supplemented experiments. In the case of sterile fermentation a biomass concentration of 16.86g/L was obtained at 72h of fermentation whereas it was 4.92g/L in 5%v/v methanol supplemented fermentation (Figure 37). The low biomass without methanol supplementation and nonsterile fermentation (case 2, Figure 37) is due to the overgrowth of contaminants. The CFU count suggests that only two log cycle increase (from 1.69 X 10^3 to 5.5 X 10^5 i.e. control 2, Table 34) of yeast cells from 0h to 72h of fermentation. However, in the case of 2% v/v and 3%v/v methanol supplemented experiments the biomass concentrations was 14.1 and 13g/L, respectively (Figure 37) and the CFU was noticed to increase by four log cycle (Table 34) at 72h of fermentation. In the case of 3 and 4%v/v methanol supplemented experiments the biomass obtained was 14.1 and 12 g/L respectively, with very few or no contamination at 72h. The above results clearly state that the increasing methanol concentration affects significantly the cell proliferation. However, it is feasible to control the contaminants if the concentration of methanol is 3%v/v or above. The reduction in biomass concentration at 4 to 5%v/v methanol supplemented fermentation is due to methanol inhibition. Meantime, it is clearly reflected in the lipid concentration as well. In the control (sterile condition, no methanol added), a lipid concentration of 7.5g/L was obtained, which is 44.48%w/w of cell dry mass. However, approximately 36.8% reduction in overall lipid concentration was noted in 5% v/v methanol (2.76 g/L lipid) supplemented experiment.

Table 34 Comparison colony forming units and probability of contamination of sterile and non-sterile cultivation conditions in the 2L flask.

Cultivation condition	CFU/mL (0h)	CFU/mL (72h)	Contamination +/-
Control 1, sterile	1.72 $x10^3$	1.41 $x10^8$	No contamination
Control 2, Non-Sterile, without methanol	1.69 x 10^3	5.5 $x10^5$	Yeast was masked by bacteria
1% methanol	1.66 x 10^3	4.3 x107	Yeast was masked by bacteria
2% methanol	1.56 x 10^3	7.8 x107	Yeast was masked by bacteria
3% methanol	1.53 x 10^3	1.15 x107	Very few start to observe at 96h
4% methanol	1.50 x 10^3	6.7 x107	No contamination
5% methanol	1.76 x 10^3	5.6 x 10^5	No contamination

Interestingly in 1 to 4% v/v methanol supplemented experiments, the lipid concentration was noted to be approximately similar (between 4.16 to 4.9g/L). As mentioned above the low lipid content in 1 and 2%v/v methanol supplemented experiments resulted due to the contamination. Conversely, in 3 and 4% the cells accumulated more lipid compared to the control condition (i.e. in control 44.48%w/w and 49.39 and 45.95%w/w in 3 and 4%v/v methanol supplemented experiments, respectively The above-obtained results indicate that growth was inhibited by methanol, however, the lipid synthesis was not inhibited greatly by the addition of methanol to the medium. Considering the obtained results, the sterile fermentation is ideal for biomass and lipid production, however, the current experiments prove that the fermentation can be conducted without sterilization by supplementing methanol to control the contaminants. Methanol is a simple alcohol (C1), which is widely used in recombinant protein production. However, it is known as a growth inhibitor when the concentration exceeds above 3% v/v in the cultivation

medium (Woon-Yong et al., 2011). Many organisms are known to have high methanol tolerance and the ability to convert methanol into products (Jinyong Yan et al., 2014). In the present investigation, *Y.lipolytica* SKY7 was tested for its methanol tolerance in a crude glycerol supplemented medium. Due to the inhibitory effect of methanol on *Y.lipolytica* SKY7 cell growth, it is used to restrict the growth of contaminants. Interestingly, *Y.lipolytica* SKY7 tolerates methanol concentration up to the tested limit (5%v/v). From the results obtained from this study and the existing reports, it can be concluded that methanol can be used to control the contaminants in a pure culture based biomass and lipid production by *Y.lipolytica*.

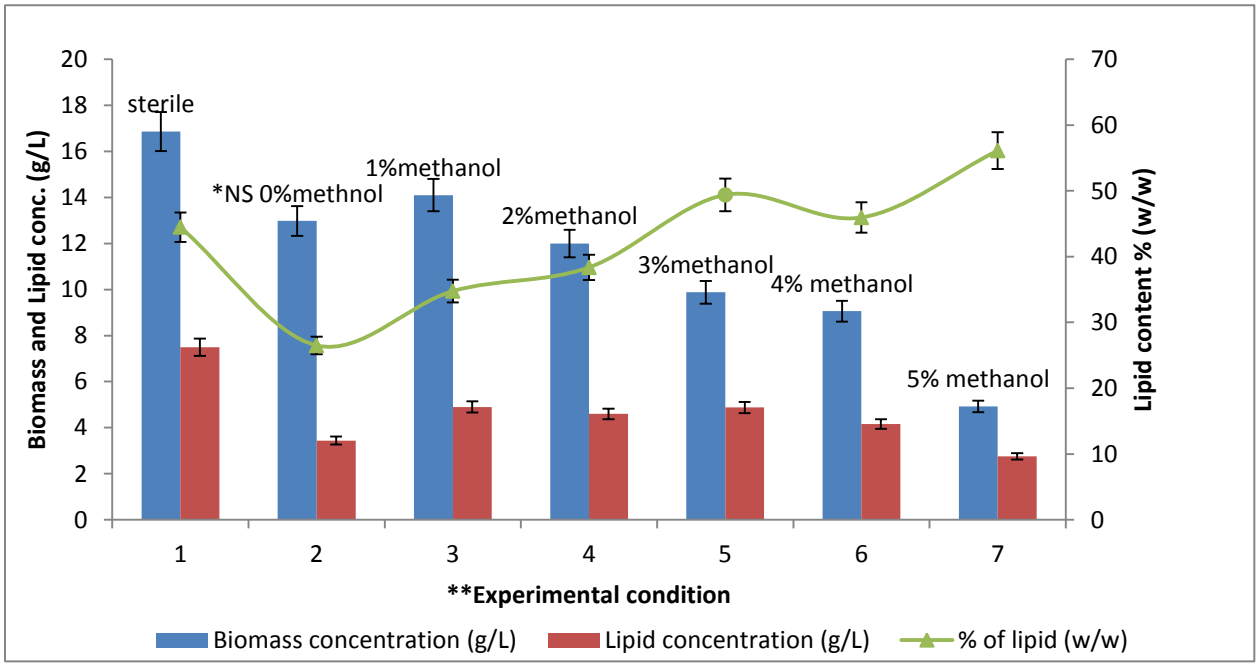

Figure 37 Variation of biomass concentration and lipid content of biomass at 72h of fermentation with different methanol concentration in the medium. *NS- nonsterile. **Fermentation conditions (1) sterile, (2), nonsterile 0% methanol added, (3) nonsterile 1% methanol, (4): 2% methanol, (5); 3% methanol, (6); 4% methanol, (7); 5% methanol

2.2. Non-sterile methanol supplemented fermentation on bench scale fermenters.

Based on the results obtained from flask studies the 3%v/v methanol supplemented experiment was conducted in the 15L fermenter and the results are presented in Figure 38. The CFU data shows that one log cycle increase in cell number (*Y.lipolytica*) at the end of fermentation (72h). From the early stage of fermentation, the cells were noted to grow slowly. Up to 18h of fermentation, there was very little change in the CFU. The slow increase in the cell number could be due to the addition of methanol and with the high soap concentration in the fermentation

media as explained in previously. However, after 18h of fermentation the CFU of *Y.lipolytica* started to increase slightly. This could be due to the acclimatization of the cells in new fermentation conditions. Meantime in the fermentation, the contaminants started to appear and overtook the growth of *Y.lipolytica*. The CFU data of contaminant shows a rapid growth from 18h onwards. The occurrence of contamination after 18h suggests that there was evaporation loss of methanol or adaptation of the contaminants towards methanol in the fermentation.

Due to the overgrowth of contaminants, the glycerol consumption increased after 24h (Figure 38). Approximately, 20g/L glycerol was consumed from 24h to 72h of fermentation. However, a significant increase in biomass or lipid content was not observed. A maximum biomass concentration was observed at 54h (6g/L) and lipid concentration was 2.16g/L, which is 36%w/w of the total cell mass (contaminants + *Y.lipolytica*). To ensure the probable reasons for the occurrence of contamination during the fermentation, the crude glycerol was diluted in sterile (autoclaved) distilled water and tap water separately. The diluted samples in sterile distilled water and tap water alone were plated on YPD agar plates. Interestingly, crude glycerol diluted with sterile tap water did not show contaminant growth. However, the non-sterile tap water and crude glycerol diluted with non-sterile tap water exhibited a slimy colony. From the above observations, it was concluded that the contamination appeared due to the use of non-sterile tap water for preparing the medium. The crude glycerol alone did not show any contaminants due to the presence of high glycerol and a considerable amount of methanol. This further confirms that the crude glycerol can be used without any sterilization if the tap water is treated properly prior to the fermentation.

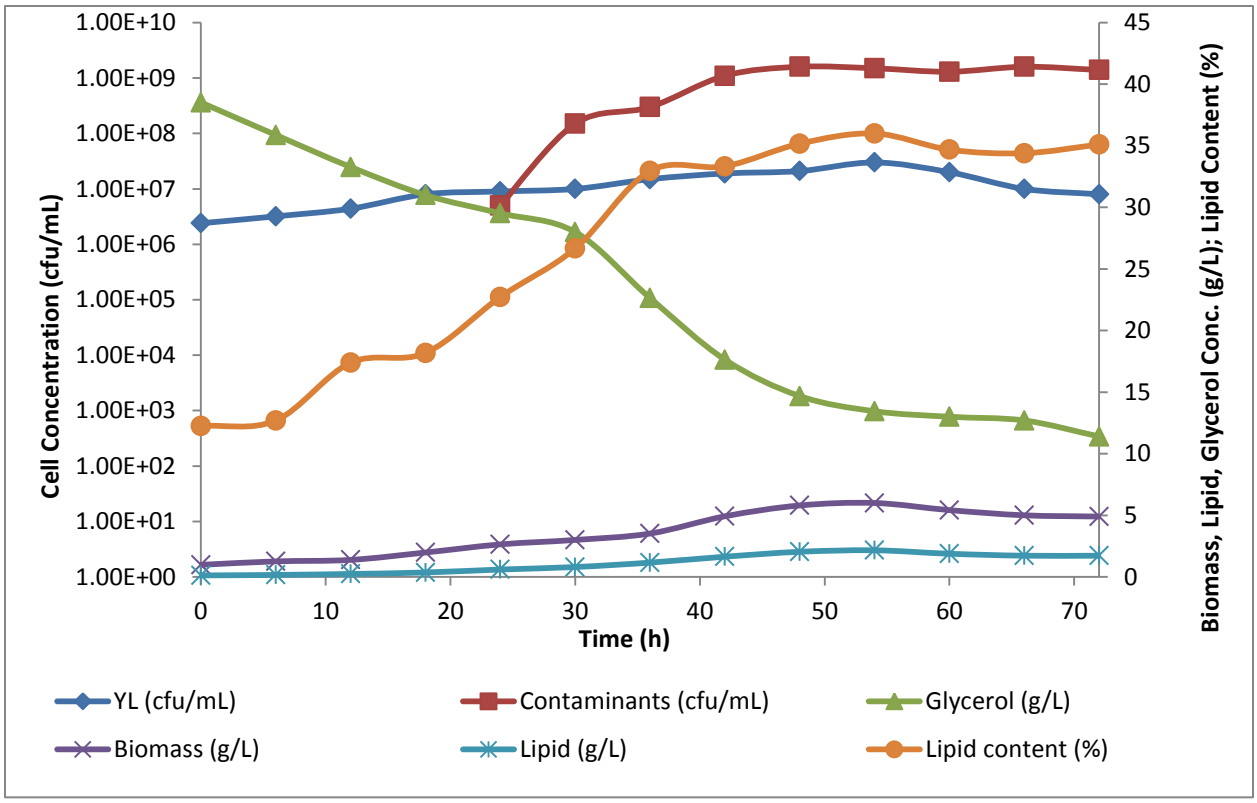

Figure 38 Time course analysis of biomass, lipid, glycerol and colony forming units of Y.lipolytica and the contaminants in 3% methanol supplemented non-sterile fermentation

2.3. UV treated tap water as diluent for media preparation for methanol based non-sterile fermentation.

The studies conducted in a 15L scale fermenter and as reported in the last section clearly established that the possibility of invading the fermentation process by contaminants was higher than flask level. It was also observed that the contamination can originate from tap water used to prepare the media. Considering this fact, a new strategy was developed to eliminate or suppress the contaminants growth for a prolonged period of time by UV (ultraviolet) treatment. The results are presented in Figure 39. A short lag period of 6h was evident from the fermentation profile (Figure 39). However, from 6h onwards the biomass production was accelerated. At the end of fermentation (72h), 17.55±1.7g/L biomass was produced, which is approximately 3 times higher than the non-UV treated tap water used for media preparation. Meanwhile, the CFU data shows that there is a 3 log cycle increase in *Y.lipolytica* cell count within 42h of fermentation. However, from 42h onwards, the CFU count of *Y.lipolytica* increased slightly (Table 35). This indicates the growth of *Y.lipolytica* was halted due to the aging of the cells. Meantime the

contaminants started to emerge in the fermentation at 42 h. A slimy colony was noticed in the fermentation, which grows comparatively slowly compared to the non-UV treated fermentation. This could be due to the low availability or exhaustion of micro and macro nutrients in the culture medium at 42h. At the end of fermentation, the contaminant count was noted as $4.4X10^5$ CFU/ml, which is 4 log cycle lower than the previous fermentation ($1.4X10^9$CFU/ml). Four times higher lipid concentration was observed in UV treatment (8.8±0.7g/L) than non-UV treatment (2.16g/L), which represents 49.3%w/w of lipid per gram of cell dry mass. The biomass and lipid production in the present study was 2.3 and 2 times, respectively compared to the other recent reports available on *Y.lipolytica* under nonsterile conditions (Taskin et al., 2015). Comparing the cultivation conditions in the present case the cultivation temperature was maintained optimum with methanol tolerant strain. However, Taskin and co-workers used a cold-adapted strain for the fermentation. Though both culture conditions have a negative impact on biomass synthesis, superior biomass synthesis was obtained in the present case of methanol tolerant strain. Meanwhile, the culture parameters such as pH, temperature, and dissolved oxygen are critical parameters in maintaining a monoculture. To date, the literature shows that the optimal temperature to achieve a high growth it is necessary to maintain the temperature at 28^oC and other factors in the optimal range (pH 5.5 to6.5) (Beopoulos et al., 2008; Papanikolaou & Aggelis, 2002). However, no reports were available to control the contaminants in lipid production in non-sterile condition using methanol as a control agent.

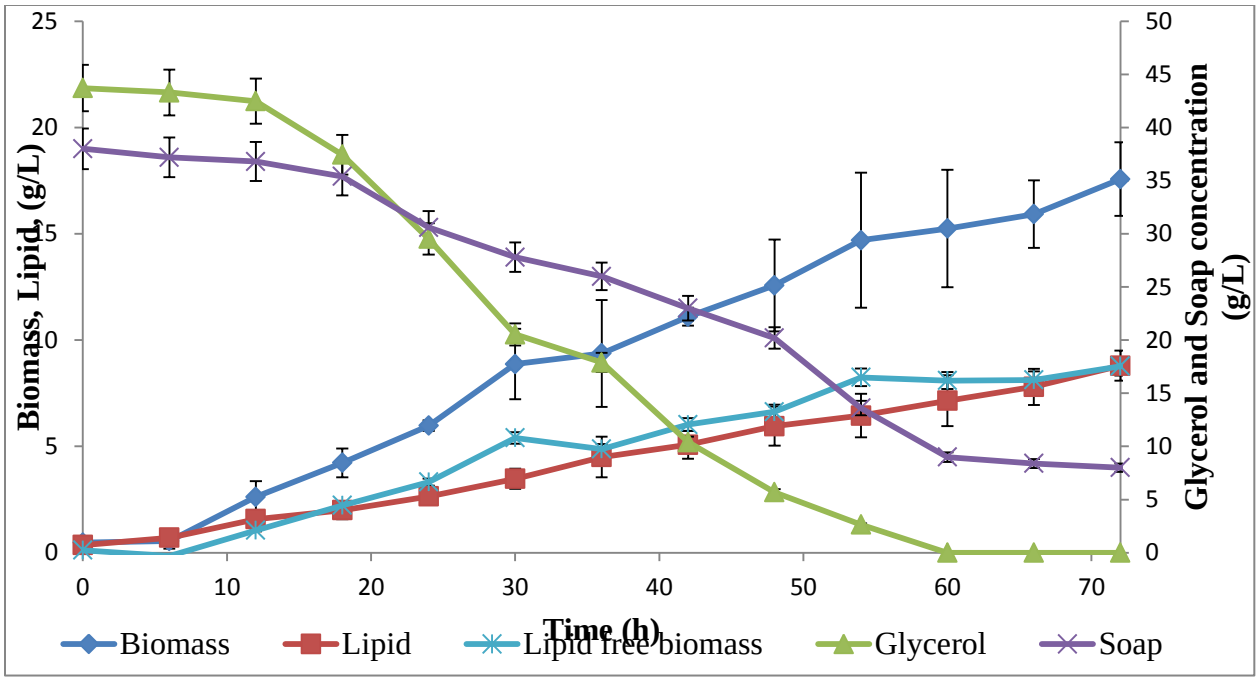

Figure 39 Time course of biomass, lipid, and glycerol and soap concentration during fermentation using UV treated water to dilute crude glycerol solution and supplemented with 3%v/v methanol to suppress the contaminants

Table 35 CFU data of non-sterile fermentation prepared with UV treated tap water

Sample (h)	**(CFU/mL)**	
	Y.lipolytica	Contaminants
0	1.50E+05	
6	1.89E+05	
12	2.20E+06	
18	3.60E+06	
24	9.40E+06	
30	1.32E+07	
36	3.89E+07	
42	2.26E+08	1.10E+04
48	2.91E+08	8.60E+04
54	2.93E+08	1.82E+05
60	3.80E+08	2.10E+05
66	5.84E+08	2.60E+05
72	5.82E+08	4.40E+05

2.4. Glycerol and soap assimilation and lipase production

Due to the high soap content of the crude glycerol stock solution (Table 33), the final concentration of soap in the fermentation media was 38g/L. In other words, to obtain 40g/L glycerol per liter of fermentation media 133g/L crude glycerol was required. Hence, 133g of crude glycerol can yield 36.5g/L soap (ie, 133 X0.274=36.5). As seen above, due to the low biomass production during the early stage of fermentation the substrate consumption was also low. The glycerol utilization was insignificant until 18h of fermentation (Figure 39). The soap started to deplete rapidly after 18h (until 18 h depleted only 2.4g/L) of fermentation. Glycerol depletion was rapid and higher than soap after 18h of fermentation (Figure 39), which indicate glycerol was the preferred substrate over soap when two are present in the fermentation medium. In the case of glycerol, it was completely consumed within 60h of fermentation and soap remained until the end of fermentation (residual soap 8g/L at 72h). The uptake of glycerol is mediated through passive transportation. The TAG synthesis from glycerol ultimately occurs through de novo synthesis (Beopoulos et al., 2009; Nicaud, 2012). However, the assimilation of soap and FFA requires an additional mechanism in terms of biocatalysts such as lipases (Nicaud, 2012; Papanikolaou & Aggelis, 2011). Lipase catalyzes the hydrolysis of carboxylate ester bond and releases free fatty acids (FFAs). The FFAs further transported into the cell by unknown transporters or by FFA-1 to the cytoplasm. Thus, transported FFAs are further converted into acetyl CoA by fatty acyl-CoA synthase (Papanikolaou & Aggelis, 2011).

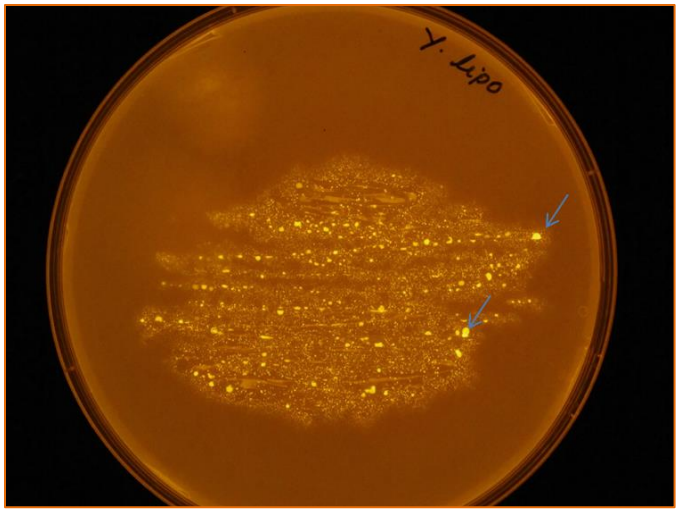

Figure 40 Lipase plate assay. The orange-yellow halos represent the extracellular lipase production by Y.lipolytica on 0.001%w/v Rhodamine supplemented selection plate

To confirm the assimilation of soap and FFA, the capacity of *Y.lipolytica SKY7* to produce extracellular lipase was determined by plating and culturing the pure strain on YPD agar supplemented with Rhodamine (0.001%w/v). A high degree of orange halo around the colonies observed under UV light confirmed the extracellular lipase production by the strain (Figure 40). Further, production of extracellular lipase activity by *Y.lipolytica* SKY7 during the fermentation process was examined (Figure 41). The results showed that a maximum lipase activity of 13.6U/ml was attained at 36h of fermentation. The enzyme activity was noticed to decrease after 36h of fermentation. The decrease in lipase activity after 36h can be correlated with the nutrient limitation and the production of proteases during the growth phase of *Y.lipolytica* (Dalmau et al., 2000). The protease produced could degrade the lipase enzyme decrease the lipase activity. The above results confirmed that the isolate *Y.lipolytica* SKY7 has high potential to assimilate simultaneously multiple carbon sources and produces multiple products in a single fermentation.

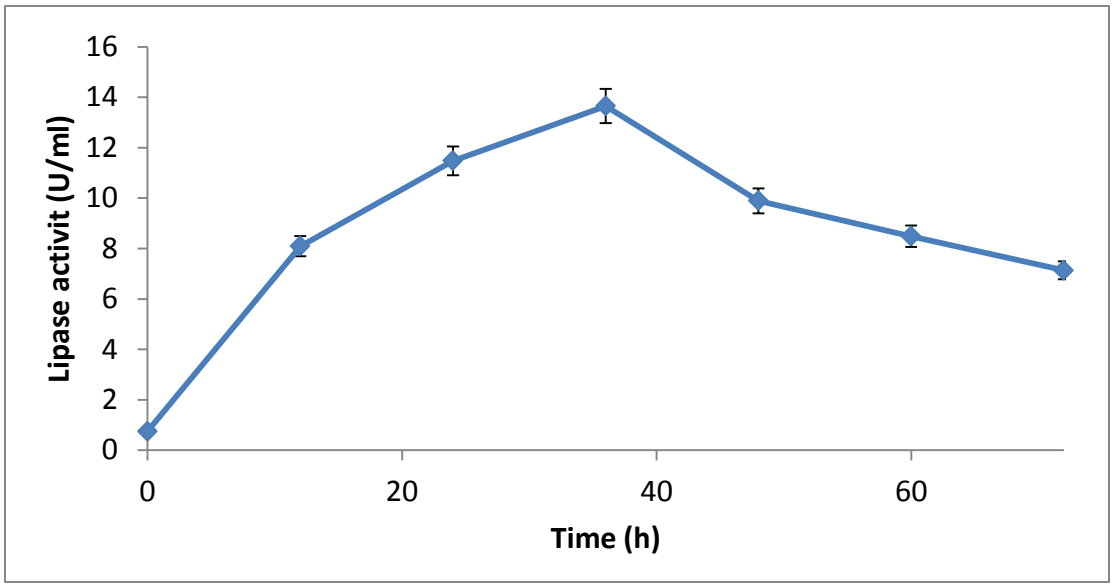

Figure 41 Variation of lipase activity in the medium with fermentation time under UV treated tap water added.

2.5. Yield of biomass and lipid production of UV treated tap water added fermentation (15L)

The yield of biomass and product is yet another factor to evaluate the process with respect to the conversion of substrate into product. An overall cumulative biomass yield (Biomass+ lipid) of 29.35g/ C mole was obtained at the end of fermentation. The overall yield profile (Figure 42) shows a high yield biomass conversion (121.14g/ C mole consumed) at 12h of fermentation. However, when the fermentation progresses the biomass yield declined significantly. From 18h onwards the yield of biomass remained approximately similar throughout the fermentation. On the other hand, the lipid-free biomass yield shows an increasing trend until 30h of fermentation and then it started to decline slowly. A maximum lipid free biomass yield of 52.68g/ C mole consumed was obtained at 12h of fermentation. From the yield of lipid-free biomass, it is evident that the substrate into biomass conversion was decelerated. As described above this could be due to two main reasons. Primarily the age of culture and secondly the availability of nutrients. The lipid yield shows a similar trend like biomass. At initial stage (12h) of fermentation, a high substrate to lipid conversion was noticed. This could be due to the initial lag, the cells might accumulate slight concentration of lipids or other probable reason could be an error in measurement due to low biomass concentrations. From 18h onward the Yp/s (yield of lipid) shows a very low increase. At the end of fermentation, an overall lipid yield of 14.4g/C mole was

achieved. Comparing with the sterile fermentation reported for the same strain approximately 58.0% decrease 1n lipid conversion was perceived (15.1g/L in sterile fermentation) (Kuttiraja et al., 2015). Concurrently the profile of Yp/x (yield of lipid per gram of biomass) showed an interesting trend. From 12h to 30h of fermentation the Yp/x showed a declining trend. This was due to the biomass synthesis during 12 to 30h of fermentation. Further, when the biomass synthesis halted the lipid accumulation accelerated. In other words at 30h, the Yp/s was 10.8g/C mole consumed, and in short time period, it increased to 12.7g/C mole consumed at 36h of fermentation. At 72h, the Yp/x reached 0.49g/g, which indicates that 49.3% of the cell mass constitutes the lipid.

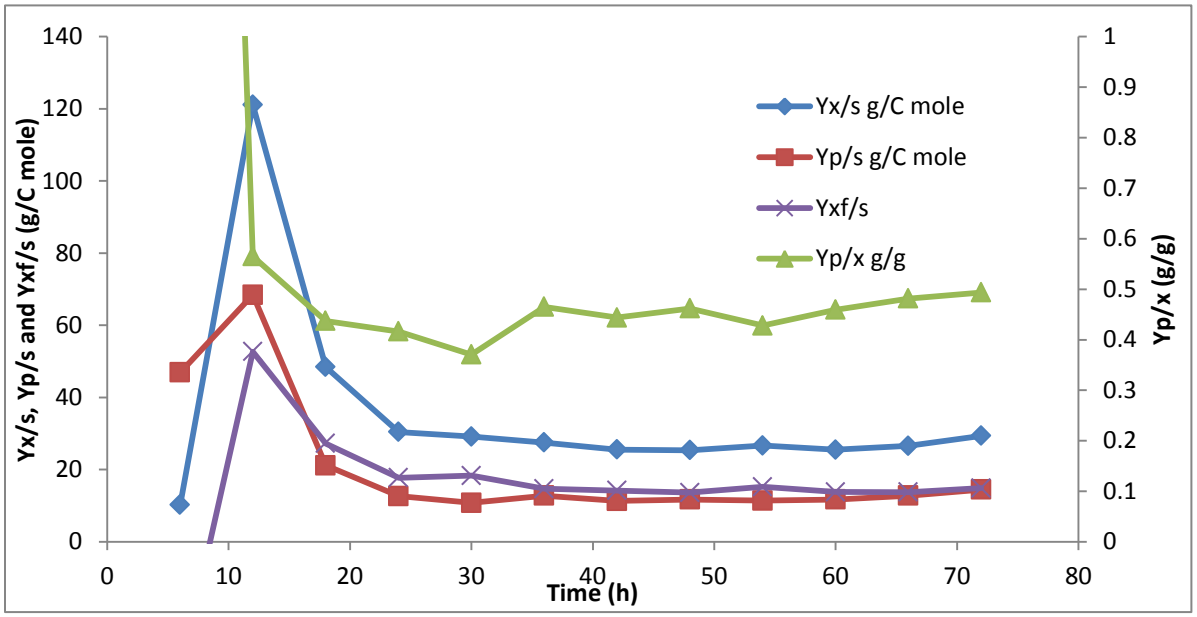

Figure 42 The yield of biomass, lipid free biomass (Yxf/s) and lipid per C moles consumed and yield of lipid per gram of biomass.

2.6. Growth and substrate consumption rates on nonsterile condition (UV treated tap water)

As seen from the above results the growth was comparatively low due to low inhibition of methanol. Due to this reason, the specific growth rate was also comparatively low when compared with the sterile condition. A maximum specific growth rate was attained at 18h ($0.073h^{-1}$) and decreased thereafter (Fig 43). The low specific growth rate in the presence of methanol (compared to other studies i.e. RSM optimized study the μ was $0.18h^{-1}$ at 12h) was obtained due to the inhibition of methanol on biomass growth in the early stage of fermentation.

From the literature, it is evident that a methanol concentration of 1%v/v was reported to improve the biomass concentration in microalgal cultivation. But it does not change either the lipid production or the composition of lipid. However, when the methanol concentration was increased, the algae cell growth was significantly reduced (Woon-Yong et al., 2011). Similar results were obtained for single cell protein production from methanol as a sole carbon source and the study indicated that a methanol concentration more than 3%w/w potentially toxic to the yeast cells. When growth was accelerated the substrate consumption also increased. It is clearly evident that at 6 to 18h of fermentation, the substrate consumption rate also accelerated (Figs 44). However, when the specific growth rate declined (i.e. after 18h, Fig 43&44), the substrate utilization rate also declined. From 30h to 54h of fermentation the substrate consumption rate decreased slowly. This could be due to low biomass production. However, the lipid production was notably stable during this period. Thus, the consumed substrate mainly converted into lipid and other by-products namely citric acid or CO_2. It has been well known that *Y.lipolytica* is a potential strain for citric acid production (Avila-Neto et al., 2014). Moreover, previous reports have were suggested that methanol can be used to induce citrate production (Shetty, 2015).

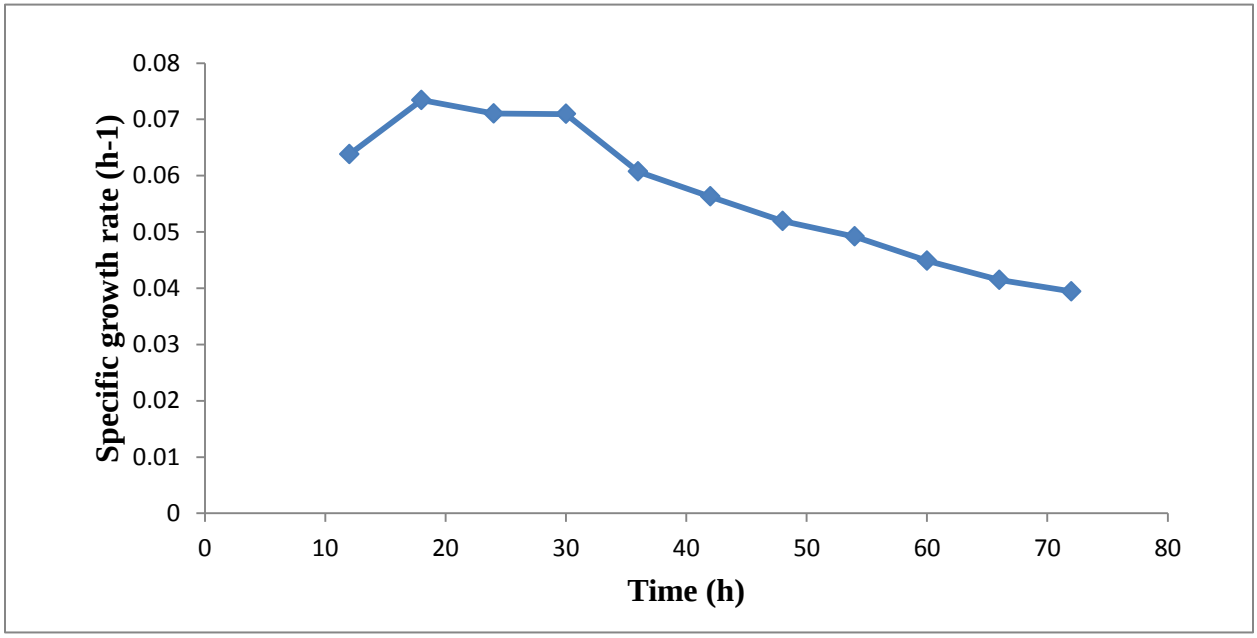

Figure 43 Specific growth rate of Y.lipolytica in the case of UV treated nonsterile fermentation supplemented with 3%v/v methanol.

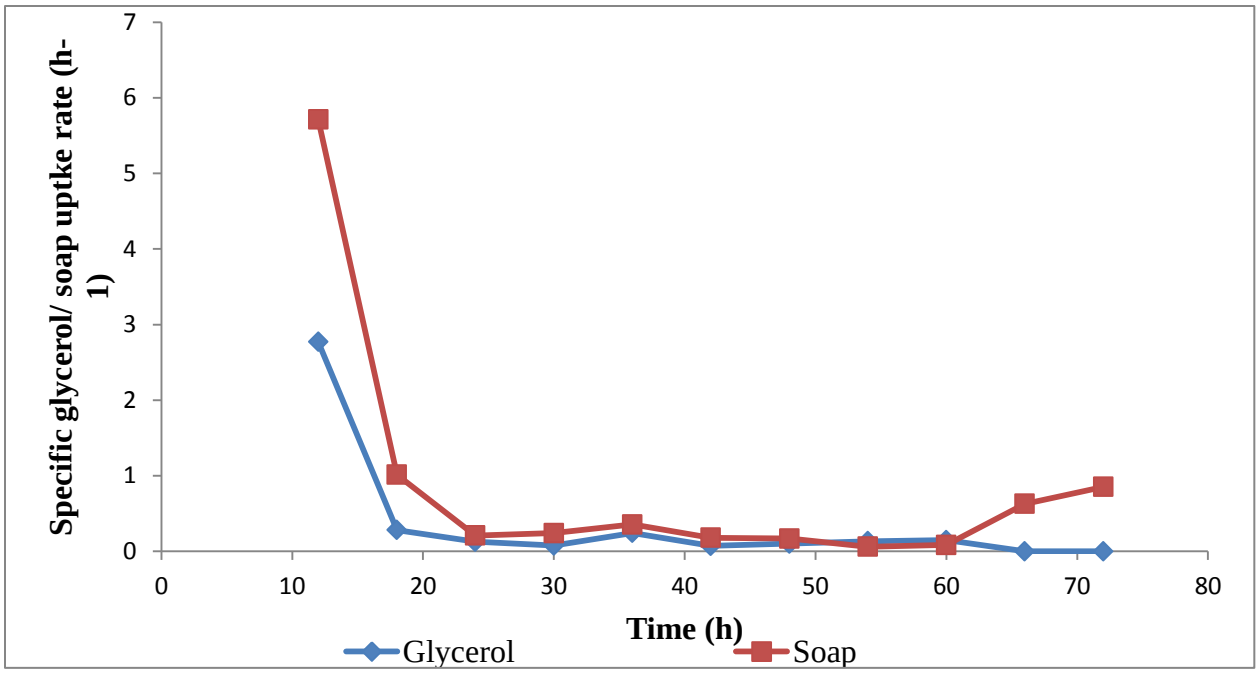

Figure 44 The specific substrate utilization rate of Y.lipolytica on UV treated nonsterile fermentation with 3%v/v methanol added medium.

3. Conclusion

The current study demonstrates the control of contaminants during monoculture for lipid production. Increasing the methanol concentration inhibits cell growth. However, *Y.lipolytica* SKY7 tolerates methanol concentration up to 5%v/v. Methanol concentration of 3%v/v shows effective control of contamination up to 96h in flask studies. The experimental results from the 15L reactor with 3%v/v methanol supplemented fermentation showed the possibility of contamination within the first 18h of fermentation due to the lack of assimilation of methanol and/or evaporation loss of methanol from the fermentation medium. However, when the UV treated tap water was used to dilute the crude glycerol solution the control over contaminants was prolonged up to 42h, which ultimately improved the growth of *Y.lipolytica* and lipid production. As a result, a 17.57±1.7g/L biomass and 8.8±0.7g/L lipid concentration were recorded at 72h of fermentation. The use of UV treated tap water increased three times biomass and 4 times lipid concentration compared to the use of tap water without UV treatment. It was concluded that use of methanol in the fermentation can control the unwanted contaminants for a prolonged period. Eventually, this new method of non-sterile fermentation will improve the

overall economy of microbial oil production by eliminating the high energy demanding steam sterilization process.

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References

Attia, A.M.A., Hassaneen, A.E. 2016. Influence of diesel fuel blended with biodiesel produced from waste cooking oil on diesel engine performance. *Fuel*, **167**, 316-328.

Avila-Neto, P.M., da Silva, G.P., Lima, C.J.B., De Paula, F.C., Contiero, J. 2014. Evaluation and optimization of growth and citric acid production by yarrowia lipolytica nrrl y-1095 using glycerol as carbon source as an alternative to use biodiesel byproduct. *Journal of Experimental Biology and Agricultural Sciences*, **2**(1), 25-31.

Barth, G., Künkel, W. 1979. Alcohol dehydrogenase (ADH) in yeast. II. NAD+-and NADP+-dependent alcohol dehydrogenases in Saccharomycopsis lipolytica. *Z. Allg. Mikrobiol*, **19**, 19:381–390.

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**(6), 692-6.

Beopoulos, A., Mrozova, Z., Thevenieau, F., Le Dall, M.T., Hapala, I., Papanikolaou, S., Chardot, T., Nicaud, J.M. 2008. Control of lipid accumulation in the yeast Yarrowia lipolytica. *Appl Environ Microbiol*, **74**(24), 7779-89.

Bondioli, P., Della, B., Laura. 2005. An alternative spectrophotometric method for the determination of free glycerol in biodiesel. *European Journal of Lipid Science and Technology*, **107**(3), 153-157.

Chatzifragkou, A., Papanikolaou, S. 2012. Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes. *Appl Microbiol Biotechnol*, **95**(1), 13-27.

Coelho, M.A.Z., Amaral, P.F.F., Belo, I. 2010. Yarrowia lipolytica: an industrial workhorse. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, **2**, 930-944.

Da Silva, G.P., Mack, M., Contiero, J. 2009. Glycerol: a promising and abundant carbon source for industrial microbiology. *Biotechnol Adv*, **27**(1), 30-9.

Dalmau, E., Montesinosa, J.L., Lottib, M., Casasa, C. 2000. Effect of different carbon sources on lipase production by Candida rugosa. *Enzyme and Microbial Technology*, **26**, 657–663.

Fei, Q., Chang, H.N., Shang, L., Choi, J.-d.-r. 2011. Exploring low-cost carbon sources for microbial lipids production by fed-batch cultivation of Cryptococcus albidus. *Biotechnology and Bioprocess Engineering*, **16**(3), 482-487.

Gerpen, J.V. 2005. Biodiesel processing and production. *Fuel Processing Technology*, **86**(10), 1097-1107.

Jinyong Yan, Xianliang Zheng, Du, L., Li, S. 2014. Integrated lipase production and in situ biodiesel synthesis in a recombinant Pichia pastoris yeast: an efficient dual biocatalytic system composed of cell free enzymes and whole cell catalysts. *Biotechnology for Biofuels*, **7**(1).

Krainer, F.W., Dietzsch, C., Hajek, T., Herwig, C., Spadiut, O., Glieder, A. 2012. Recombinant protein expression in Pichia pastoris strains with an engineered methanol utilization pathway. *Microbial Cell Factories*, **11**(22).

Kuttiraja, M., Krishna, S., Dhouha, A., Tyagi, R.D. 2015. A substrate-based approach for the selection of oil-bearing heterotrophs from nitrogen-deficient soil for lipid production. *Appl Biochem Biotechnol*, **175**(4), 1926-37.

Leung, D.Y.C., Wu, X., Leung, M.K.H. 2010. A review on biodiesel production using catalyzed transesterification. *Applied Energy*, **87**(4), 1083-1095.

Machado, J., F. R. S., , Michelon, M., Burkert, J.F.M., Dalcanton, F., Burkert, C.A.V., Furlong, E.B. 2015. Biomass production by Yarrowia lipolytica as a source of lipids: bench scale cultivation on raw glycerol-based medium. *International Food Research Journal* **22**(3), 1253-1260.

Mario Pagliaro, M.R. 2010. The Future of Glycerol : Edition 2.

Meesters, 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**.

Nicaud, J.M. 2012. Yarrowia lipolytica. *Yeast*, **29**(10), 409-18.

Papanikolaou, S., Aggelis, G. 2002. Lipid production by Yarrowia lipolytica growing on industrial glycerol in a single-stage continuous culture. *Bioresour Technol*, **82**(1), 43-9.

Papanikolaou, S., Aggelis, G. 2011. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. *European Journal of Lipid Science and Technology*, **113**(8), 1031-1051.

Petrache, H.I., Tristram-Nagle, S., Harries, D., Kucerka, N., Nagle, J.F. 2006. Swelling of phospholipids by monovalent salt. *Journal of Lipid Research*, **47**, 302-309.

Prim, N., Sánchez, M., Ruiz, C., Pastor, F.I.J., Diaz, P. 2003. Use of methylumbeliferyl-derivative substrates for lipase activity characterization. *J Mol Catal B Enzym*, **22**, 339–346.

Samul, D., Leja, K., Grajek, W. 2014. Impurities of crude glycerol and their effect on metabolite production. *Ann Microbiol*, **64**, 891-898.

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. *Biotechnology for Biofuels*, **7**(1), 1-11.

Shetty, V.G. 2015. Production and optimization of citric acid by aspergillus niger using molasses and corncob. *International Journal of Pharmacy and Pharmaceutical Sciences*, **7**(5), 152-157.

Tan, H.W., Abdul Aziz, A.R., Aroua, M.K. 2013. Glycerol production and its applications as a raw material: A review. *Renewable and Sustainable Energy Reviews*, **27**, 118-127.

Taskin, M., Saghafian, A., Aydogan, M.N., Arslan, N.P. 2015. Microbial lipid production by cold-adapted oleaginous yeastYarrowia lipolyticaB9 in non-sterile whey medium. *Biofuels, Bioproducts and Biorefining*, **9**(5), 595-605.

Tchakouteu, S.S., Kalantzi, O., Gardeli, C., Koutinas, A.A., Aggelis, G., Papanikolaou, S. 2015. Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency. *J Appl Microbiol*, **118**(4), 911-27.

Venkataramanan, K.P., Boatman, J.J., Kurniawan, Y., Taconi, K.A., Bothun, G.D., Scholz, C. 2012. Impact of impurities in biodiesel-derived crude glycerol on the fermentation by Clostridium pasteurianum ATCC 6013. *Appl Microbiol Biotechnol*, **93**(3), 1325-35.

Woon-Yong, C., Sung-Ho Oh, Yong-Chang Seo, Ga-Bin Kim, Do-Hyung Kang, Shin-Young Lee, Kyung-Hwan Jung, Jeong-Sub Cho, Ju-Hee Ahn, Geun-Pyo Choi, Lee, H.-Y. 2011. Effects of Methanol on Cell Growth and Lipid Production from Mixotrophic Cultivation of Chlorella sp. *Biotechnology and Bioprocess Engineering* **16**, 946-955.

Chapter-VII

Research Article-8

A biochemical approach to improving the biomass and lipid production using biotin and intermediate supply of ammonium sulfate

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RÉSUMÉ

Une approche biochimique basée sur la biotine et sur l'alimentation stratégique de l'ammonium afin d'améliorer la production de lipides dans *Y. lipolytica SKY7* a été testée dans un milieu de fermentation à base de glycérol. Dans des conditions de fermentation contrôlées et déficitaire en azote, une concentration de biomasse et de lipide de 26.57±0.02g/L and 10.4±0.39g/L ont été atteint respectivement avec 38.5% p/p de lipide de la masse total des cellules. Lorsque la biotine avec une alimentation intermédiaire d'ammonium a été ajoutée, la concentration de biomasse et de lipides s'est améliorée par rapport au control. En 72 heures et avec une supplémentation en biotine, et une alimentation stratégique en ammonium, une concentration de biomasse et de lipide de 27.77±0.07g/L et 12.71±0.06g/L a été atteinte. Cependant, lorsque le milieu de fermentation a été enrichie avec de la biotine seule la concentration de biomasse et de lipides a atteint 27,85 ± 0,35 g / l et 15,74 ± 0,15 g / l, respectivement. L'augmentation de la teneur en lipides suggère que la biotine pourrait être l'un des principaux éléments qui peuvent déclencher la synthèse des lipides, même après 48 heures de fermentation. Dans le cas du contrôle, le rendement de biomasse (Yx / s) était de 42.88g / C mol et le rendement lipidique (Yp / s) était de 16.97g / C mol, alors qu'avec la biotine et l'alimentation stratégique de l'ammonium, les facteurs de rendement se sont légèrement améliorés de Yx/s 44.27/C mole et Yp/s 20.53g/C mole. Toutefois, comparé aux deux autres conditions de fermentation où la biotine a été ajoutée (100µg/L), le Yx/s s'est amélioré en arrivant à 47.16g/C mole et le Yp/s à 28.01g/C mole. A l issu de cette étude, nous pouvons clairement conclure que l'addition de la biotine au milieu de fermentation peut améliorer de manière significative la production de lipides.

Mots-clés: huile de cellule unique, la biotine, l'alimentation stratégique, le glycérol brut, et *Y. lipolytica*

Abstract

A biochemical approach based on biotin and strategic feeding of ammonium to enhance the lipid production in *Y.lipolytica* SKY7 was tested in glycerol-based fermentation media. In nitrogen deficient control fermentation conditions a biomass and lipid concentration of 26.57±0.02g/L and 10.4±0.39g/L were achieved respectively with 38.5%w/w lipid of total cells mass. When biotin with intermittent ammonium feed was added the biomass and lipid concentration was slightly improved than the control. At 72h with supplementation of biotin and strategic ammonium feeding a biomass and lipid concentration of 27.77±0.07g/L and 12.71±0.06g/L was achieved. However, when the fermentation medium was fortified with biotin alone the biomass and lipid concentration reached 27.85±0.35g/L and 15.74±0.15g/L, respectively. The increase in the lipid content suggests that biotin could be one of the main elements, which can trigger the lipid synthesis even after 48h of fermentation. In the case of the control, the biomass yield (Yx/s) was 42.88g/C mole and lipid yield (Yp/s) was 16.97g/C mole, whereas with biotin and strategic ammonium feeding the yield factors slightly improved to Yx/s 44.27/C mole, Yp/s 20.53g/C mole. However, compared to the other two conditions in biotin (100µg/L) added fermentation the Yx/s was improved to 47.16g/C mole and Yp/s was 28.01g/C mole. From this study, it can be clearly concluded that the addition of biotin to the fermentation medium can significantly improve the lipid production.

Keywords: Single cell oil, Biotin, Strategic feeding, crude glycerol, and *Y.lipolytica*

Introduction

Single cell oil (SCO) production and its application as renewable energy, pharma, and food industry have become an important area of research in the recent decade. Microbes from the kingdom of bacteria, fungi (mold and yeast), and algae were widely explored for SCO production. Microbes which can accumulate more than 20% (CDW) TAG are known as oleaginous organisms [1]. However, few of the organisms gained more attention within the research community due to their high ratio of TAG production. These microbes include *Lipomysis starkey, Rhoditurella glutines, Rhodosporidium toruloides Yarrowia lipolytica, Mortirella issabellina, Mucour, Trichosporon fermentans, and Cunninghamella echinulate* [2-10]. It is expected that in near future SCO will become one of the most promising energy sources due to the energy crisis and clean energy acts throughout the globe. However, the SCO production has not yet been a practical reality due to the high cost of the feedstocks and other operational costs[11]. In the above context, the current biodiesel (energy) production is ultimately derived from food waste such as used cooking oil, animal fat, and to a certain extent algal oil [12]. However, the purity and availability of used oil are a negative factor in terms of the production of high-quality biodiesel.

Many studies have been conducted to find alternate low-cost renewable resources for biodiesel. Among them, lignocellulosic biomass, food waste, municipal waste, and agro-food industrial waste and the crude glycerol from biodiesel, soap, and ethanol industry were studied extensively [13-15]. Even though the substrate cost has been brought down considerably, the process to make them suitable for microbial fermentation (or pretreatment) is one of the major concerns. Alternative to the low-cost substrates, various modes cultivation (fed-batch, continuous and sequential batch) were studied by researchers to improve the biomass and lipid concentration in heterotrophic microbes. Mainly glucose and glycerol were used as a substrate in fed-batch or continuous mode of fermentation. In a fed-batch mode using glycerol as carbon source, a biomass concentration of 118g/L with 25%w/w lipids attained [16]. In the case of glucose as a substrate, a 106.5g/L biomass with 67.5%w/w lipid was attained at 134h of fermentation [17]. Though a high biomass and the lipid concentration was attained, the overall low conversion efficiency of the substrate to lipid and biomass , low productivity and long fermentation time may negatively affect the process.

Glycerol has been identified as one of the favorite platform chemicals for food, chemical, pharma industry [18]. Glycerol can be used as a carbon source for the green synthesis of industrially important chemical compounds through bioprocess. One such product is SCO produced through microbial fermentation. However, the conversion of glycerol into SCO needs a control in nutrient supply to modify biochemical events towards lipid accumulation. For this purpose, mainly the deficiency of nutrients such as nitrogen, phosphate, magnesium, etc. is employed by various researchers [19]. The limiting nitrogen supply in lipid synthesis is well-studied subject [19, 20]. In the case of nitrogen limitation the Krebs cycle enzyme isocitrate dehydrogenase (IDH) was deactivated due to the unavailability of NH_4^+ ions. In such conditions, the generated iso-citrate accumulates and that triggers the equilibration of isocitrate into citrate through aconitase. Once the citrate starts to accumulate inside the mitochondria the citrate malate transporters get activated and transports the citrate from mitochondria to the cytoplasm. In an oleaginous organism, the presence of ATP citrate lyase (ACL) converts the citrate into acetyl coA. This acetyl coA further used for TAG synthesis. Even though ACL plays a crucial role in converting the citrate into acetyl coA the first committed step in TAG synthesis begins when the acetyl CoA was converted into malonyl-CoA by AcetylCoA carboxylase (ACC) [1, 21]. However, the biochemical events in TAG biosynthesis were revealed, the mass production of SCO from heterotrophic microbes is still economically not feasible due to the low biomass and lipid productivity. Mainly, the nutrient limitation reduces the biomass production at the early stages of fermentation. At the same time, the intracellular enzymes such as ACL and ACC activity was reported to be declined with depletion of nitrogen concentration of the media [22]. There are many attempts that were made to improve the biochemical events responsible for TAG synthesis by genetic engineering. In some cases, the genetically modified organisms were able to accumulate over 80% of TAG [23]. However, biomass production and lipid productivity were comparatively low with conventional microbes.

At this scenario improving the lipid accumulation is not the only factor to be targeted. Biotin is one of the important growth factors, which is mainly involved in fatty acid biosynthesis. Biotin regulates the key enzyme ACC (Acetyl-coA carboxylase). The conversion of acetyl-CoA formed in the cytoplasm has been converted into Malonyl-CoA by ACC. In this biochemical event, biotin binds as a cofactor with carboxylase carrier protein (known as Biotin carboxylase carrier protein complex), which is further processed by biotin carboxylase and transfers the carboxyl

group to Malonyl-CoA. The above-mentioned step is the first committed key step in fatty acid biosynthesis. Hence, biotin plays the key role in regulating the ACC activity. Once the malonyl-CoA is formed by this pathway the final destination of formed Malonyl-CoA is a fatty acid or TAG formation. To date, very few studies have been reported on the influence of externally added biotin in lipid synthesis in yeast and higher fungi. A study conducted by Kaneda (1966) reported a significant increase in fatty acid concentration and the increase in the chain length of the fatty acid in *B. subtitles* strain. Considering all the biochemical facts and the need of improvement of the overall process, the current research is devoted to studying the effect of biotin and intermediate nitrogen supplementation to improve the biomass and lipid accumulation.

In general, biotin is one of the important cofactors for microbial growth [24]. However, very few reports were devoted to study the influence of the importance of biotin in TAG synthesis [25]. Meantime the nitrogen concentration in the fermentation media is a critical factor that could extend the lipid synthesis on lipogenic phase. Due to these reasons biotin supplementation in the media and an intermittent nitrogen supply was attempted to increase the biomass and lipid concentration in batch cultivation.

1 Materials and methods

1.1 Culture and medium of batch culture

In the present study *Y.lipolytica* SKY7 was used to produce biomass and lipid on crude glycerol-based fermentation medium. The culture was preserved on YPD agar slants (Yeast extract 10/L, Dextrose 20/L, peptone 5g/L and agar 20g/L). The pre-inoculum was prepared in YPD broth (Yeast extract10g/L, Dextrose 20g/L, and peptone 5g/L). The inoculum was prepared in YPG medium (Yeast extract 10g/L, Peptone 5g/L, and crude glycerol 20g/L). The glycerol stock used in the current study was noted to have high soap content. The original composition of crude glycerol is presented in Table 36. The fermentation media was fortified with 50g/L crude glycerol. The YL strain used in this research grows in the hydrophobic substrate without inhibition until final concentration reaches 5%w/w. The medium was supplemented with $(NH_4)_2SO_4$-1.25g/L, yeast extracts 1g/L, KH_2PO_4 2.7g/L, Na_2SO_4 1g/L, and $MgSO_4$ 0.5g/L. The flask containing media was autoclaved at 121^oC for 15minutes. The sterilized media was inoculated with 5% v/v *Y. lipolytica* SKY7 inoculum. The fermentation was carried out in an incubating shaker at 28^oC and 180 rpm for 72h. The initial pH was adjusted to 6.5 in all cases.

1.2 Impact of supplementation of biotin on biomass and lipid production

Four different concentrations (10, 25, 50 and 100µg/L) of biotin was tested in the current study. All experiments were conducted in 2L Erlenmeyer's flask with each working volume of 700mL. All fermentations were conducted in duplicates. The samples were collected at each 12h interval to determine the biomass, lipid, glycerol and soap concentrations. The media composition, inoculum preparation, and fermentation conditions were same as described in section 1.1.

1.3 Fermentation in 15L bench scale reactor

Based on the results obtained from the flask studies, fermentation was conducted in a 15L fermenter. The fermentation media contains 52.98g/L glycerol with 13.3g/L soap. The medium was supplemented with $(NH_4)_2SO_4$ 1.25g/L, yeast extracts 1g/L, KH_2PO_4 2.7g/L, Na_2SO_4 1g/L, and $MgSO_4$ 0.5g/L. The fermentation media was fortified with optimal biotin concentration (100µg/L). The final working volume was maintained at 10L. The samples were collected at every 6h interval and stored at 4^0C until it was used for further analysis. The fermentation working volume was 10L (Biogenie, Quebec, Canada) (9.5L media+0.5L inoculum). The fermenter was equipped with programmable logic control (PLC) system, which included a devolved oxygen (DO) probe, antifoam, mixing, aeration, temperature(28^0C). the fermentation pH was maintained at 6.5. The above parameters were maintained using the program ifix 3.5, Intellution, USA. The pH meter (Mettler Toledo, USA) was calibrated using buffers pH 4 and 7.0 (VWR, Canada). The DO probe was calibrated to zero using sodium sulfate and to 100% by purging air in distilled water. A well grown (18h) pure inoculum (5% v/v) was transferred aseptically to the fermentation media. Samples were collected at each 6h intervals to analyzed the biomass, lipid, and residual glycerol content.

1.4 Biotin and intermittent pulse $(NH_4)_2SO_4$ supplementation

Based on our previous reactor studies the depletion of nitrogen in the fermentation media was assessed. It showed a rapid depletion in nitrogen concentration in the fermentation media from 6 to 18h of fermentation and a concomitant decrease in cell growth during the same time. Based on this observation, $(NH_4)_2SO_4$ was supplemented at three different fermentation time of 12, 18 and 24h with 100, 50 and 25mM/L concentration, respectively. The fermentation media contains 53.69g/L glycerol with 11.6g/L soap. The medium was supplemented with $(NH_4)_2SO_4$ 1.25g/L, yeast extracts 1g/L, KH_2PO_4 2.7g/L, Na_2SO_4 1g/L, and $MgSO_4$ 0.5g/L. The fermentation was

conducted up to 72h. Samples were collected at 6h intervals and kept at 4^0C until it was used for further analysis.

1.5 Analytical methods

1.5.1 Lipid extraction and measurement

Fermented broth (25 mL) was used for biomass and lipid quantification. The collected samples were centrifuged at 4000rpm for 10 minutes and the supernatant was collected in a clean tube. The biomass was washed twice with distilled water and the cell pellets were collected by centrifugation. Each sample (the washed pellet) for biomass quantification was transferred to a clean pre-weighed aluminum pan and it was kept at 105^0C until it reached a constant weight. A chloroform and methanol based conventional method of lipid extraction was employed for lipid quantification [26]. Wet sample was mixed with 15mL of 2:1 chloroform: methanol mixture and zirconium beads (0.7mm) were added to the mixture and the cells were disrupted by bead beater for 3 minutes (BioSpec Products, Bartlesville, OK, USA). The samples after bead beating were filtered through Whatman filter paper with a pore size of 0.45µm. The residual solids, thus obtained were re-suspended in 1:1 chloroform-methanol mixture and the procedure was repeated. The resulting filtrates were pooled in a pre-weighed glass tube and the solvent was evaporated under low nitrogen pressure. The residual samples were further dried at 60^0C in a hot air oven until the sample reached a constant weight. The weight, thus recorded represented the amount of lipids in that sample.

1.5.2 Glycerol measurement:

Glycerol in the centrifuged supernatant was estimated based on the chemical method. Pure glycerol (Sigma-Aldrich, Canada) was used as the calibration standard [27]. The soap and FFA was extracted by petroleum ether as described by Hu et al [28]. Citric acid was estimated by the chemical method [29].

Lipase activity was measured spectrophotometrically by the method described by Prim et al [30]. Lipase activity was measured as the ability to hydrolyze the para-nitrophenyl palmitate (pNPP) by the enzyme present in the fermentation broth. 300µL of fermentation broth was added to 2.7mL of 1mM pNPP solution prepared in 50mM Tris buffer containing 50mM NaCl, 0.4% (w/v) Triton X-100 and 0.1% (w/v) Gum arabica. The pH was maintained at 8 and the reaction

was carried out at 37^0C for 15-minutes. The absorbance readings were recorded at 405nm in a spectrophotometer. Standards were prepared by diluting appropriate concentrations of para-nitrophenol (pNP). One unit of lipase activity was defined as the amount of enzyme required to hydrolyze and release 1µM of pNP per milliliter per minute.

The specific growth rate was calculated according to the following equation

$$\mu = (\frac{1}{x})\frac{dx}{dt} \quad (1)$$

Where µ is the specific growth rate, x is the concentration of biomass (g/L) and t is the fermentation time.

The yield of biomass with respect to the substrate was calculated as gram biomass produced per mole of carbon (mole-C) consumed during the fermentation process. The mole of C consumed was calculated from the amount of glycerol and soap consumed (i.e. soap contains 16 C atom per mole with molecular weight 278, whereas glycerol contains three carbon atoms per mole with molecular weight 92.09. The total moles of carbon consumed were obtained by adding mole C consumed for soap and glycerol.

The yield of biomass and lipid were calculated based on the carbon moles used by the organism since glycerol and soap were present in the medium. The calculations were made according to the following equation.

$$Yx/s = \frac{g\ of\ biomass\ produced}{Total\ moles\ of\ carbon\ Consumed} = \frac{g\ of\ biomass\ produced}{C\ moles\ consumed\ from\ glycerol + C\ moles\ consumed\ from\ Soap} \quad (2)$$

$$Yp/s = \frac{g\ of\ lipid\ \ produced}{Total\ moles\ of\ carbon\ Consumed} = \frac{g\ of\ lipid\ produced}{C\ moles\ consumed\ from\ glycerol + C\ moles\ consumed\ from\ Soap} \quad (3)$$

2 Results and discussion

2.1 Evaluation of biotin concentration on biomass and lipid production

The variation of biomass concentration with fermentation time at various initial biotin concentrations in the medium is presented in Figure 45. The fermentation conducted without

biotin supplementation served as the control. In the control experiment, the biomass concentration reached maximum (14.3 g/L) at 36h fermentation and after that, there was no significant increase. At 10 µg/L biotin concentration in the fermentation media, the biomass concentration increased slowly but attained almost similar value (14.5g/L) to that of the control. However, at 50µg/L biotin concentration the final biomass concentration increased (maximum biomass 16.25 g/L at 72h). In the case of 100µg/L biotin concentration, the biomass concentration (18.55 at 60h and 18.65 g/L at 72h) was notably higher than the other conditions.

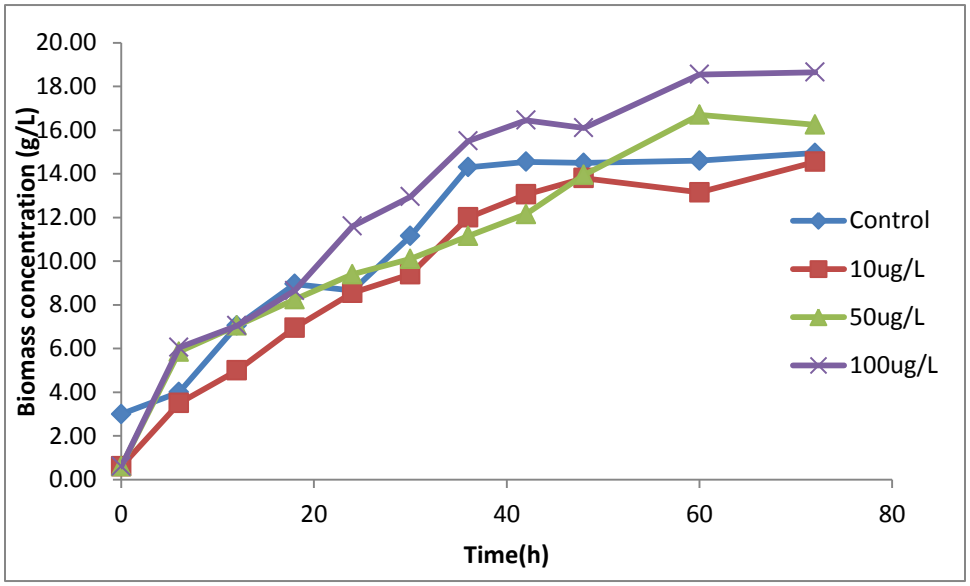

Figure 45 Comparison of biomass concentration at different biotin concentration

An increase in lipid concentration was noticed at the end of fermentation in biotin supplemented experiments compared to the control (Figure 46). At 10µg/L biotin in the medium, the increase in lipid concentration was insignificant compared to the control (only 0.5g/L increase in lipid). However, at 50 and 100 µg/L biotin in the medium, the increase of lipid concentration was significantly higher at 7.35 and 9.5g/L, respectively (Figure 46). These results confirm that the addition of biotin 50 µg/L or higher could improve the lipid production by the strain used (SKY7).

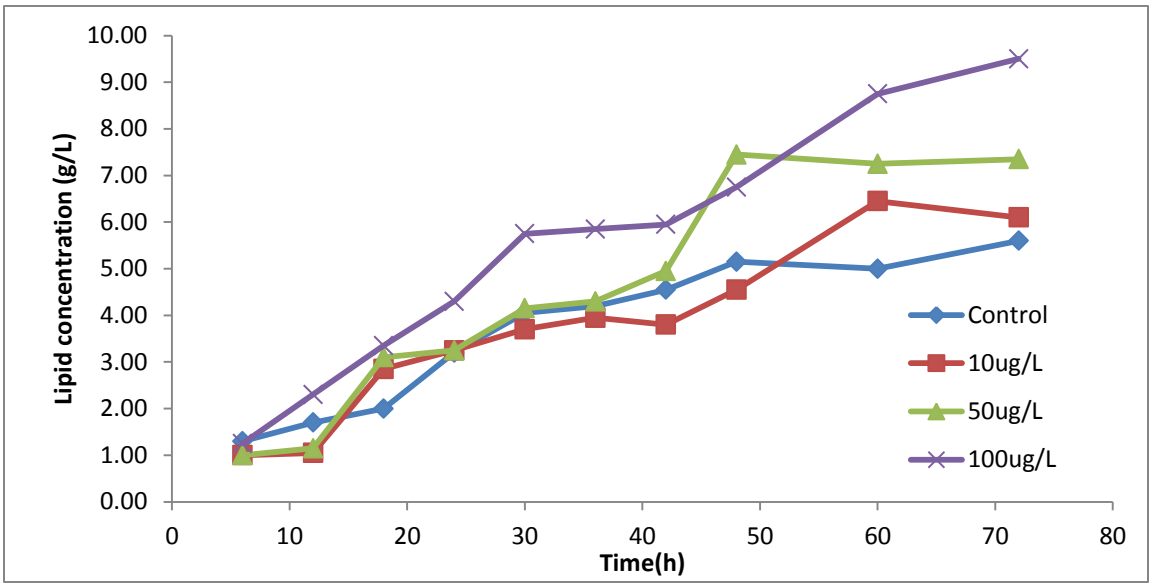

Figure 46 Effect of biotin on lipid concentration

An increasing trend in glycerol consumption was observed with increased biotin concentration in the medium (glycerol consumption was 22.9, 28.7, 31.4 and 30.2 at 0, 10, 50 and 100 µg /L biotin, respectively) (Figure 47). When the biomass concentration was compared with respect to glycerol consumption, a similar biomass increment was obtained. However, a higher lipid concentration was observed compared to the control experiments in biotin supplemented studies (Fig 46). This explains the glycerol consumed was most efficiently converted into lipid in the case of biotin supplemented studies than non-biotin supplemented studies.The increased biomass and lipid concentration and relatively higher consumption of glycerol and in biotin-supplemented experiments suggest that biotin could be one of the important trace elements required for TAG synthesis. [25].

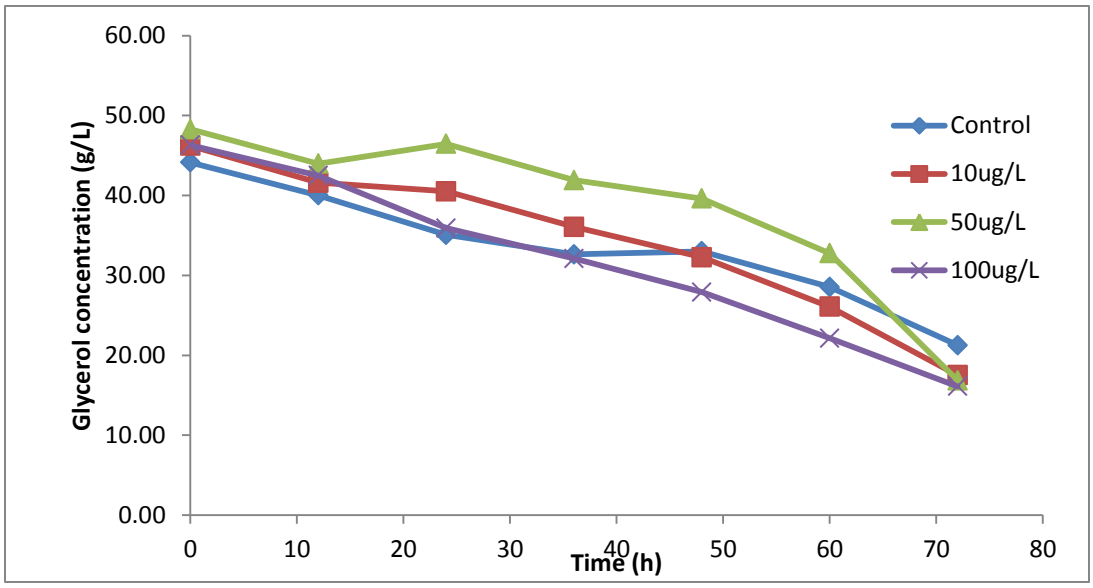

Figure 47 Time course analysis of glycerol concentration on control and biotin spplimented studies

2.2 Comparison of biomass in biotin and diammonium sulfate supplemented experiments with control at 15L scale

Based on the flask studies 100µg/L biotin supplementation was chosen to test in 15L bench scale fermenter. In order to compare the results a control experiment without biotin supplementation was also conducted. A very slight difference in biomass concentration was obtained in the three experiments (1; Control, 2; Biotin supplementation 100µg/L, 3; Biotin and pulse feed of ammonium sulfate) shows there was no impact on biomass accumulation in biotin and additional ammonium sulfate addition during fermentation (Figure 48). The highest biomass concentration of 27.85±0.35 and 27.77±0.07 g/L was found with the addition of biotin and biotin along with pulse feed of (NH4)2SO4 at 72h. The control biomass concentration was 26.57±0.02g/L at 72h. The biomass concentration started slightly decreasing in the control experiment after 60h of fermentation. In order to calculate the lipid-free biomass, the lipid concentration was subtracted from the total biomass and is presented in Figure 49. The lipid-free biomass concentration was comparatively lower at 6 to 24h in pulse feed of (NH4)2SO4 along with biotin experiments than the control and biotin alone supplemented experiments. As the fermentation progressed, the biomass increased, reached a maximum and then decreased. A sharp decline in lipid-free biomass concentration was noticed in the case of only biotin supplementation (Figure 49). The decline in lipid-free biomass was slightly slower in biotin along with pulse feed (NH4)2SO4 supplementation case, whereas the depletion of lipid-free biomass in the control was not

significant. Further, increase in lipid-free biomass occurred mainly from 6 to 24 h of fermentation (Figure 49). The increase in biomass concentration in this period was slightly varied for all three conditions (17.56 to 20.12g/L). From these observations, it can be concluded that the addition of biotin or biotin along with (NH4)2SO4 may improve the biomass concentration.

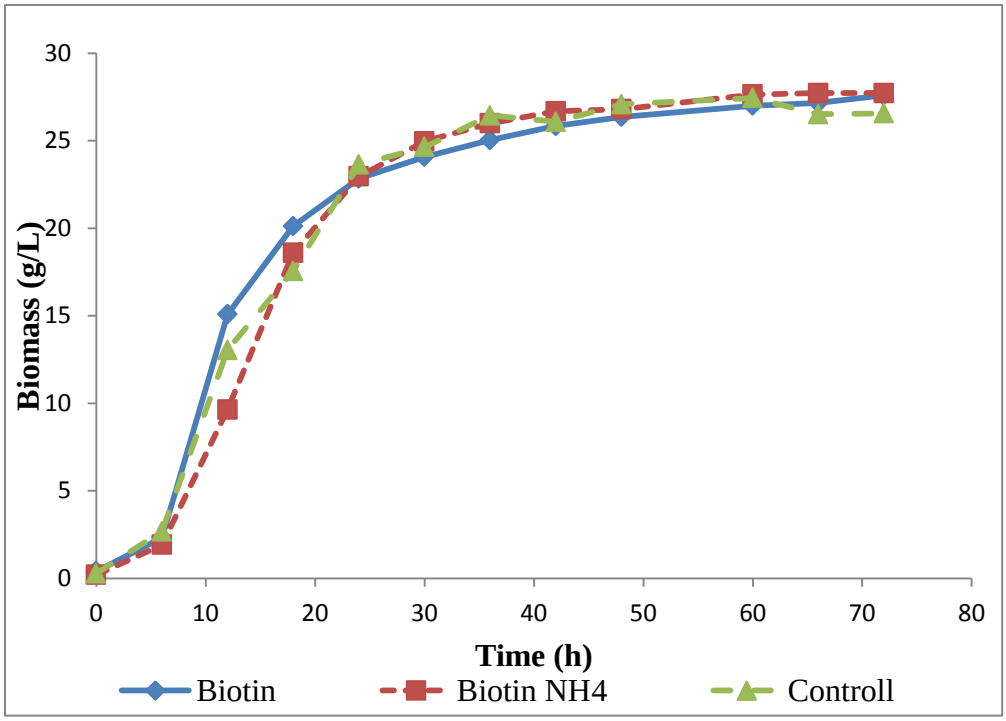

Figure 48 A evolution of biomass in biotin, biotin with intermediate supplementation of diamminium salt and control studies

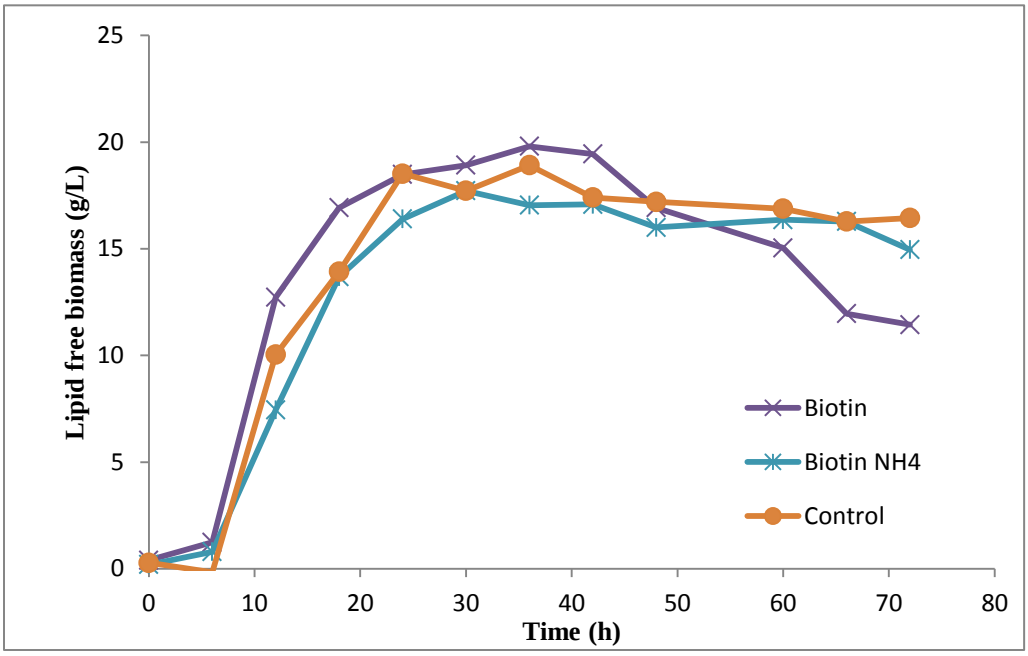

Figure 49 Comparison of lipid-free biomass in biotin with intermediate supplementation of diammonium salt and control studies

2.3 The impact of biotin and $(NH_4)_2SO_4$ fortification on lipid accumulation at 15L scale

A comparative lipid production in 15L fermenter under controlled conditions is presented in Figure 50. In all cases, the lipid production started from the early stage of the fermentation, which can be explained due to the presence of soap and FFA (hydrophobic substrate). In the present study, the fermentation media contains 11 to 13g/L soap as a second carbon source along with glycerol (Figure 51). It has been reported that cultivation of *Y.lipolytica* on hydrophobic substrates accumulates lipid in cells along with the cellular growth [31]. However, in other cases (i.e. hydrophilic substrates), the lipid synthesis happens through de novo synthesis where the lipid accumulation is reported to occur after the depletion of main nutrients (usually nitrogen) in the fermentation medium [1]. Higher lipid accumulation of 15.73±0.01g/L at 72h of fermentation with 57.3%w/w lipid of total cell dry mass was perceived in the biotin supplemented experiment. In the case of biotin fortification along with pulse feed of $(NH_4)_2SO_4$ experiment, lipid concentration of 12.71±0.06g/L at 72h with 46.5%w/w lipid of the total dry cell mass was recorded. The lower lipid concentration 10.4±0.39g/L lipid was obtained in the control experiment, which represented 38.5%w/w lipid of the total cell dry mass. In the case of biotin supplemented experiments, the lipid accumulation was significantly increased from 36h of fermentation (an increase in 11g/L lipid concentration), whereas in the case of biotin with pulse

feed of $(NH_4)_2SO_4$, the lipid accumulation occurred mainly from 6 to 48h of fermentation and thereafter the lipid synthesis decreased. The cessation of lipid production at 48h in the control experiment could be due to a low availability of nutrients (nitrogen and biotin) led to the reduction of intracellular metabolism towards lipid biosynthesis.

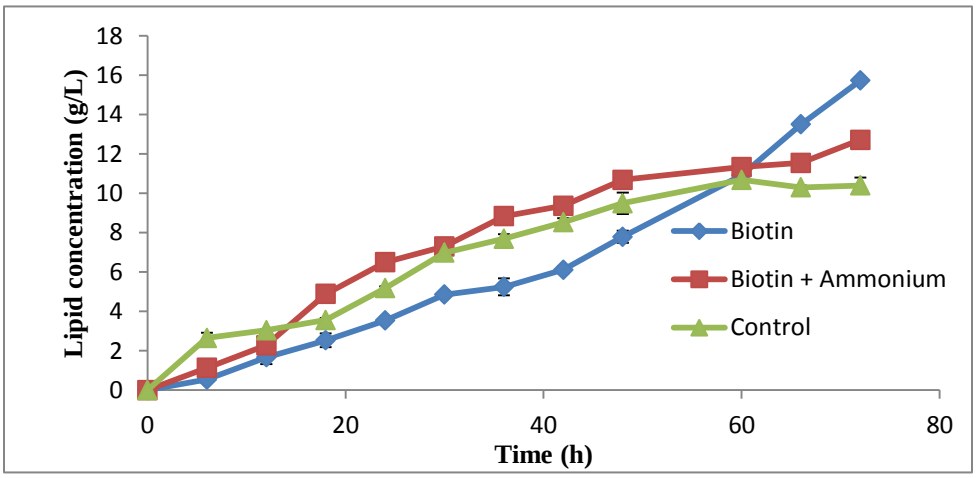

Figure 50 Effect of biotin on lipid production at reactor level

The results of soluble nitrogen concentration in the medium suggest that the assimilable nitrogen was available until 24h of fermentation because after that the nitrogen concentration in the medium remained approximately unchanged until the end of fermentation (Figure 52A). In the case of biotin supplemented experiments, the soluble nitrogen decreased until 12h and after that stayed unchanged (Fig 52A). However, the glycerol and soap were assimilated by the organism even after 24h (Figure 51A & B) and was converted to lipid and other metabolites (not presented). The decline in the lipid concentration towards the end of fermentation could be due to the low glycerol and soap concentration in the control experiment (soap 0.7g/L and glycerol 1.02g/L at 72h). Meantime, approximately similar soap and glycerol concentrations were found in biotin and nitrogen puls feed experiments. However, the lipid concentration was noticed to increase in two cases but not in the control. The increase in the lipid concentration in biotin and puls feed cases can be explained from the citric acid concentrations. The back utilization of citric acid in the case of control was notably lower than the biotin and nitrogen added fermentations. Due to the back utilization of citric acid, the lipid concentration in the biotin supplemented studies was considerably increased (Figure 53). When the carbon concentration goes below the critical concentration, the microbes tend to utilize the reserve materials for energy. In the control,

the decline in lipid concentration after 60h could be due to utilization of stored lipid by the yeast cells. However, in the case of biotin along with pulse feed of $(NH_4)_2SO_4$ the addition of biotin and nitrogen to the fermentation media anticipated to change the intracellular metabolism as reported by Shuib et al.,[32] compared control experiment. One of the examples of change in the metabolism is the consumption of the product (citric acid) as a carbon source (Figure 53). This can be further explained by the lipid concentration obtained. When the additional nitrogen was supplied, the lipid concentration stayed highest until 48h and the final lipid concentration (at 72h) was noticed to be slightly higher than the control experiments but comparatively lower than biotin supplemented experiments. The increasing trend of lipid accumulation, which is evident from the lipid concentration (Figure 50) suggests that the late fermentation stage (66h) favors lipid production. It explains that intermediate feeding of ammonium alone may not improve the lipid accumulation. Biotin along with ammonium and other metal ions and carbon may improve the lipid production[32].

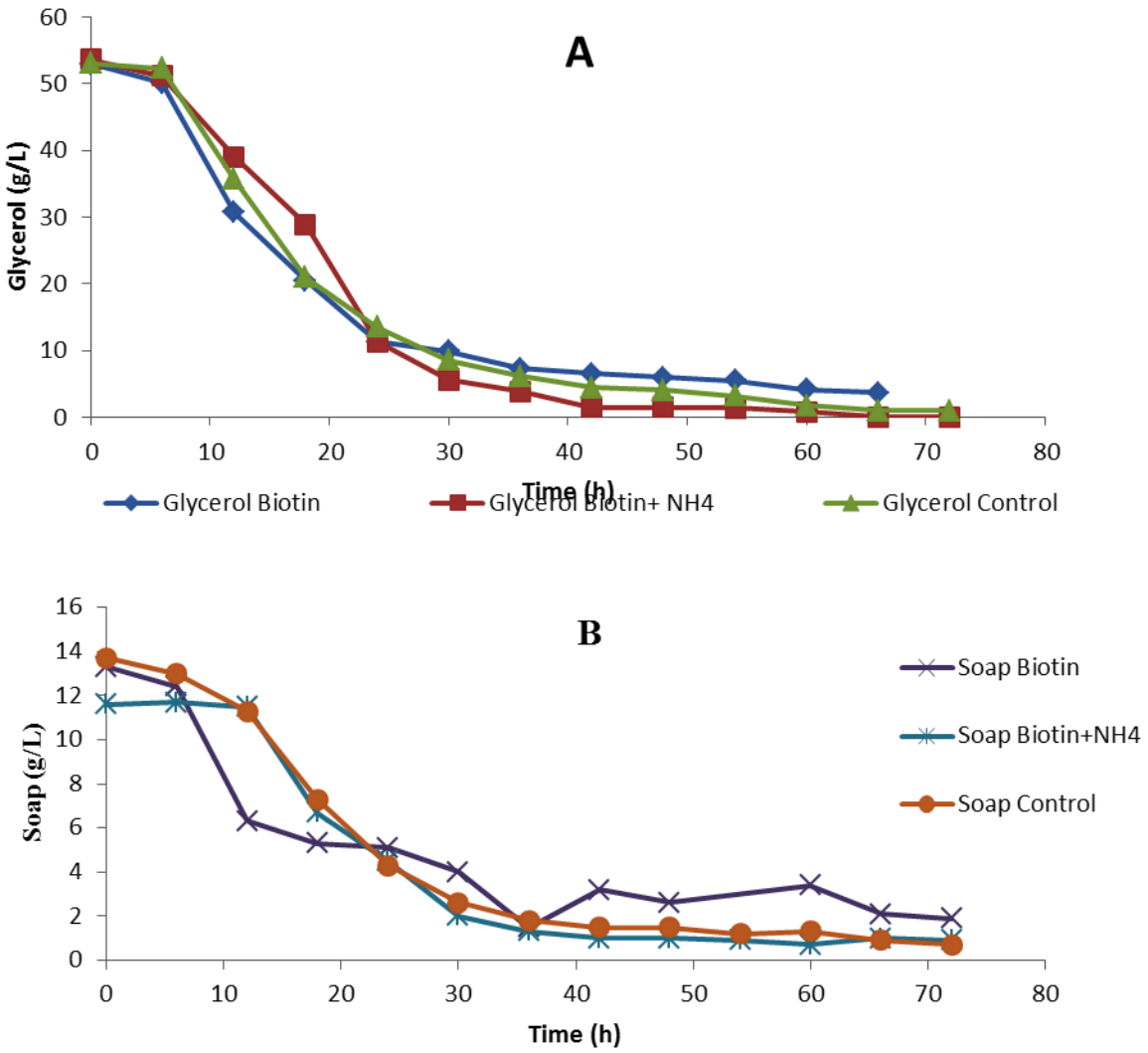

Figure 51 Time course analysis of Glycerol (A) and soap (B)

In the case of biotin alone supplemented studies, the nitrogen concentration decreased rapidly than the other two conditions. The nitrogen concentration reached a stable concentration at 24h in the control and biotin along with $(NH_4)_2SO_4$ supplied studies. However, in the case of biotin alone supplemented experiments the nitrogen concentration reached an approximate stable concentration at 12h. The low nitrogen concentration in the fermentation media and the addition of biotin may lead to a higher lipid production at a late stage (36h and after) of fermentation. This fact is further strengthened from the increase in lipid concentration from 66h onward in pulse nitrogen and biotin supplied studies, whereas lipid concentration declined in the control

experiment. Moreover, the earlier studies [25] suggest that the biochemical events contributed by biotin in lipid synthesis also confirms its importance in lipid synthesis.

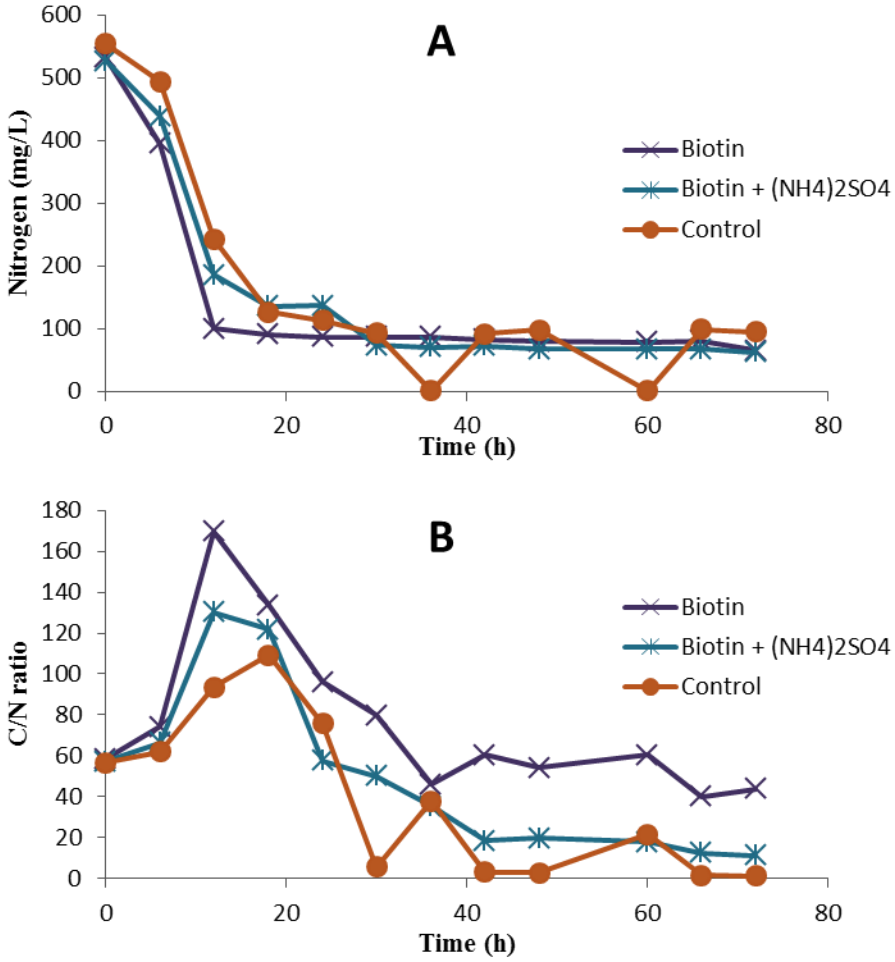

Figure 52 Time course analysis of nitrogen content in fermentation broth and (B) the evolution of C/N ratio during fermentation

2.4 Citric acid production in different fermentation conditions

The maximum citric acid concentration of 8.04g/L was obtained at 60h of fermentation in the control experiment (Figure 53), whereas 3.88g/L citric acid was obtained at 42h in the case of biotin added with $(NH_4)_2SO_4$ experiments. In the case of biotin alone supplemented studies a maximum citric acid concentration of 3.72g/L was obtained at 60h of fermentation. In the case of biotin added with $(NH_4)_2SO_4$ study, the citric acid concentration was noticed to decrease from 42h onwards. A decrease in citric acid concentration in the control and biotin supplemented experiments was observed from 60h of fermentation. The high citric acid in the control run and low in the other cases suggest that most of the assimilated carbon in biotin suppled experiments

was converted to lipid and biomass. The high citric acid concentration in the control experiments can occur due to the shift in unfavorable C/N ratio during fermentation (Figure 51B). The C/N ratio calculated and presented in figure51B supports the fact that in the control a low C/N ratio is suspected to be the cause of low lipid production and high citric acid production. The higher C/N ratio is evident in biotin supplemented study. (Fig 52B). Intermediate nitrogen addition also resulted in a lower C/N ratio than biotin alone. When nitrogen is added intermittently as pulse, the extra carbon is also required to be added to the media. The other possible metabolic reason for higher lipid production could be the supply of biotin that may lead to an increase in the ACC activity, which might have increased the conversion efficiency of acetyl-CoA into malonyl-CoA. It can be hypothesized that when the acetyl-CoA is converted into malonyl-CoA in an efficient way, the accumulation of citrate in the cytoplasm will be regulated by an optimal concentration. In other words, the enzyme ACL may not be inhibited by high citrate concentration. Thus, the produced citrate may convert into lipids efficiently. However, the low activity and feedback inhibition of ACL may lead to the accumulation of citrate in the cytoplasm and when the concentration of citrate exceeds the critical limit, the citrate will be secreted out to the external media.

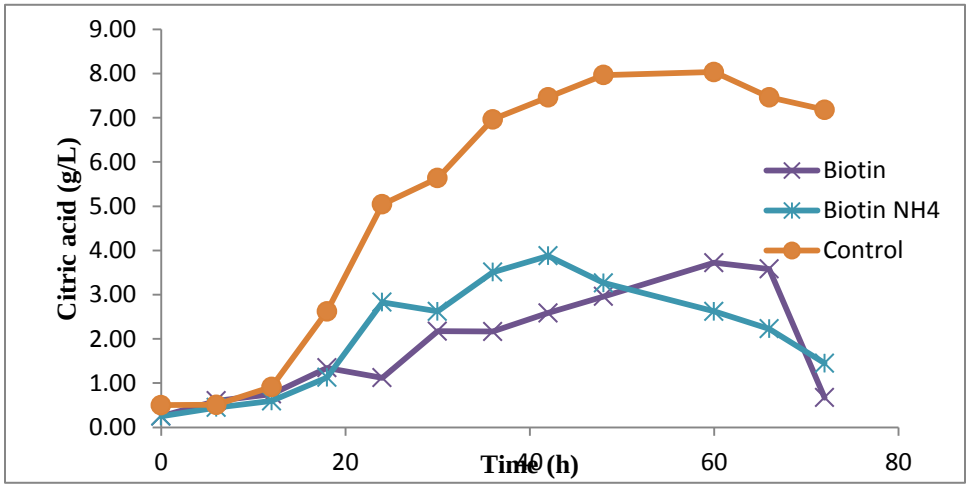

Figure 53 Production of citric acid in different cultivation conditions

2.5 Lipase production by *Y.lipolytica* SKY7 on high soap containing fermentation medium

Lipase production by *Y.lipolytica* is one of the trade characteristics of this strain when it is grown on hydrophobic substrates. Lipase production under different experimental conditions is presented in Figure 54. Lipase activity of 16.6U/mL was observed at 12h of fermentation when

biotin was supplemented in the medium. The consumption of soap was slow at 0 to 6h (Fig 51B), whereas an increase in the lipase activity was substantial during this time. As the lipase activity increased, the soap utilization also increased. In the case of biotin and $(NH_4)_2SO_4$ fortification study, the lipase activity was found 16.7U/mL at 18h of fermentation. Correspondingly, the soap utilization notably occurred from 12 to 24h of fermentation; the soap concentration decreased from 11.3 to 4.3 g/L during this time, which may be due to lower lipase activity. A moderate increase in lipase activity was noticed from 0 to 18h (9.25 U/mL) of fermentation in the control. When we compare the soap utilization in control experiments with the other two conditions, the soap utilization was higher in control than biotin but lower in biotin along with nitrogen pulse feed. The soap degardation in biotin along with nitrogen added study was comparatively low due to the low lipase activity at 0 to 12h of fermentation. A rapid decline in the lipase activity was evident from Figure 54. It has been reported that the lipase production in *Y.lipolytica* was notably increased with late exponential growth (12 to 18h) and early stationary phase. After reaching the maximal level the decline in the lipase activity was due to the proteolysis, catalyzed by proteases produced during the growth phase by this strain [33]. The decline in lipase activity can occur due to the substrate limitation, and other fermentation conditions.

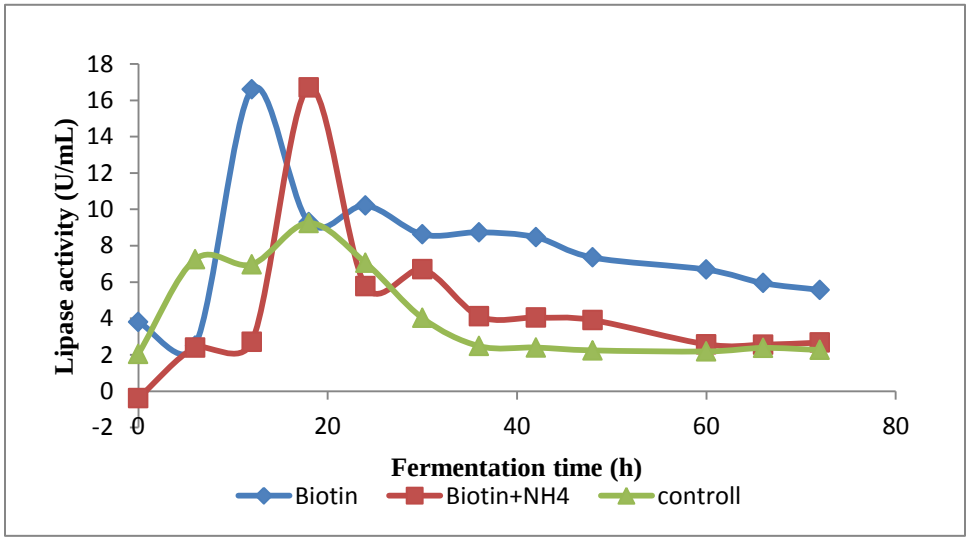

Figure 54 Lipase productions in different cultivation condition

2.6 Comparison of Yield of biomass and lipid

The yield of biomass (Yx/s or gram biomass produced/C mole consumed) shows a high substrate to biomass conversion in the control followed by biotin along with pulse nitrogen feed

experiments (Figure 55). In the case of biotin alone, the Yx/s stayed approximately same throughout the fermentation (Figure 55). In other two cases (control and biotine+pulse nitrogen), the yield of biomass decreased until 18h of fermentation and after that stayed almost constant. However, at the end of fermentation, the Yx/s value was slightly higher in the case of biotin supplemented study. It could be explained due to higher accumulation of lipid in biomass. To evaluate the actual biomass yield, the lipid-free biomass yield (lipid-free biomass produced per C mole consumed) was calculated and presented in Figure 56. The Yxf/s in the case of biotin added study stayed higher than the control and biotin along with pulse nitrogen added studies until 48h of fermentation. The maximal Yxf/s values in all three conditions were observed at 12h of fermentation. At the end of the fermentation (72h) the lipid-free biomass yield in case of control and biotin along with pulse nitrogen was 25.91 and 23.47 g/ C mole, respectively. The lowest lipid free biomass yield was observed for biotin supplemented experiment at 72h (19.14g/C mole). The Yxf/s data shows that the lipid accumulation occurred from the early stage of fermentation. Further the stable Yx/s from 24h and decrease in the Yxf/s clearly suggest the total increase in biomass after 24h of fermentation in all cased were mainly contributed by the lipid production.

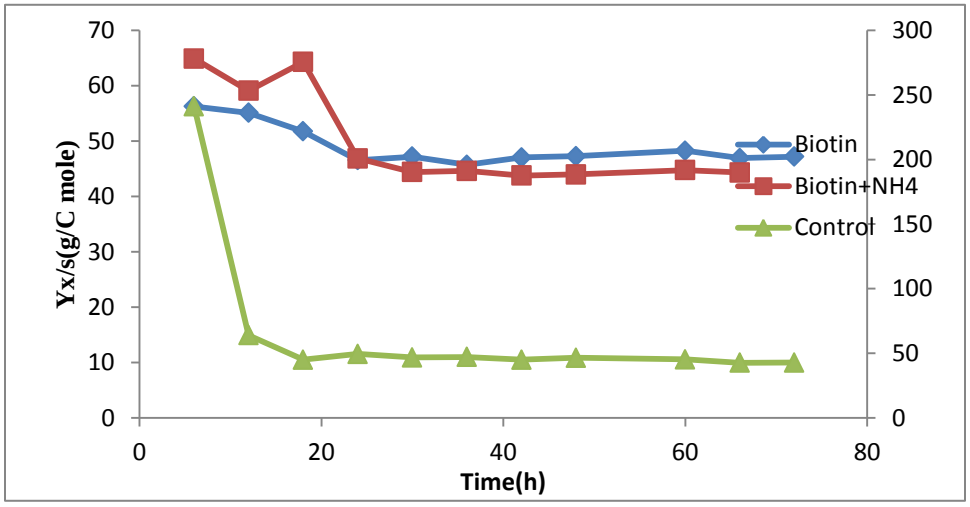

Figure 55 The yield of cumulative biomass at different time intervals

In general, the TAG synthesis was reported to occur after the cell growth decreases. However, when the hydrophobic substrate is available in the fermentation media the lipid synthesis can simultaneously occur or the cells start accumulating lipid along with the cellular growth. When the fermentation media was supplied with biotin, the specific growth rate (μ) was noticed to

reach its maximum value of 0.223h^{-1} at 12h of fermentation (Figure 58). From 12h onward, a rapid decline in μ shows that the growth was substantially decreased after 12h of fermentation. In the case of biotin with pulse nitrogen feed study, μ reached 0.187h^{-1} at 12h, which is comparatively lower than the biotin supplemented study. When the above conditions were compared with the control, μ value of 0.212h^{-1} was attained at 12h. Based on all these observations, it can be concluded that the maximum specific growth was attained within the first 12h of fermentation for all three conditions studied.

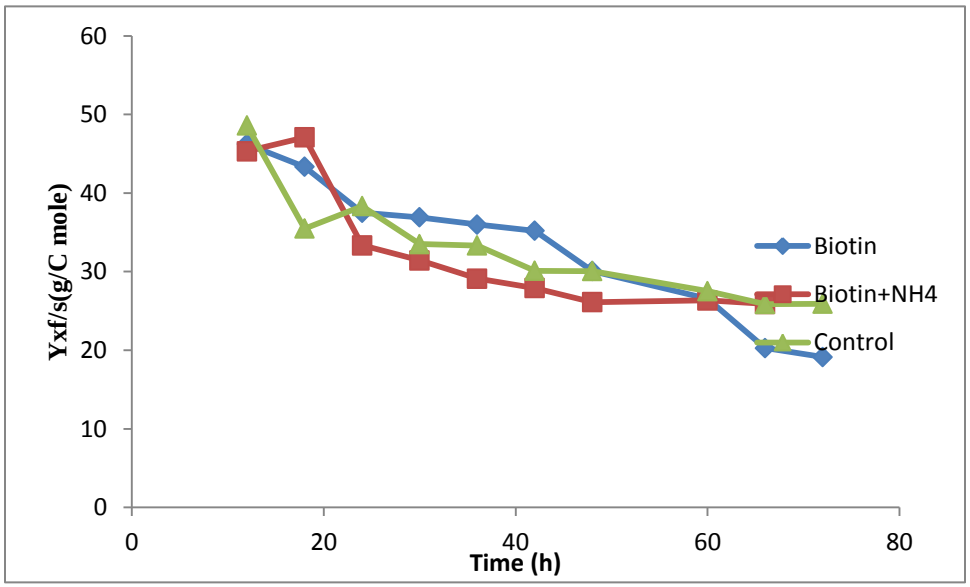

Figure 56 Yield of lipid-free biomass at different time points

The Yp/s (substrate to lipid conversion g lipid produced/ C mole consumed) shows a high lipid conversion in the case of control and biotin with pulse nitrogen feed experiments (Figure 57). In the case of control experiment, the Yp/s value was noticed to gradually increase from 18h onwards. The Yp/s value became approximately stable (16.44 g/ C mole) after 48h of fermentation, which also reflected in total lipid concentration (i.e. the lipid concentration reached 9.88g/L at 48h and after that there was no significant increase in lipid concentration). However, in the case of biotin along with pulse nitrogen feed experiment a slightly higher Yp/s was observed at 12 to 24h. Thereafter, a slight increase was evident from the figure 58. A maximum Yp/s value of 20.53g/C mole was attained at 72h of fermentation. In the case of biotin alone study, the Yp/s value was initially lowest compared to the other cases, started increasing from 36h onwards, surpassed at 48 h and finally reached maximum value 28.01g/C mole at 72h of fermentation. A similar trend was observed in the case of Yp/x (g lipid per g of biomass data not

shown). The higher Yp/x in the case of biotin supplemented study shows a high substrate to lipid conversion, which could be due to the results of an increase in ACC activity due to the addition of biotin as previously mentioned.

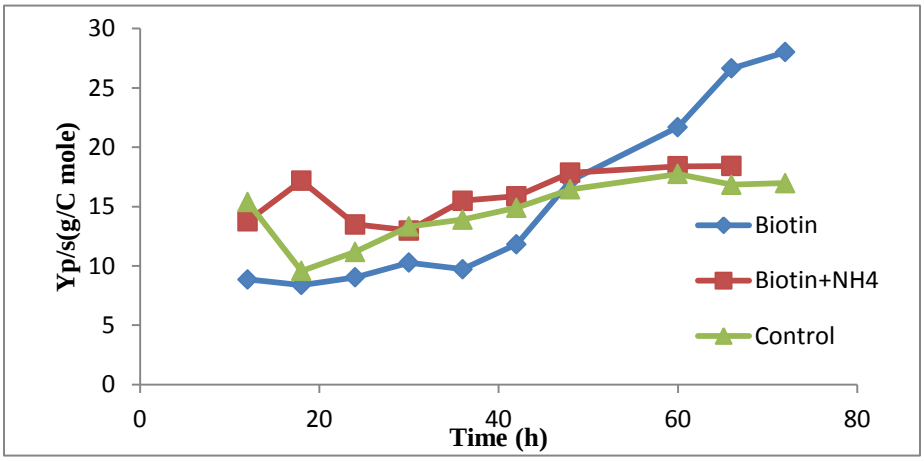

Figure 57 Yield of lipid in biotin and biotin with intermediate diammonium salt added and control experiments at different time scale

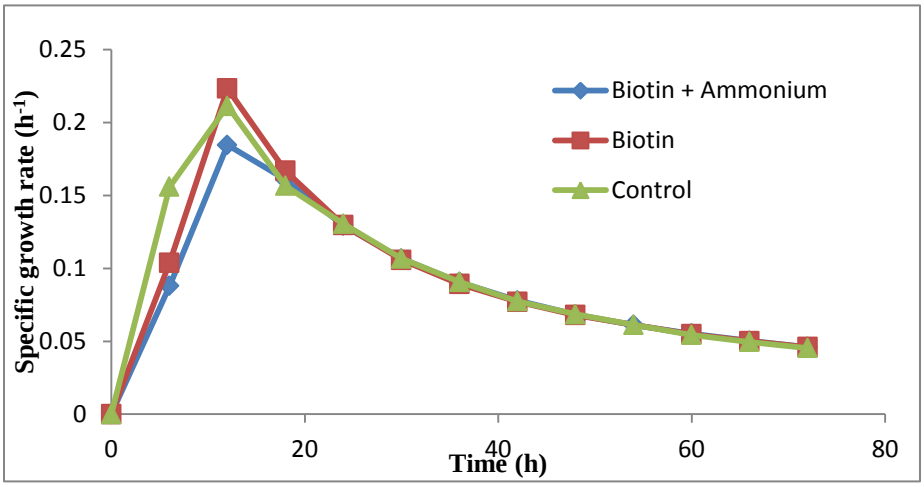

Figure 58 specific growth rate of biotin and biotin with intermediate diammonium salt added and control experiments at different time scale

3 Conclusion and summary

A comparison of biotin alone and biotin along with pulse $(NH_4)_2SO_4$ supplementation was studied. The results revealed that the addition of biotin to the fermentation medium significantly improved the lipid production. The presence of soap in the fermentation media lead to higher lipid accumulation in *Y.lipolytica* SKY7. The current study also showed that using a mixture of

glycerol and soap could lead to the production of multiple products in a single fermentation namely lipid, citric acid, and lipase and this finally add value to the process options. Based on the current study an average biomass concentration ranging from 26.57±0.02 to 27.85±0.35g/L was obtained. Highest lipid concentration of 15.73±0.15g/L was produced in biotin supplemented study. Based on the results obtained it is concluded that the addition of biotin leads to a high lipid concentration and lipid yield. The biotin along with pulse nitrogen supply had a lesser impact on lipid accumulation compared to the control. Further, improvements in the controlled supply of nitrogen during the fermentation need to be studied in order to improve the biomass and lipid concentration in batch fermentation.

Acknowledgement

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References

1. C. Ratledge and J. P. Wynn, in *Advances in Applied Microbiology*, 2002.
2. A. Beopoulos, J. Cescut, R. Haddouche, J. L. Uribelarrea, C. Molina-Jouve and J. M. Nicaud, *Progress in lipid research*, 2009, **48**, 375-387.
3. S. Papanikolaou and G. Aggelis, *European Journal of Lipid Science and Technology*, 2011, **113**, 1031-1051.
4. S. S. Tchakouteu, O. Kalantzi, C. Gardeli, A. A. Koutinas, G. Aggelis and S. Papanikolaou, *Journal of applied microbiology*, 2015, **118**, 911-927.
5. Z. Zhu, S. Zhang, H. Liu, H. Shen, X. Lin, F. Yang, Y. J. Zhou, G. Jin, M. Ye, H. Zou and Z. K. Zhao, *Nature communications*, 2012, **3**, 1112.
6. C. Saenge, B. Cheirsilp, T. T. Suksaroge and T. Bourtoom, *Process Biochemistry*, 2011, **46**, 210-218.
7. Zhiwei Gong, H. Shen, X. Yang, Q. Wang, H. Xie and Z. K. Zhao1, *Biotechnology for Biofuels*, 2014, **7**.
8. S. Fakas, S. Papanikolaou, A. Batsos, M. Galiotou-Panayotou, A. Mallouchos and G. Aggelis, *Biomass and Bioenergy*, 2009, **33**, 573-580.
9. L. Zhao, H. Zhang, L. Wang, H. Chen, Y. Q. Chen, W. Chen and Y. Song, *Bioresource technology*, 2015, **197**, 23-29.
10. D. Gao, J. Zeng, Y. Zheng, X. Yu and S. Chen, *Bioresource technology*, 2013, **133**, 315-321.
11. L. Yang, Z. Zhu, W. Wang and X. Lu, *Bioresource technology*, 2013, **150**, 1-8.
12. A. Gnanaprakasam, V. M. Sivakumar, A. Surendhar, M. Thirumarimurugan and T. Kannadasan, *Journal of Energy*, 2013, **2013**, 1-10.
13. S. Octave and D. Thomas, *Biochimie*, 2009, **91**, 659-664.
14. A. H. Mondala, R. Hernandez, T. French, L. McFarland, J. W. Santo Domingo, M. Meckes, H. Ryu and B. Iker, *AIChE Journal*, 2012, **58**, 1279-1290.

15. S. Papanikolaou, I. Chevalot, M. Galiotou-Panayotou, M. Komaitis, I. Marc and G. Aggelis, *Electronic Journal of Biotechnology*, 2007, **10**, 0-0.
16. P. Meesters, G. N. M. Huijberts and G. Eggink, *Applied microbiology and biotechnology*, 1996, **45**.
17. L. Y. Zhu, M. H. Zong and H. Wu, *Bioresource technology*, 2008, **99**, 7881-7885.
18. T. W. a. G. Petersen, A. A. a. J. Bozell, J. Holladay, J. White and A. Manheim, 2004, **1**.
19. S. Wu, C. Hu, G. Jin, X. Zhao and Z. K. Zhao, *Bioresource technology*, 2010, **101**, 6124-6129.
20. C. N. Economou, G. Aggelis, S. Pavlou and D. V. Vayenas, *Biotechnology and bioengineering*, 2011, **108**, 1049-1055.
21. A. Beopoulos, T. Chardot and J. M. Nicaud, *Biochimie*, 2009, **91**, 692-696.
22. A. A. Hamid, N. F. Mokhtar, E. M. Taha, O. Omar and W. M. W. Yusoff, *Annals of Microbiology*, 2010, **61**, 463-468.
23. J. Blazeck, A. Hill, L. Liu, R. Knight, J. Miller, A. Pan, P. Otoupal and H. S. Alper, *Nature communications*, 2014, **5**, 3131.
24. W. R. Streit and P. Entcheva, *Applied microbiology and biotechnology*, 2003, **61**, 21-31.
25. T. Kaneda, *Canadian Journal of Microbiology*, 1966, **12**, 501.
26. J. Folch, M.Lees and G.H.Slane-Stanley, *J. Biol. Chem*, 1957, **226**, 497–509.
27. P. Bondioli and B. Della, Laura, *European Journal of Lipid Science and Technology*, 2005, **107**, 153-157.
28. S. Hu, X. Luo, C. Wan and Y. Li, *Journal of agricultural and food chemistry*, 2012, **60**, 5915-5921.
29. J. R. Marier and M. Boulet, *Journal of Dairy sciences*, 1958, **41**, 1683-1692.
30. N. Prim, M. Sánchez, C. Ruiz, F. I. J. Pastor and P. Diaz, *J Mol Catal B Enzym*, 2003, **22**, 339–346.
31. A. Beopoulos, Z. Mrozova, F. Thevenieau, M. T. Le Dall, I. Hapala, S. Papanikolaou, T. Chardot and J. M. Nicaud, *Applied and environmental microbiology*, 2008, **74**, 7779-7789.
32. S. Shuib, W. N. Nawi, E. M. Taha, O. Omar, A. J. Kader, M. S. Kalil and A. A. Hamid, *TheScientificWorldJournal*, 2014, **2014**, 173574.
33. E. Dalmau, J. L. Montesinosa, M. Lottib and C. Casasa, *Enzyme and Microbial Technology*, 2000, **26**, 657–663.

Chapter-VIII

Research Article-9

Diversity and Composition of Bacterial Communities Vary in Sludge of Different Pulp and Paper Industrial Wastewater

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To be submitted

Résumé

Les eaux usées de l'industrie de pâte à papier sont considérées comme l'un des polluants majeurs en raison de sa haute teneur en carbone et d'autres éléments nutritifs. Afin d'éliminer la charge nutritive élevée de l'eau usée, les industries utilisent des méthodes de traitement chimiques ou biologiques. Dans le cas du traitement biologique, un certain nombre de microbes interagissent ensemble pour éliminer les polluants. Cependant, la connaissance approfondie des communautés microbiennes dans les boues papetières reste limitée. L'approche biotechnologique actuelle comme le séquençage Illumina MiSeq des amplicons PCR a facilité l'analyse microbienne dans des environnements complexes. En Employant cette technique sur les boues issues des eaux usées des industries papetières permettrait d'améliorer les connaissances sur la communauté microbienne ainsi que sa structure. Dans cette étude, la structure de la communauté bactérienne a été caractérisée par séquençage NGS à haut débit de PCR avec amplification du gène ARNr 16S dans différentes boues d'eaux usées de pâtes à papiers. La structure de la communauté microbienne variait par rapport au processus de transformation. L'analyse comparative de la littérature actuelle a montré un grand potentiel de production de lipides à partir des boues, aussi bien que de biopolymères, des acides organiques, des exopolysaccharides et une production d'enzyme à intérêt industriel.

Mots clés : boues de Pâtes à papiers, eaux usées, communauté microbienne, lipides microbiens, enzymes, acides organiques, biopolymères

Abstract

Pulping industry waste water has classified as one of the major pollutants due to its high carbon and other nutrient content. In order to remove the high nutrient load from the waste water, industries employ either chemical or biological treatment methods. In the case of biological treatment, a number of microbes are functioning together to remove the pollutants. However, knowledge about the microbial communities in pulp and paper mill sludge is limited. In a biotechnological perspective sludge characterized by elevated biodiversity would represent more interest for bioprospection, in addition, to being more resistant to stress-induced by fermentation operation. In this study, the structure of bacterial community was characterized by high-throughput sequencing of PCR-amplified 16S rRNA gene in different pulp and paper and wastewater sludges. The structure of the microbial community varied based on the pulping process and Kraft pulping process displayed the highest level of biodiversity. The comparative analysis of the current literature showed a great potential of sludge in lipid, biopolymer, organic acids, exopolysaccharides and industrial important enzyme production.

Keywords; *Pulp and paper sludge, wastewater, microbial community, microbial lipids, enzymes, organic acids, biopolymers.*

Introduction

Pulp and paper industry is ranked as one of the most polluting industry. In Canada, pulp and paper industry is accounting for major part of water and common air pollutants (Ince et al., 2011). In general, the pulping process in known as one of the highest water demanding process which utilizes up to 60 m^3 of water to produce every ton of paper. Based on the pulping process the COD and BOD of the wastewater release may reach from 20 to 110 kg/ton (Thompson et al., 2001).

In early days, the wastewater treatment was based on chemical methods. At present, due to the development of advanced biological methods, the wastewater is treated with biological methods (Bajpai, 2015). Biological treatments of wastewater are efficient, environmentally friendly and therefore they are widely employed in food, agro, pulping and, petrochemical industries. To eliminate the high nutrient load of wastewater discharged from pulp and paper industry, activated sludge (AS) process was engaged. In general, after the primary settling process, the waste water stream is released into an activated sludge treatment reactor. The primary solids are disposed of separately. In activated sludge (AS) process, the nutrients and the pollutants in the wastewater are removed by a mixture of mutually benefited microbes. The current literature shows it is efficient to remove 50% to 70% of total BOD/COD, nitrate, phosphate, suspended solid, absorbable organic halides (AOH) (Arcos-Hernandez et al., 2010; Chaudhary S et al., 2002). The primary secondary sludge is composed of a number of microbial species. The presences of different toxic pollutants acclimatize these microbes to withstand the unforgiving environment. In order to degrade the toxicants, these microbes produce different classes of enzymes (Moreau et al., 2015; Tyagi et al., 2014). Moreover, the microbes work with a mutual interaction. The microbes involved in AS process are efficient in nutrient and pollutants removal. However, the high carbon to nitrogen ratio (50-over 200:1) in pulp and paper wastewater makes the pollutants and nutrient removal process tedious (Tucker, 2005). To overcome the low efficiency of nutrient and pollutants removal in the process, the additional nitrogen source is added to enhance microbial activity, which results in elevation of biodiversity in activated sludge.

The high carbon to nitrogen ratio prevailing in AS is a well-known condition for microbial biosynthesis of various value-added products such as polyhydroxyalkanoate, lipids and, extracellular polysaccharides. Indeed, microbes accumulate the excess carbon into storage

materials such as polymers, lipids, glycogens, carbohydrates or different carboxylic acids such as citric acid, acetic acid propionic acid butyric acid, and biogas under these conditions (Bengtsson et al., 2008; Hori et al., 2011; Reis et al., 2003; Sun et al., 2014; Wang & Yu, 2007; Wu et al., 2012). A great deal of effort has been invested in harnessing the nutrient and energy potential of AS for the synthesis of biomass, but very little is known about the diversity and the structure of microbial communities thriving in this ecological niche.

In the present study, we explored the biodiversity and the composition of the microbial community in AS from different pulp and paper mill wastewater using high-throughput sequencing of PCR-amplified bacterial 16S rRNA gene. Indeed, based on the microbial community and its structural analysis it can be possible to enrich (bioaugmentation) specific microbes displaying the potential to synthesize different valued added products by changing the operational parameter to enhance the biological nutrient removal process.

1. Materials and methods

1.1. Collection of sludge samples

Samples were collected from different industries with the different pulping process (Kraft, TMP, and CTMP). The samples (activated sludge) were collected in 20L tanks and transported to the lab and then kept in 4^0C until it was taken for sludge DNA extraction. The detailed description of samples is presented in Table 36.

Table 36 Description of sludge samples and the pulping process involved

*CTMP	Kraft	**WAS-Kraft (Thunder bay)	Dolbeau
A84	A87	A89	A91
C86	B88		B92
B90			A93
			B94

* CTMP- chemical thermal and mechanical pulping, **WAS- waste activated sludge. B 90- CTMP sludge at low suspended solids added with nutrients, A9- samples with low suspended solids and added with nutrients, B92- nutrients and citric acid added, A93- fermentation with low suspended solids, B94-fermentation with High suspended solids.

1.2. DNA extraction methods

One ml of each sample was transferred to a sterile Eppendorf tube and centrifuged at 10,000 × g for 10 min. The supernatant was discarded and the pellet of each sample was suspended in 1ml of 0.1M Tris buffer (pH 8). 5µg of lysozyme was added to the solution and the sample was well mixed by vortex. The resulting suspension was kept at 4°C for a period of 12h. After the incubation, 1ml of chloroform-isoamyl alcohol was added and the sample was centrifuged at 10,000 × g for 20 minutes and the upper phase was transferred to a new tube. 0.6 mL of isopropanol was added to the above solution and kept on ice for 20 minutes to precipitate the DNA. The sample was centrifuged again at 10,000 × g for 10 minutes and the supernatant was discarded carefully and the precipitated DNA (the pellet) was suspended in 70% v/v ethanol and centrifuged at 10,000 × g for 10 minutes to remove the excess salts. The samples were further suspended in TE (100 mM EDTA, 10 mM TRIS, pH 8.0) buffer, RNase was added and the precipitation step was repeated to obtain purified DNA. The purified genomic DNA (gDNA) samples were aliquoted in nuclease-free water to get a final concentration of 40 ng/µl.

1.3. PCR amplification and sequencing of 16S rRNA gene

PCR-amplification of the V1-V3 regions of 16S rRNA, libraries preparation and Illumina MiSeq 250 bp paired-ends sequencing reactions were performed by the technical staff of Centre d'Innovation Génome Québec et Université McGill, resulting in 27,661,770 raw sequences. Paired-end reads were merged using the software Flash (Magoc & Salzberg, 2011) with minimum and maximum overlap length between the two reads of 20 and 250 bases, respectively. The maximum proportion of mismatched base pairs tolerated in the overlap region was set to 0.3. The merged reads (2,062,080) were processed using the UPARSE pipeline (drive5, Tiburon, California) (Edgar, 2013): truncating reads to a uniform length of 420 (representing the length of more than 97,5 % of the reads). Reads were dereplicated and then sorted by abundance. Unique reads (1,464,986) were then clustered at 97% identity using the greedy algorithm of UPARSE with a minimum of two sequences for the OTU to be considered. Then 233 chimeric OTU were removed using UCHIME drive5 run against the gold standard reference database. The remaining 1,158 non-chimeric OTUs were assigned using the Greengenes database (version gg_13_8). Reads were rarefied to the sequencing effort of the smallest 16S rRNA gene library (7,378 reads) to avoid biases in comparative analysis introduced by the sampling depth, giving a final number

of 598 OTUs. Raw sequences were deposited in the Sequence Read Archive of the National Center for Biotechnology Information under the Bioproject SUB1332015.

1.4. Statistical analysis

The richness and evenness of bacterial community in sludge samples were analyzed using the software R, as previously described (Peck et al., 2016, Microbial Biotechnology) .

2. Results

From the OUT (Operational Taxonomic Unit) table, it is concluded that 598 OTUs were present in the given sludge samples. To avoid the discrepancies in the results in figures representing the abundance of microbes, OTUs representing in less than 1% relative abundance were eliminated from the dataset for biodiversity and taxonomic analyses.

2.1 Microbial diversity of sludge samples

Rarefaction analysis unveiled that sequencing effort was sufficient to cover the whole microbial communities (Figure 59). Further, the Shannon index was calculated and presented in Table 37 for the samples analyzed. It shows a high microbial diversity (3.12±0.38) in the case of Kraft sludge. Meantime in the case of CTMP, it was 2.8±0.56 and in TMP 2.06±0.77. Based on the Shannon index, the microbial diversity in Kraft sludge was notably higher than the other two processes. Moreover, the rarefaction curves also support the same findings. The abundance of dominant species in the samples was represented by Simpson index. In the present study, the high Simpson index value was obtained in the case of Kraft sludge followed by that the CTMP process and the low values were in the case of TMP process.

Table 37 Shannon and Simpson Diversity index

	Alpha diversity	
Sample	**Shannon**	**Simpson**
Kraft	3.12±0.38	0.88±0.04
CTMP	2.8±0.56	0.85±0.08
TMP	2.06±0.77	0.64±0.18

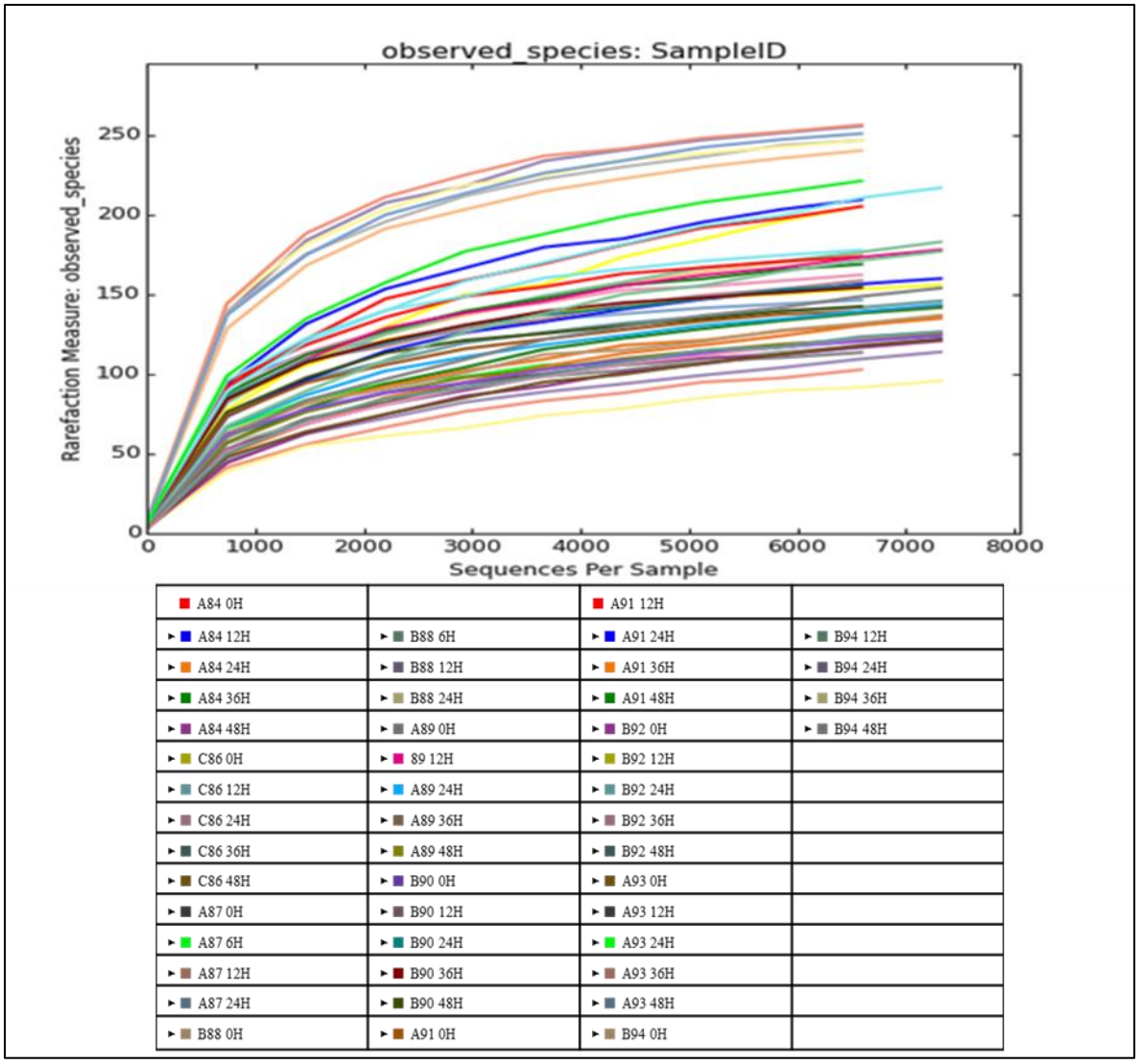

Figure 59 Rarefaction of Observed taxonomic units (OUT's)

2.2. Phylogenetic analysis of observed OTU

Microbial communities were dominated by members of the *Proteobacteria.* The variation in OTU is presented in the dendrogram (Figure 60). Based on the difference in the microbial diversity the samples were clustered and presented as a dendrogram. The analysis shows that the samples were segregated in 4 different nodes based on the difference in the microbial diversity. The difference in the microbial diversity obtained can be correlated with the pulping process used in the industry. In other words, the environment where these microbes evolved has a strong influence in the microbiome structure.

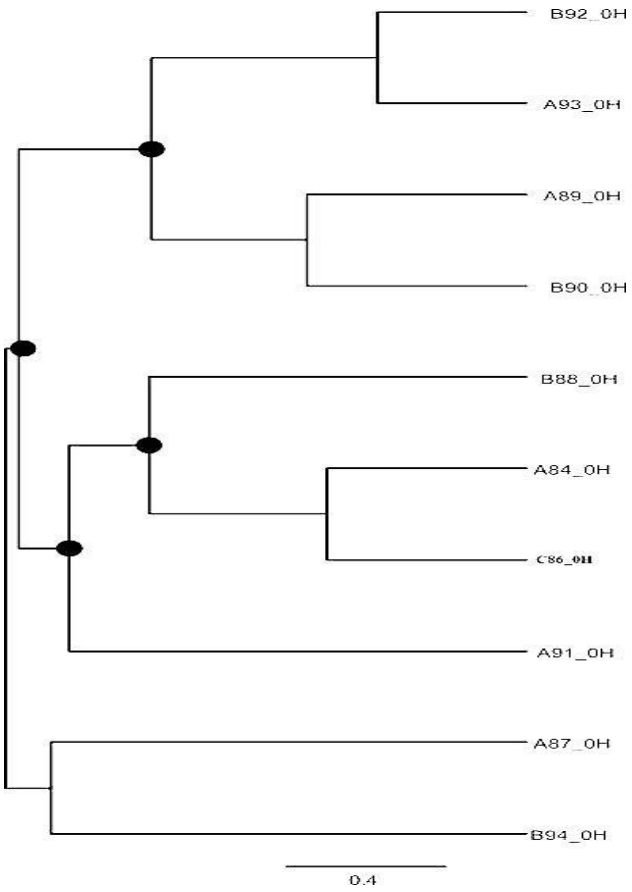

Figure 60 Dendrogram showing the similarity of microbiome in different samples

The Kraft sludge A87 and B94 noticed to have a similar microbial community. The dendrogram shows A91 (Dolbeau) sludge is segregated from all other samples into a single node and wich is close resemblance with 86, A84 nd B88. However, the other samples (B90, A89, A93, and B92) were clustered in a single node. The difference in the microbial community in this node is differentiated into two different groups that are B90 and A89 into a group with more similar OTUs and A93 and B92 with similar OTUS. In the present experimental data shows the variance in the OUT is highly varied in A93 and B92 compared with B94 and A87. This difference in microbial structure could occur due to the variation in the pulping process and the type of wood and the environmental conditions. However, the current analysis shows the differences in the OTUs were varied within the same samples and at the same time it has similarities with other process samples. For example, B94 and A87 were from Dolbeau and kraft process. However, the OTUs are similar. This confirms the diversity of microbial communities within the pulp and paper mill sludges can be similar in composition irrespective of the pulping process. This can

occur mainly due to the presence of common components found in the sludge (Cellulose, Hemicellulose, Lignin, and the chemicals used for pulping).

The assigned taxa for the OTUs shows the major communities found in this study are *Bacillus, Acinetobacter, Zoogloea, Pseudomonas, Methylobacillus, Ramlibacter, Curvibacter, Giesbergeria, Comomonas, Piscinibacter, Janthinobacterium, Aquaspirillumm, Nitrosococcus, Acidispharea, Methylocladium. Giesbergeria, Pisinibacter* are two new genus found in this study, which shows a close relation to lipid, EPS, and biopolymer producing organisms. The other observation revealed the presence of sulfate- and nitrate-reducing groups in sludge such as *Sulfuricurvum and Nitrosomonas*. The other important genus of organisms like *Cloacibacterium* is placed distant to the biopolymer producers. Nonetheless, a high relative abundance of *Cloacibacterium* is found in Thunder Bay sludge and has been reported for hydrocarbon degradation.

2.3 The significance of microbial molecular indicator species on pulp and paper mill wastewater sludge

The microbial community obtained in the pulp and paper mill wastewater sludge mainly belonged to six phyla. Based on the indicator species analysis (Figure 61) the most prominent one was *Proteobacteria* followed by that *Firmicutes, Actinobacteria, Bacteroidetes, Verrucomicorobia, and Chloroflexi*. However, other OTUs from another phylum (*Synergistetes, Planctomycetes, Armatimonadetes, Lentisphaere, Spirochaetes, Chlorobi and Thermotogae*) were also found in the samples shows the richness of the microbial community in the sludge. Even though the OTUs from *Proteobacteria* were found in high abundance, OTUs from *Actinobacteria* is the only phylum found in most of the samples. In the case of indicator species, only OTU 10 and OTU 20 were found with significant abundance. When comparing the assigned OTU with the taxonomic classification it belonged to the genera Hippea and Bacillus. Further, the ecological importance of these two microbes was analyzed. In the case of Hippea, it is known to inhabitants in the environment such as elemental sulfur and it exhibit growth in environments containing organic acids such as acetate, succinate, casamino acid etc. in general the kraft pulping employed salts of sulfates to enhance the pulping process. This proves the presence of high sulfate content in the sludge as well. Meantime the hippea is known to grow at low pH and the pulp and paper wastewater sludge from kraft process ranges from 1.5 to 10. The genus

bacillus is known to secrete different extracellular enzymes. Here in the present case, the presence of cellulose and hemicellulose and lignin make the environment suitable for this genera.

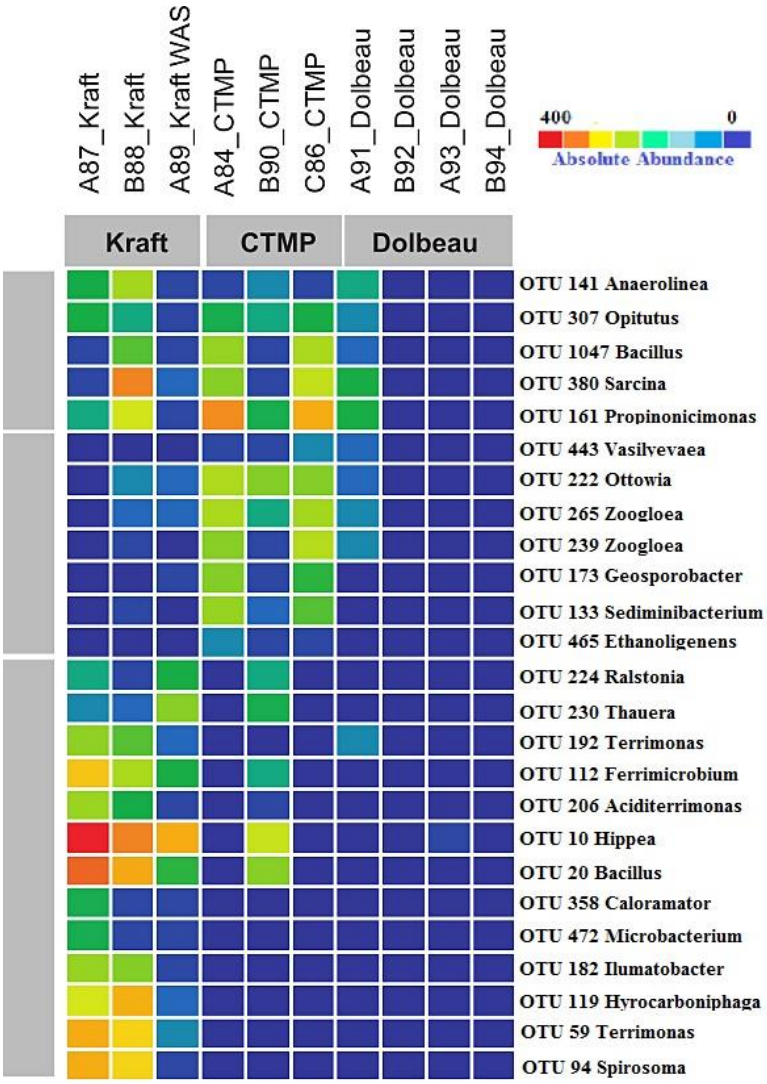

Figure 61 Heat map of OUT

3. Taxonomical classification of microbial community

3.1. CTMP process (A84, B90, and C86)

In CTMP process, secondary sludge microbes from the phylum of proteobacteria dominated, followed by *Bacteroidetes*. The other phyla found in the CTMP sludge were *Firmicutes, Actinobacteria, and Verrucomicrobia*. Further, the sequences were identified to genus level and the results were shown (Figure 62 A,B &C) for the sequence more than 1% in abundance. In the case of A84 the microbial community constitutes 13% of *Acidisphaera, 23% Parabacteroides, 10% of Nitrosococcus, 8% of Curvibacter* and others were found in 5% or less of the total

community. At the same time, the C84 also shows similar dominance genous in the fermentation. However the abundance ratio was different from A84 and in C86; *Acidisphaera, 41%, Parabacteroides, 7% of Nitrosococcus, and 5% of Curvibacter* was observed. Interestingly in the case of B90, the abundance of *Parabacteriodes* 35% , Cloacibacterium 25% ,and *Herbaspirillum* 7% was the dominant general. The change can occur due to the variation in sampling time. However, the presence of similar microbial community (even in low abundance) in all samples shows the uniform nature of microbiome in CTMP process sludge.

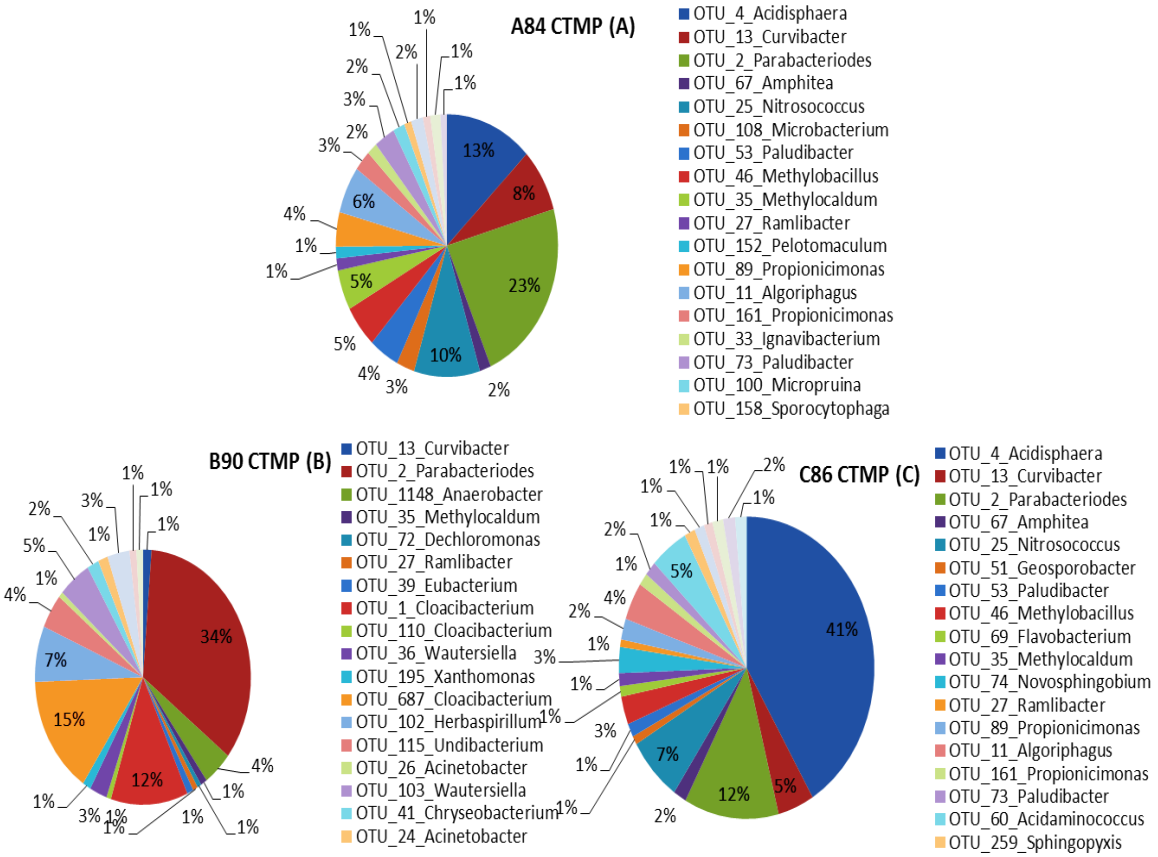

Figure 62 The difference in relative abundance of CTMP sludge

3.3. Kraft sludge

Taxonomic classification of the observed OTUs in kraft sludge found to have different microbiome from CTMP process. In the case of A87, the dominant OYUs belonged o the genera of (Figure 63 A, B & C), *Janthanobactterium 23% Acidisphaera 6%, Sulfuricurvum 21%, and Hippea 6%.* However, the microbial abundance of B88 was different from A87. The most dominant genera in B88 was from *Pseudomonas 22%, Eubacterium 19%, Parabacteriodes 14% and Acidispheara 8%.* Whereas in the case of activated kraft sludge (A89) the *Cloacibacterium*

was found as the most dominant genera contributing 76% of the total microbial community. Parabacteriodes was the second dominant genera with 7% of the total microbial community. The most abundant sequence found in the case of A87 (*Sulficurvum, Hippea)* confirms that the high availability of sulfate in the sludge due to the use of sulfate in kraft pulping process. Further, *Cloacibacterium* is known for its bioremediation capacity of Poly Aromatic Hydrocarbon (PAH) degradation of oil contaminated soil. This could be due to the presence of high phenolic compounds in the Thunder Bay sludge (20.5% w/w Lignin).The other microbial abundance of Kraft sludge obtained from *Acidispheara , Eubacterium Methylocladum , Paludibacter , Anaerobacter , Curvibacter , Nitrosococcus , and Bacillus .*

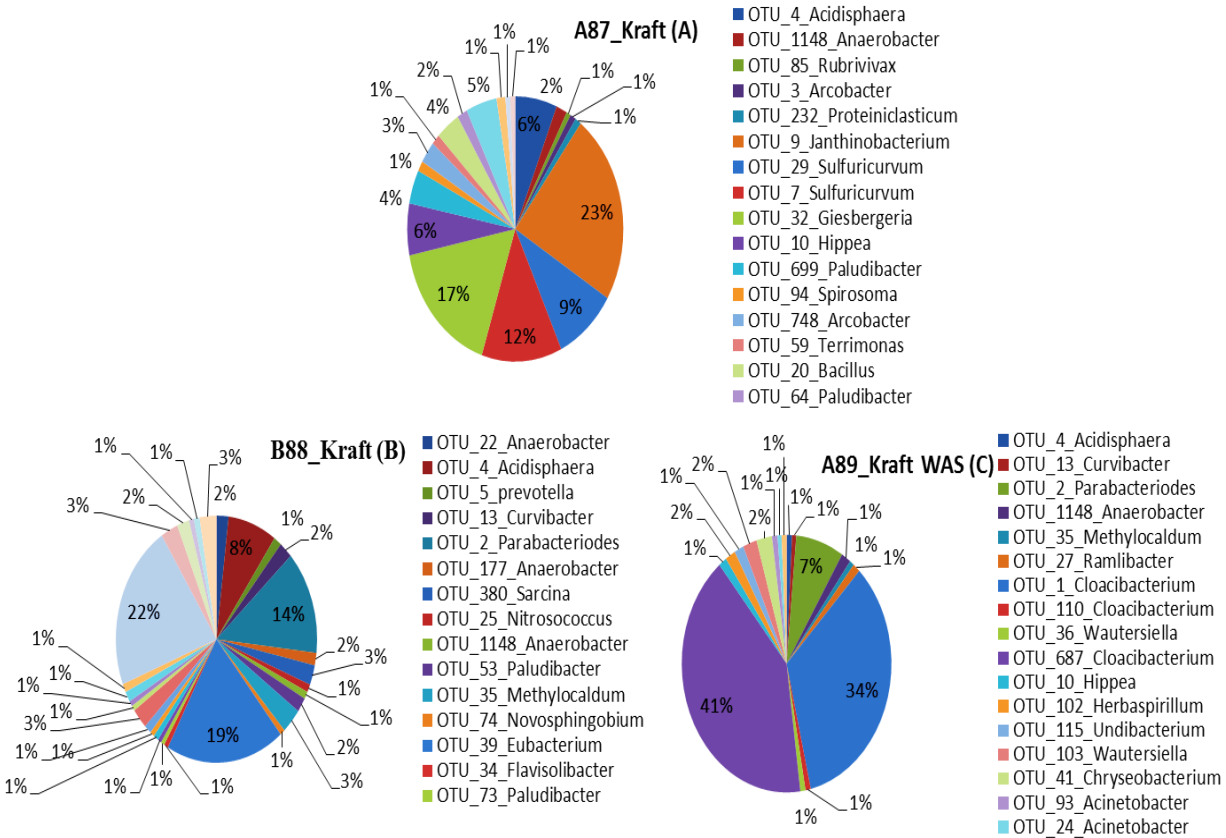

Figure 63 Difference in relative abundance of OTUs in kraft sludge

3.6. TMP (Dolbeau)

The relative abundance of the microbial community in TMP (Dolbeau) sludge is presented in 64 A,B, C & D. The most prominent genera in A 91 were *Anaerobacter 28% Ramlibacter 7%, Adhaeribacter 5% Proteiniclastricum 11% and Ethanoligenens 18%.* However, in the case of B92 and A93 *Cloacibacterium*, was found as the most prominent genera with 79% and 77%

relative abundance. Followed by that *Flavobacterium* was with 7% and 8% relative abundance. I mean time the total microbiome analysis of B94 shows the presence of other genera such as *Commomonas*, *Anaerobacter* in common. In the case of B94 *Arcobacter* 64% *Parabacteriodes* 9%, *Arcobacter* 77%, and *Janthinobacterium* 9% was found as the prominent genera.

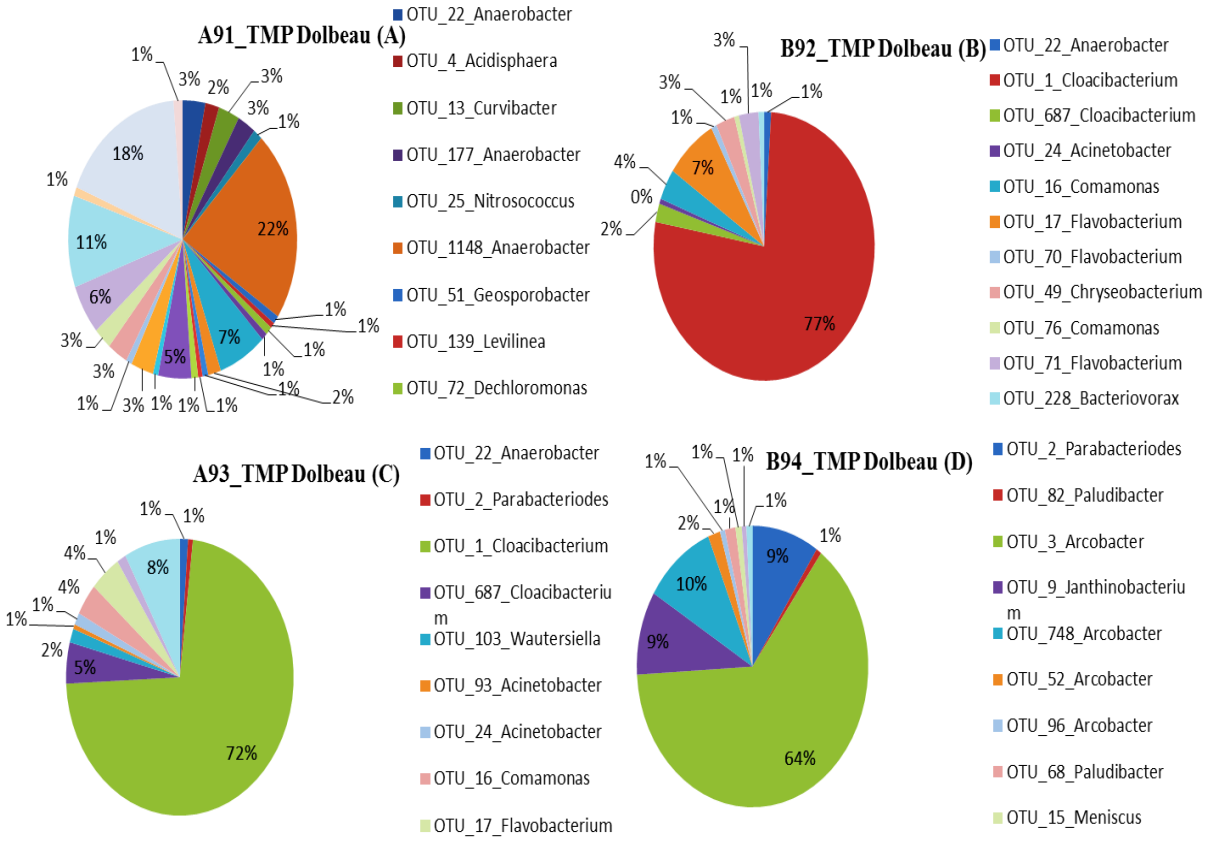

Figure 64 The relative abundance of OTUs in TMP (Dolbeau) Sludge

4. Discussion

The current study reveals the composition of the natural microbial community involved in pulp and paper industry sludge treatment. The phylogenetic analysis showed the similarities between the microbial communities in the different sludge samples share a common microbiome. However, depends on the pulping process and the nature of the sample (composition of the sludge) a small variation in the microbial community was observed. In other words, the CTMP, kraft, and TMP process have shared a single node denotes the similarities in the OTUs obtained. However as stated above based on the composition some genera such as *Arcobacter* and *Cloacibacterium* in TMP process sludge, *Janthinobacterium, Sulfuricurvum, Pseudomonas and Coloacibacterium* in the case of kraft sludge. In CTMP process, it was *Parabacteriodes, Acidispheara, and Coloacibacterium*. *Coloacibacterium* is one of the genera which are shared

most common in all the samples and which are known to present in the phosphate removal process as well as in hydrocarbon degradation sites. The other important genera such as *Sulfuricurvum and Hippea* were important in sulfur removal. It can conclude from the pulping process that the use of salts of sulfur leads to the accumulation of sulfur in the waste water stream.

Based on the bacterial community presents in the different pulp and paper sludge obtained from the different pulping process, we can expect that individual sludge would have the capacity to produce different value added products namely, lipids, biopolymers, industrial important enzymes, exopolysaccharides (EPS) and great potential of bioremediation capacity. Table 38 shows a list of bacteria detected in the sludge samples that were reported for the production of different value-added products in literature.

Table 38 Application of microbes found in different pulp and paper industry wastewater sludge

Organism (Genus)	**Product**	**Reference**
Curvibacter	Biopolymer and Phosphate accumulation	(Kamika et al., 2014; Martin Koller et al., 2010)
Nitrosococcus	Denitrification	(Agot et al., 2001)
Geosporobacter	Aromatic hydrocarbon degradation	(Lin et al., 2014)
Dechloromonas	Polyphosphate accumulation	(Kamika et al., 2014)
Novosphingobium	Polyromantic hydrocarbon degradation	(Han et al., 2013)
Kitasatospora	Antifungal agent	(Young et al., 1999)
Ramlibacter	*β*-glucosidase	(Wang, 2012)
Arthrobacter	Lipid production	
Sulfurospirillum	Perchloroethylene reduction, sulfur reduction	(Kodama et al., 2007)
Janthinobacterium	Chitinase and Violacein production	(Pantanella et al., 2007)
Cloacibacterium	Organic acids and enzyme production	(Hyun et al., 2014)
Rubrivivax	Biopolymer, EPS, and 5-aminolevulinic acid production	(Martin Koller et al., 2010; Supalak & Poonsuk, 2013)
Pseudomonas	Lipid, biopolymer, EPS production	(Martin Koller et al., 2010)
Proteiniclasticum	Acetate, propionate, iso butyrate, and proteolytic enzymes	(Zhang et al., 2010)
Acinetobacter	Wax, EPS, lipase production, polyphosphate accumulation and Aromatic hydrocarbon degradation	(Alvarez & Steinbuchel, 2002; Zheng et al., 2011)
Flavobacterium	Organic acid production and polyphosphate accumulation	(Kim et al., 2006)

Paludibacter	Propionate production	(Ueki et al., 2006)
Aquaspirillum	Biopolymer production	(Martin Koller et al., 2010)
Comamonas	Biopolymer production and polyphosphate accumulation	(Martin Koller et al., 2010)
Zoogloea	Biopolymer, and EPS production	(Bala Subramanian et al., 2010; Martin Koller et al., 2010)
Acidaminococcus	Acetic and butyric acid and Glutaconate Co-A transferase production	(Wolfgang et al., 1981)
Acdisphaera	Fe (Iii) reduction	(Shipeng et al., 2013)
Bacillus	Lipid, biopolymer, organic acids, enzymes	(Prajapati et al., 2014)
Methylocaldum	Methane production	(Kizilova et al., 2013)
Methylobacter	Methane production	(Kizilova et al., 2013)
Micropruina	Biopolymer and Glycogen accumulation	(Tomoyoshi et al., 2000)
Cupriavidus	Biopolymer production	(Martin Koller et al., 2010)
Enterobacter	Biopolymer production	(Nighat, 2012)
Prevotella	EPS and Exoxylanase, B-1,4 Xylosidase production	(Ales et al., 1995; Yamanaka et al., 2011)
Propionicimonas	Propionate production	(Bae et al., 2006)

4.1. Lipid production from pulp and paper wastewater sludge

Triacylglycerides (TGA) is one of the main storage compounds in higher organisms (Eukaryotes). These are non-polar, water insoluble, high calorific energy reserve materials, synthesized by microbes in the presence of high carbon with low nutrient content in the media. However, in the case of bacteria, TAG has not been considered as a common storage compound. To date, a very few bacteria were identified to store TAG as energy reserve material. The main prokaryotic genus producing TAG and wax as storage compounds includes *Acinetobacter, Mycobacterium, Streptomyces, Rhodococcus, Pseudomonas, and Nocardia* . Based on the data obtained from 16S rDNA sequencing, the main lipid producers present in the pulp and paper industry wastewater were from the genus of *Pseudomonas, Acinetobacter, Bacillus, and Arthrobacter*. Comparing the above four genus *Acinetobacter* is one of the common genus found in all sludge samples. Based on the existing data, it has been revealed that the microbes from the phylum *Proteobacteria, Verrucomicrobia, Bacteriodetes, Firmicutes, and Actinobacteria* were reported to be found in municipal wastewater sludge samples. When these sludge samples were used for the fermentation the total lipid content was reached to 17.5% CDW (Mondala et al., 2012). Comparing the microbial community present in the raw activated sludge from the municipal wastewater treatment plant and the pulp and paper industry wastewater, similar microbial phyla were dominated in both. Moreover, the presence of identified lipid producers

confirms that the pulp and paper industry wastewater sludge could be one of the future sources for microbial lipid production.

4.2. Biopolymer and organic acid production from pulp and paper wastewater sludge.

Biopolymer from wastewater sludge could be yet another possible value-added product could be produced by sludge born microbes. Technically the pulp and paper wastewater sludge composed of high COD and low in macronutrients such as nitrogen phosphorus, magnesium etc. This physicochemical characteristic of sludge makes it as a suitable carbon source for biopolymers such as PHA (Polyhydroxyalkanoates), Glycogens, PLA (Poly-lactate) etc. Based on the microbial community analysis (table 36), it has been identified that the major biopolymer producing microbial genera such as *Curvibacter, Rubrivivax, Pseudomonas, Acinetobacter, Aquaspirillum, Comamonas, Zoogloea, Bacillus, Methylobacter, Micropurina, Cupriavidus, and Enterobacter* were found in all the sludge samples analyzed (Martin Koller et al., 2010). Based on the microbial variations observed during different stages of fermentation, it can be assumed that at the initial stage of fermentation mainly the acidogenic genus such as *Acinetobacter, Proteiniclasticum, Cloacibacterium, Flavobacterium, Acidaminococcus, and Bacillus*, were found to produce a carboxylic acid. Among them, *Acidaminococcus and Proteiniclasticum* were mainly known for acetic acid, butyric acid, and propionate production (Wolfgang et al., 1981; Zhang et al., 2010). The biopolymers such as PHA/PHB and PLA production were already been established on the above-mentioned organic acids. The work carried out by Dias et al 2007 confirmed the feeding of excess acetate significantly improved the polymer production and the propionate feeding limited to approximately 0.17C-mol (Dias et al., 2008). This clearly indicates the mutual relationship between the microbial consortia present in the activated sludge sample. That is the polymer or lipid producing organisms could utilize the organic acids synthesized by the non-polymer producing bacteria. On another hand, the cost of the bioplastics production solely depends on the cost of raw materials that used for its production. Using the waste water sludge and microbes present in it could eliminate the need for inoculum preparation and at the same time, it reduces the negative impact of wastewater sludge on the environment.

4.3. EPS and industrial enzyme production from pulp and paper industry wastewater sludge.

Bio-flocculant is yet another value-added product can be produced from pulp and paper industry wastewater. Nutrient-limited condition with high carbon concentration was reported to be the

suitable condition for EPS production in *Escherichia, and Pseudomonas* genus(Sutherland, 2001). Moreover, the organic acid such as acetate, propionate, pyruvate, succinate was reported to a good carbon source for EPS production(Sutherland, 2001). The known EPS producers found in the analyzed wastewater sludge samples were *Rubrivivax, Pseudomonas, Acinetobacter, Zoogloea, Bacillus,* and *Prevotella* (Bala Subramanian et al., 2010; Geisinger & Isberg, 2015; Lim et al., 2007; Steunou et al., 2013; Yamanaka et al., 2011). In sludge disposal, bulking and dehydration is a primary concern. Sludge bulking can occur due to filamentous or non-filamentous microbial growth in sludge. At present scenario, chemical flocculants were used on the industrial scale. The previous studies on municipal wastewater sludge show the potential microbial consortium able to produce EPS were reported (Bala Subramanian et al., 2010). When these microbes were isolated and grown in mineral media resulted in higher than what observed in the consortium. This could be due to a high competition in the mixed ecosystem. Providing an appropriate importance to EPS production condition to the sludge could lead to an economic bio-flocculent production from waste streams from pulp and paper industry wastewater.

Meanwhile, the sludge born microbes were compared with the existing industrially important enzyme-producing microbes. Interestingly several microbes with the novel enzyme production were identified based on the available literature. The main reason for this ability is the presence of different complex organic compounds in the pulp and paper industry sludge (namely-cellulose, hemicellulose, and lignin). Based on the work reported by Berlemont and Martiny (2013) the phyla *Actinobacteria, Bacteroidetes Acidobacteria, Firmicutes, Proteobacteria* and *Thermotogae* have processed the putative genes responsible for endo and exocellulase production (Renaud & Adam, 2013). The other industrial enzymes such as chitinase from *Janthinobacterium (Kielak et al., 2013),* lipase and urease from *Cloacibacterium (Hyun et al., 2014),* lipase from *Acinetobacter (Zheng et al., 2011),* glutaconate-CoA transferase from *Acidaminococcus* (Wolfgang et al., 1981), exoxylanase, B-1,4 xylosidase from *Prevotella* (Ales et al., 1995) and amylase from *Bacillus* (Prajapati et al., 2014) were reported earlier. Moreover, other enzymes such as peroxidases were also a possible industrial important enzyme can be produced from waste substrates.

Other than the above-mentioned applications, microbes found in the sludge's were shown useful for the removal of phosphate, nitrate, polychloethylene, sulfur, and polyaromatic carbon reduction (Table 38).

5. Summary and conclusion

The microbial community analysis of pulp and paper mill waste water sludge shows the countless potential of different value added product formation and a rich biodiversity which works in a mutual relation. The microbial community structure was noticed to vary according to the fermentation time and the change in the microbial community is hypothesized due to the change in the metabolites present in the medium at different time point. The metabolites produced by the microbes could be used for the value-added product formation by sludge microbes. Further, the gene specific 16S rRNA gene analysis could reveal the possibilities of sludge utilization/ disposal methods in an effective and environmentally friendly way.

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References

Agot, A., Janne, B.U., Ingolf, F.N. 2001. Comparative phylogeny of the ammonia monooxygenase subunit A and 16SrRNA genes of ammonia-oxidizing bacteria. *FRMS Microbiology Letters*, **205**, 237-242.

Ales, G., Jennifer, M., Anne, S.D., Harry, J.F. 1995. A Xylan Hydrolase Gene Cluster in Prevotella ruminicola B14: Sequence Relationships, Synergistic Interactions,and Oxygen Sensitivity of a Novel Enzyme with Exoxylanase and ・-(1,4)-Xylosidase Activities. *Appl. Environ. Microbiol*, **61**(8), 2958-64.

Alvarez, H.M., Steinbuchel, A. 2002. Triacylglycerols in prokaryotic microorganisms. *Appl Microbiol Biotechnol*, **60**(4), 367-76.

Arcos-Hernandez, M.V., Gurieff, N., Pratt, S., Magnusson, P., Werker, A., Vargas, A., Lant, P. 2010. Rapid quantification of intracellular PHA using infrared spectroscopy: an application in mixed cultures. *J Biotechnol*, **150**(3), 372-9.

Bae, H.S., Moe, W.M., Yan, J., Tiago, I., da Costa, M.S., Rainey, F.A. 2006. Propionicicella superfundia gen. nov., sp. nov., a chlorosolvent-tolerant propionate-forming, facultative anaerobic bacterium isolated from contaminated groundwater. *Syst Appl Microbiol*, **29**(5), 404-13.

Bajpai, P. 2015. Generation ofWaste in Pulp and PaperMills. *Management of Pulp and Paper MillWaste,*, **Chapter 2**(15), 9-17.

Bala Subramanian, S., Yan, S., Tyagi, R.D., Surampalli, R.Y. 2010. Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering. *Water Res*, **44**(7), 2253-66.

Bengtsson, S., Hallquist, J., Werker, A., Welander, T. 2008. Acidogenic fermentation of industrial wastewaters: effects of chemostat retention time and pH on volatile fatty acids production. *Biochemical Engineering Journal,*, **40**(3), 492–499.

Chaudhary S, Rohella R, Manthan M, N, S. 2002. Decolorization of Kraft paper mill effluent by white rot fungi. *J Microbiol*, **38**, 221-224.

Dias, J.M., Oehmen, A., Serafim, L.S., Lemos, P.C., Reis, M.A., Oliveira, R. 2008. Metabolic modelling of polyhydroxyalkanoate copolymers production by mixed microbial cultures. *BMC Syst Biol*, **2**, 59.

Edgar, R.C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, **10**(10), 996-998.

Geisinger, E., Isberg, R.R. 2015. Antibiotic modulation of capsular exopolysaccharide and virulence in Acinetobacter baumannii. *PLoS Pathog*, **11**(2), e1004691.

Han, M.G., André, O.H., Ahmad, Y.A.R., Kok, G.C., Michael, A.S. 2013. Comparative genomic analysis of six bacteria belonging to the genus Novosphingobium: insights into marine adaptation, cell-cell signaling and bioremediation. *Gan et al. BMC Genomics 2013, 14:431*, **14**(431), 1-14.

Hori, K., Ichinohe, R., Unno, H., Marsudi, S. 2011. Simultaneous syntheses of polyhydroxyalkanoates and rhamnolipids by Pseudomonas aeruginosa IFO3924 at various temperatures and from various fatty acids. *Biochemical Engineering Journal*, **53**(2), 196-202.

Hyun, D.W., Shin, N.R., Kim, M.S., Kim, J.Y., Kim, P.S., Oh, S.J., Whon, T.W., Bae, J.W. 2014. Cloacibacterium haliotis sp. nov., isolated from the gut of an abalone, Haliotis discus hannai. *Int J Syst Evol Microbiol*, **64**(Pt 1), 72-7.

Ince, B.K., Cetecioglu, Z., Ince, O. 2011. Pollution Prevention in the Pulp and Paper Industries. *Environmental Management in Practice*, 223-246.

Kamika, I., Coetzee, M., Mamba, B.B., Msagati, T., Momba, M.N. 2014. The impact of microbial ecology and chemical profile on the enhanced biological phosphorus removal (EBPR) process: a case study of Northern Wastewater Treatment Works, Johannesburg. *Int J Environ Res Public Health*, **11**(3), 2876-98.

Kielak, A.M., Cretoiu, M.S., Semenov, A.V., Sorensen, S.J., van Elsas, J.D. 2013. Bacterial chitinolytic communities respond to chitin and pH alteration in soil. *Appl Environ Microbiol*, **79**(1), 263-72.

Kim, B.Y., Weon, H.Y., Cousin, S., Yoo, S.H., Kwon, S.W., Go, S.J., Stackebrandt, E. 2006. Flavobacterium daejeonense sp. nov. and Flavobacterium suncheonense sp. nov., isolated from greenhouse soils in Korea. *Int J Syst Evol Microbiol*, **56**(Pt 7), 1645-9.

Kizilova, A., Yurkov, A., Kravchenko, I. 2013. Aerobic Methanotrophs in Natural and Agricultural Soils of European Russia. *Diversity*, **5**(3), 541-556.

Kodama, Y., Ha le, T., Watanabe, K. 2007. Sulfurospirillum cavolei sp. nov., a facultatively anaerobic sulfur-reducing bacterium isolated from an underground crude oil storage cavity. *Int J Syst Evol Microbiol*, **57**(Pt 4), 827-31.

Lim, D.-J., Kim, J.-D., Kim, M.-Y., Yoo, S.-H., Kong, J.-Y. 2007. Physicochemical Properties of the Exopolysaccharides Produced by Marine Bacterium Zoogloea sp. KCCM10036. *J. Microbiol. Biotechnol*.

Lin, J., Hao, B., Cao, G., Wang, J., Feng, Y., Tan, X., Wang, W. 2014. A study on the microbial community structure in oil reservoirs developed by water flooding. *Journal of Petroleum Science and Engineering*, **122**, 354-359.

Magoc, T., Salzberg, S.L. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, **27**, 2957–2963.

Martin Koller , Aid Atlić , Miguel Dias , Angelika Reiterer , Braunegg, G. 2010. Microbial PHA Production from Waste Raw Materials. *Microbiology Monographs*, **14**.

Mondala, A.H., Hernandez, R., French, T., McFarland, L., Santo Domingo, J.W., Meckes, M., Ryu, H., Iker, B. 2012. Enhanced lipid and biodiesel production from glucose-fed activated sludge: Kinetics and microbial community analysis. *AIChE Journal*, **58**(4), 1279-1290.

Moreau, A., Montplaisir, D., Sparling, R., Barnabé, S. 2015. Hydrogen, ethanol and cellulase production from pulp and paper primary sludge by fermentation with Clostridium thermocellum. *Biomass and Bioenergy*, **72**, 256-262.

Nighat, N. 2012. Biosynthesis of polyhydroxybutyrate in Enterobacter sp. SEL2 and Enterobacteriaceae bacterium sp.PFW1 using sugar cane molasses as media. *African Journal of Biotechnology*, **11**(14).

Pantanella, F., Berlutti, F., Passariello, C., Sarli, S., Morea, C., Schippa, S. 2007. Violacein and biofilm production in Janthinobacterium lividum. *J Appl Microbiol*, **102**(4), 992-9.

Prajapati, V.S., Trivedi, U.B., Patel, K.C. 2014. A statistical approach for the production of thermostable and alklophilic alpha-amylase from Bacillus amyloliquefaciens KCP2 under solid-state fermentation. *3 Biotech*, **5**(2), 211-220.

Reis, M.A.M., Serafim, L.S., Lemos, P.C., Ramos, A.M., Aguiar, F.R., M., V.L.M.C. 2003. Production of polyhydroxyalkanoates by mixed microbial cultures. *Bioprocess and Biosystems Engineering*, **25**(6), 377–385.

Renaud, B., Adam, C.M. 2013. Phylogenetic Distribution of Potential Cellulases in Bacteria. *Appl. Environ. Microbiol*, 1545-54.

Shipeng, L., Karuna, C., Marco, R., Sandor, N., Manesh, B.S., Thomas, R.N., Robert, L.H., Kirsten, K. 2013. Insights into the Structure and Metabolic Function of Microbes That Shape Pelagic Iron-Rich Aggregates ("Iron Snow"). *Applied and Environmental Microbiology* **73**, 4272–4281.

Steunou, A.S., Liotenberg, S., Soler, M.N., Briandet, R., Barbe, V., Astier, C., Ouchane, S. 2013. EmbRS a new two-component system that inhibits biofilm formation and saves Rubrivivax gelatinosus from sinking. *Microbiologyopen*, **2**(3), 431-46.

Sun, R., Xing, D., Jia, J., Zhou, A., Zhang, L., Ren, N. 2014. Methane production and microbial community structure for alkaline pretreated waste activated sludge. *Bioresour Technol*, **169**, 496-501.

Supalak, S., Poonsuk, P. 2013. Characterization of a newly isolated Rubrivivax benzoatilyticus PS-5 with self-flocculation property and optimization pathway for 5-aminolevulinic acid production. *African Journal of Biotechnology*, **12**(16), 2069-2081.

Sutherland, I.W. 2001. Microbial polysaccharides from Gram-negative bacteria. *Int. Dairy J*, **11**, 663.

Thompson, G., Swain, J., Kay, M., Forster, C. 2001. The treatment of pulp and paper mill effluent: a review. *Bioresour Technol*, **77**, 275-286.

Tomoyoshi, S., Yoichi, K., Shunsuke, M., Satoshi, H., Takaaki, S., Wen-Tso, L., Kazunori, N. 2000. Micropruina glycogenica gen. nov., sp. nov., a new Gram-positive glycogen-accumulating bacterium isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology* **50**, 201-207.

Tucker, P. 2005. Co-composting paper mill sludges with fruit and vegetable wastes.

Tyagi, S., Kumar, V., Singh, J., Teotia, P., Bisht, S., Sharma, S. 2014. Bioremediation of Pulp and Paper mill Effluent by Dominant Aboriginal Microbes and Their Consortium. *Int. J. Environ. Res*, **8**(3), 561-568.

Ueki, A., Akasaka, H., Suzuki, D., Ueki, K. 2006. Paludibacter propionicigenes gen. nov., sp. nov., a novel strictly anaerobic, Gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan. *Int J Syst Evol Microbiol*, **56**(Pt 1), 39-44.

Wang, J., Yu, H.Q. 2007. Biosynthesis of polyhydroxybutyrate (PHB) and extracellular polymeric substances (EPS) by Ralstonia eutropha ATCC 17699 in batch cultures. *Appl Microbiol Biotechnol*, **75**(4), 871-8.

Wang, L. 2012. Ramlibacter ginsenosidimutans sp. nov., with Ginsenoside-Converting Activity. *Journal of Microbiology and Biotechnology*, **22**(3), 311-315.

Wolfgang, B.K., Ulrike, D., Roswitha, S. 1981. Glutaconate Co A-Transferase from Acidaminococcus fermentans. *Eur J Biochem*, **118**, 315-321.

Wu, S.C., Liou, S.Z., Lee, C.M. 2012. Correlation between bio-hydrogen production and polyhydroxybutyrate (PHB) synthesis by Rhodopseudomonas palustris WP3-5. *Bioresource Technology*, **113**, 44-50.

Yamanaka, T., Yamane, K., Furukawa, T., Matsumoto-Mashimo, C., Sugimori, C., Nambu, T., Obata, N., Walker, C.B., Leung, K.P., Fukushima, H. 2011. Comparison of the virulence of exopolysaccharide-producing Prevotella intermedia to exopolysaccharide non-producing periodontopathic organisms. *BMC Infect Dis*, **11**, 228.

Young, R.C., Kee, C.S., Hye, K.M., Dae, Y.S., Jin, S.N., Jongsik, C., Kyung, S.B. 1999. Kitasatospora cheerisanensis sp. nov., a new species of the genus Kitasatospora that produces an antifungal agent. *lnternational Journal of Systematic Bacteriology*, **49**, 753-758.

Zhang, K., Song, L., Dong, X. 2010. Proteiniclasticum ruminis gen. nov., sp. nov., a strictly anaerobic proteolytic bacterium isolated from yak rumen. *Int J Syst Evol Microbiol*, **60**(Pt 9), 2221-5.

Zheng, X., Chu, X., Zhang, W., Wu, N., Fan, Y. 2011. A novel cold-adapted lipase from Acinetobacter sp. XMZ-26: gene cloning and characterization. *Appl Microbiol Biotechnol*, **90**(3), 971-80.

CHAPITRE IX

CONCLUSIONS ET RECOMMENDATIONS

CONCLUSIONS

Le succès de la production d'huile microbienne à partir de microorganismes hétérotrophes dépend fortement de la capacité des microorganismes à accumuler des lipides à partir de différentes sources de carbone, le coût des matières premières ou des sources de carbone, des nutriments, les conditions d'opération et la durée de la fermentation. Compte tenu des paramètres présentés ci-dessus, la présente étude a été réalisée afin de produire des lipides pour la fabrication de biodiesel en utilisant de nouveaux isolats présentant des caractéristiques robustes comme la capacité à utiliser un large éventail de sources de carbone, la tolérance à des composants inhibiteurs, une croissance rapide et une grande accumulation de lipides dans un temps de production court. Neuf souches microbiennes différentes de bactéries, de levures et de champignons ayant un potentiel ont été isolées à partir d'échantillons de sol. Ces isolats ont été étudiés dans différentes conditions de culture pour identifier une souche susceptible de produire des lipides à partir du glycérol brut.

En se basant sur les tests initiaux à différentes concentrations de glycérol (100 et 200 g/L) et rapports C/N (100 et 200), la souche SKY7 a été identifiée comme étant une souche potentielle ayant la capacité de croitre dans les milieux de fermentation à base de glycérol brut pour la production de biomasse et de lipides. Par la suite, l'identification basée sur la technique moléculaire confirme que la souche SKY7 est *Yarrowia lipolytica*. Le profil d'utilisation des substrats (BIOLOG) montre que *Y.lipolytica SKY7* peut utiliser le glycérol et le glucose comme seule source de carbone. *Y.lipolytica* est également connu pour utiliser des substances hydrophobes, comme les acides gras libres (Free Fatty Acids), comme source de carbone. Dans les conditions expérimentales (concentration de glycérol 100 g/L avec un rapport C/N 100), *Y.lipolytica* a été en mesure de produire 42,04% p/p de lipides en 72h de fermentation. Par conséquent, *Y.lipolytica SKY7* a été choisi pour des études ultérieures.

Les paramètres de culture tels que le pH, la température, l'agitation et l'oxygène dissout (DO) sont des facteurs importants directement liés à la production de biomasse et de lipides. Dans l'étude actuelle, l'impact du pH (contrôle et sans contrôle) sur la production de biomasse et de lipides au niveau des fermenteurs a été étudié. L'expérience où le pH est contrôlé a entraîné une accumulation de lipides relativement élevé (45,5% p/p) en 60h de fermentation. Une diminution de 15% de l'accumulation de lipides a été observée avec les conditions de pH non contrôlées.

Ceci permet de conclure que le contrôle du pH au cours de la fermentation pourrait améliorer l'accumulation de lipides dans *Y.lipolytica* SKY7. Par la suite, selon les études réalisées, les paramètres importants tels que la concentration de glycérol, le rapport C/N, et le volume de l'inoculum ont été choisis pour une optimisation statistique. Une expérience complète a été élaborée et exécutée à l'aide de DOE (design expert). Les valeurs optimales obtenues à l'aide de cette expérience sont : une concentration de glycérol à 82,5 g/L, un rapport C/N 75 avec un volume d'inoculum de 6,25% v/v. Lorsque la condition ci-dessus a été reproduite au niveau du fermenteur, une biomasse de 29,5 g/L avec 50% d'accumulation de lipides a été obtenue. Le rendement de la biomasse et des lipides était 0,332 g/g et 0,179 g/g, respectivement. En se basant sur ces résultats, il a été conclu que les conditions de fermentation ci-dessus sont optimales pour *Y.lipolytica SKY7* dans des milieux de fermentation à base de glycérol.

L'étude sur l'utilisation des lixiviats de silos comme source d'azote apporte une nouvelle façon de produire de la biomasse et des lipides en utilisant les eaux usées industrielles en tant que source d'azote. Cette opération remplace les sources d'azote commerciales telles que l'extrait de levure, la peptone, les sels d'ammonium, etc. Les lixiviats de silos contiennent une DBO de 12 à 19 g/L et 1,5 à 4 g/L d'azote. Ils contiennent également des acides organiques qui peuvent agir en tant que source de carbone. Les études initiales avec les lixiviats de silos seuls ont résulté à une biomasse de 25,16 g/L (TSS) et 1,84 g/L de lipides. Lorsque le glycérol est ajouté au milieu, les concentrations de biomasse et de lipides ont été considérablement améliorées. Une concentration de biomasse de 25,82 g/L (TSS) avec une production lipidique de 15,15 g/L ont été atteintes après 51h de fermentation. Après 51h, la teneur en lipides des cellules était de 63,85% p/p. L'étude actuelle démontre une production de biomasse et de lipides élevée dans un court laps de temps. Le rendement en biomasse et en lipides (51h) sont de 83,45 g/mole de carbone consommé et 53,28 g/mole de carbone, respectivement. Par conséquent, les lixiviats de silos peuvent être l'une des sources nutritives appropriées pour la production de biomasse et de lipides. De plus, l'utilisation des lixiviats de silos permettra d'éliminer la pollution de l'environnement local.

Le méthanol est l'un des principaux constituants trouvés dans le glycérol brut obtenu comme sous-produit de la production du biodiesel. La teneur en méthanol varie dans le glycérol brut en fonction de la composition de l'huile utilisée pour la transestérification et l'efficacité de la réaction de transestérification. Le méthanol est considéré comme un inhibiteur de croissance

potentiel lorsqu'il se retrouve en forte concentration. Une expérience de fermentation non stérile profitant de la présence de méthanol dans le glycérol brut a été réalisée. Les études à l'échelle des erlenmeyers ont démontré qu'une concentration en méthanol de 3% v/v peut être utilisée dans le milieu de fermentation pour conduire un procédé exempt de contamination ou avec une faible contamination ayant une production de biomasse et de lipides importante. Lors de ces essais, il s'est révélé que *Y. lipolytica* peut croître lentement avec une concentration de méthanol de 5% v/v. Sur la base des études en erlenmeyers, des expériences en fermenteur de 15 litres à une concentration de méthanol de 3% v/v pour contrôler la croissance des contaminants ont été menées. Toutefois, la croissance de *Y.lipolytica* était lente et après 18h de fermentation, la croissance des contaminants a surpassé la croissance de *Y.lipolytica*. En raison de la contamination lors de la fermentation, la production de la biomasse et la concentration de lipides ont été significativement réduites (une biomasse de 6 g/L (54h) avec une concentration en lipides de 2,16 g/L). En raison de l'apparition de la contamination à un stade précoce de la fermentation, un traitement UV de l'eau du robinet a été effectué et l'eau traitée aux UV a été utilisé pour la préparation des milieux. Sous cette condition, des concentrations en biomasse et en lipides jusqu'à 17,57 et 8,8 g/L ont été atteintes, respectivement. Comparativement à l'essai avec l'eau non traitée aux UV, la biomasse obtenue est 3 fois plus importante, alors que dans le cas des lipides, l'augmentation est 4 fois supérieure. Suite aux résultats obtenus ci-dessus, il a été conclu que le méthanol et le traitement UV de l'eau du robinet peut s'avérer une alternative pour opérer la fermentation dans les conditions non stériles ou réduire au minimum la contamination pour produire une grande quantité de biomasse et de lipides.

Pour améliorer la teneur en lipides des cellules, une approche biochimique consistant à ajouter de la biotine a été étudiée. Les résultats obtenus à partir de l'étude suggèrent que la concentration en lipides est significativement améliorée de 10,12 g/L (témoin) à 16,16 g/L (biotine), lorsqu'il y a ajout de biotine à 100 µg/L au milieu de fermentation. Il n'y avait pas de différence significative dans la concentration de la biomasse lors des essais, ce qui suggère que, lorsque la biotine a été ajoutée, la production de lipides a été efficace par rapport au témoin. De plus, une addition intermédiaire ou par impulsion de sels de (d'ammonium) diammonium a également été testée. Les résultats démontrent une légère augmentation de la concentration en lipides et aucune augmentation significative de la concentration de la biomasse. Sur la base de l'étude ci-dessus, on peut conclure que l'addition de biotine favorisera significativement la production de lipides.

Une caractérisation de la communauté microbienne des boues des usines de pâtes et papier a été étudiée par rapport à la valeur ajoutée (lipides) du flux de déchets. La diversité des ressources microbiennes des boues des usines de pâtes et papier permet la production de lipides, d'acides organiques, des biopolymères, des enzymes industrielles, etc. Sur la base de l'analyse de la communauté, il était évident que les boues de l'industrie de pâtes et de papier pourraient être une ressource potentielle pour plusieurs applications à faible coût.

PARTIE 2

Recommandations

1) Dans l'étude actuelle, le mode de fermentation par «batch» a été utilisé. Toutefois, la fermentation de type «fed-batch» et en mode continu doivent être évalués en ce qui concerne l'augmentation de la concentration de la biomasse et des lipides.
2) La DO (oxygène dissous) est un autre facteur important qui a besoin d'être étudié. La DO a un fort impact sur la croissance et l'accumulation de lipides. Par conséquent, il doit être évalué et la relation entre la DO et la production de lipides doit être établie.
3) Des concentrations jusqu'à 200 ml/L de lixiviats de silos ont été étudiées. Toutefois, la composition des lixiviats de silos varie en fonction de la source (grains et traitement), d'où la nécessité d'évaluer des concentrations plus élevées (300 ml-500 ml/L).
4) Une étude de mise à l'échelle, avec des paramètres de culture optimales de *Y.lipolytica* SKY7 est nécessaire de démontrer l'efficacité du procédé à l'échelle industrielle. La cinétique du procédé complet doit être évaluée ainsi que l'estimation des coûts pour connaître le potentiel du procédé.
5) La production de lipase au même moment que l'utilisation de glycérol brut est un autre facteur intéressant. L'utilisation du bouillon fermenté (contenant des lipases) doit être testée comme source d'enzymes brutes pour traiter le savon contenu dans le glycérol brut.
6) La production d'acide citrique et son impact sur la production de lipides doivent être évalués. En même temps, la possibilité de récupération de l'acide citrique doit être évaluée.
7) Les enzymes intracellulaires en lien avec la production de lipides dans les essais de contrôle et supplémentés avec la biotine doivent être étudiées.
8) La possibilité d'utiliser les cellules (sans lipides) comme protéines animales doivent être évaluées puisque *Y.lipolytica* SKY7 est l'un des organismes reconnu comme étant généralement sécuritaire et qu'il produit des protéines monocellulaires et des lipides.

ANNEXES

ANNEXE 1

A substrate-based approach for the selection of oil bearing heterotrophs from nitrogen deficient soil for lipid production

(Raw Data of figures)

Figure 9 (phylogenetic tree of isolates showing evolutionary relationship with other closely relaed species (Partial DNA sequence of ITS region of isolats)

Candida membranifaciens >P13340_1_SF_A23_096.ab1 (SK)

CCTGCGGAAGGATCATTACTAGTAGTTTTAGTTTGTTGCCTGCGCTTAATTGCGCGGCGA

CAAGCAAACACCTTACACACTGTGTTTTTGTTTTATTAGAAACTTGCTTTGGTTTGGCGA

AAGCTGGGCCAAAGACTTACACAAACTTCAATTGCAAATTAGATTGTTTTAAATTTTTAT

TGTCAATTTTGTTTAGTTAATTTCAAAATAATCTTCAAAACTTTCAACAACGGATCTCTT

GGTTCTCGCATCGATAGAGAACGCAGCGAAATGCGATAAGTAATATAGATTGCAGATTTT

CGTAGATCATCGAATCTTTAGACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCT

GTTTAGCGTCATTTCTCCTTCAACCTTTGGGTTTGGTGTTAGGTAGTACTCTTATCAACT

AAGTGTTTGCTTCAAATATAATGGCAGGAGTAGCTAGTAGAACGAACTGTTATTCAATAG

A

Yarrowia lipolytica >P13340_2_SF_C23_094.ab1 (S7 LS)

GCGGAAGGACATTATTAGTTTTATCTATTTCTGTGGATTTCTATTCTATTACAGCGTCAT

TTTATCTCAATTATAACTATCAACAACGGATCTCTTGGCTCTCACATCGATAGAGAACGC

AGCGAACCGCGATATTTTTTGTAGCTTGCAGATGTAGATCATCAATCTTTAGACGCACAT

TGCGCGGTATGGCATTCCGTACCGCACGGATGGAGGAGCGTGTTCCCTCTGGGATCGCAT

TGCTTTCTTAGAATGGATTTTTTAAACTCTCAATTATTACGTCATTTCACCTCCTTCATC

CGAATTACCCGCTAGACTTAAGCATATCAATAAGCGGA

Yarrowia lipolytica >P13340_3_SF_E23_092.ab1(S7 SS)

GCGGAAGGCATTATTAGTTTTATCTATTTCTGTGGATTTCTATTCTATTACAGCGTCATT

TTATCTCAATTATAACTATCAACAACGGATCTCTTGGCTCTCACATCGATAGAGAACGCA

GCGAACCGCGATATTTTTTGTAGCTTGCAGATGTAGATCATCAATCTTTAGACGCACATT

GCGCGGTATGGCATTCCGTACCGCACGGATGGAGGAGCGTGTTCCCTCTGGGATCGCATT

GCTTTCTTAGAATGGATTTTTTAAACTCTCAATTATTACGTCATTTCCCTCCTTCATCCG

AATTACCCGCTAGACTTAAGCATATCAATAAGCGGA

Candida membranifaciens >P13340_4_SF_G23_090.ab1 (N1)

TGCGGAAGGATCATTACTAGTAGTTTTAGTTTGTTGCCTGCGCTTAATTGCGCGGCGACA
AGCAAACACCTTACACACTGTGTTTTTGTTTTATTAGAAACTTGCTTTGGTTTGGCGAAA
GCTGGGCCAAAGACTTACACAAACTTCAATTGCAAATTAGATTGTTTTAAATTTTTATTG
TCAATTTTGTTTAGTTAATTTCAAAATAATCTTCAAAATTTCACAACGGATCTCTTGGTT
CTCGCATCGATAGAGAACGCAGCGAAATGCGATAAGTAATATAGATTGCAGATTTTCGTG
AATCATCGAATCTTTAGACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTT
GAGCGTCATTTCTCCTTCAAACCTTTGGGTTTGGTGTTAGGTAGTACTCTTAGTCAACTA
AGTGTTTGCTTCAAATATAATGGCAGGAGTGTACTGGATAGTACGAACTGGTTATTCAAT
GTATTAGGTTTATCCAACTCGTTAGAGACTGGGGCAGTAAATTTCTAGTAATTGGCTCGG
CCTTATAATAACAAACTAAGTTTAGCCTCAATCAGTAAGAATACCCGCTAGCTTACATAT
CAATAACGGA

Candida membranifaciens >P13340_5_SF_I23_088.ab1 (S28 L)

AACCTGCGGAAGGACATTACTAGTAGTTTTAGTTTGTTGCCTGCGCTTAATTGCGCGGCG
ACAAGCAAACACCTTACACACTGTGTTTTTGTTTTATTAGAAACTTGCTTTGGTTTGGCG
AAAGCTGGGCCAAAGACTTACACAAACTTCAATTGCGAAATTAGATTGTTTTAAATTTTT
ATTGTCAATTTTGTTTAGTTAATTTCAAAATAACTTCAAAATTTCAACAACGGATCTCTT
GGTTCTCGCATCGATAGAGAACGCACGAAATGCGATAGTAATATAGATTGCGATTTTCGT
GAATCATCGAATCTTTAAGCACATTGCGCCCTTTGGTTCCAAAGGGCATGCGTTAGCGTC
ATTTCTCCTTAAACTTTGGGT

Candida membranifaciens >P13340_6_SF_K23_086.ab1 (A1)

GTAGTAGCCTGCGGAAGGATCATTACTAGTAGTTTTAGTTTGTTGCCTGCGCTTAATTGC
GCGGCGACAAGCAAACACCTTACACACTGTGTTTTTGTTTTATTAGAAACTTGCTTTGGT
TTGGCGAAAGCTGGGCCAAAGACTTACACAAACTTCAATTGCGAAATTAGATTGTTTTAA

ATTTTTATTGTCAATTTTGTTTAGTTAATTTCAAAATAATCTTCAAAACTTTCAACAAGG

ATCTCTTGGTTCTCGCATCGATAGAGAACGCAGCGAAATGCGATAAGTAATATAGATTGC

AGATTTTCGTAGATCATCGAATCTTTAGACGCACATTGCGCCCTTTGGTATTCCAAAGGG

CATGCCTGTTTAGGCGTCATTTCTCCTTCAAACCTTTGGGTTTGGTGTTAGGTAGTACTC

TTAGTCAACTAAGTGTTTGCTTCAAATATAATGGCAGGAGTGTACTGGATAGTACGAACT

GGTTATTCAATGTATTAGTTTATCCAACTCGTTAGAACTGGGGCATAAATTTCTAGTAAT

GTCGCTATAATAACAAACTAATTTAGC

Candida membranifaciens >P13340_7_SF_M23_084.ab1(RGA)

CTGCGGAAGGCATTACTAGTAGTTTTAGTTTGTTGCCTGCGCTTAATTGCGCGGCGACAA

GCAAACACCTTACACACTGTGTTTTTGTTTTATTAGAAACTTGCTTTGGTTTGGCGAAAG

CTGGGCCAAAGACTTACACAAACTTCAATTGCGAAATTAGATTGTTTTAAATTTTTATTG

TCAATTTTGTTTAGTTAATTTCAAAATAATCTTCAAAACTTTCAACAACGGATCTCTTGG

TTCTCGCATCGATAAGAACGCAGCGAAATGCGATAAGTAATATAGATTGCAGATTTTCGT

GAATCATCGAATCTTTAGACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTT

TAGGCGTCATTTCTCCTTCAAACCTTTGGGTTTGGTGTTAGGTAGTACTCTTAGTCAACT

AAGTGTTTGCTTCAAATATAATGGCAGGAGTGTACTGGATAGTACAACTGGTTATTCAAT

GTATTAGTTTATCCAACTCGTTAGAACTGGGGCAGAAATTTCTAGATTGTCGGCCTATAT

AACAAATA

Yarrowia lipolytica >P13340_8_SF_O23_082.ab1(S28 S)

CCTGCGGAAGGCATTATTAGTTTTATCTATTTCTGTGGATTTCTATTCTATTACAGCGTC

ATTTTATCTCAATTATAACTATCAACAACGGATCTCTTGGCTCTCACATCGATAAGAACG

CAGCGAACCGCGATATTTTTTGTAGCTTGCAGATGTAGATCATCAATCTTTAGACGCACA

TTGCGCGGTATGGCATTCCGTACCGCACGGATGGAGGAGCGTGTTCCCTCTGGGATCGCA

TTGCTTTCTTAGAATGGATTTTTTAAACTCTCAATTATTACGTCATTTCACCTCCTTCAT

CCGAATTACCCGCTAGACTTAAGCATATCAATAAGCGGAGAA

Figure 10 Glycerol assimilation with respect to biomass and lipid accumulation by SKY7 (*Y.lipolytica*)

Time t	Biomass	Lipid yeild	Carbon source concentration	Specific growth rate (U)	Lipid (product)yeild (Yps)	Biomass yeild (YXS)	Yxp
0	0.52	0	101.23	0	0	0.0051368	0
24	4.12	0.44	91.39	0.086	0.045	0.366	0.122
48	5.84	0.68	75.43	0.050	0.016	0.129	0.128
72	7.72	3.24	59.83	0.037	0.071	0.158	0.450
96	7.83	3.33	55.71	0.028	0.066	0.144	0.456
120	7.95	3.41	50.44	0.023	0.034	0.073	0.459

ANNEX 2

Elucidating the effect of glycerol concentration and C/N ratio of Lipid Production using *Yarrowia lipolytica* - SKY7

Raw Data of Figures

Figure 12, (A) biomass concentration obtained on different glycerol concentration, (B) the glycerol consumed on each fermentation and (C) lipid concentration

34.3 g/L glycerol

	BM avg	Bm stv	lip Avg	Lip Stv	gly avg	gly stv
0	0.68	0.04			34.54	0.15
6	1.58	0.11			33.40	0.29
9	1.88	0.04			32.27	0.15
12	2.45	0.35	0.82	0.18	30.10	0.00
18	3.78	0.11	0.92	0.04	28.66	0.29
24	5.10	0.14	1.15	0.21	27.73	0.15
30	5.08	0.04	1.67	0.11	26.19	0.29
36	5.38	0.11	1.80	0.14	24.12	0.58
42	6.60	0.14	1.88	0.11	21.34	0.44
48	8.60	0.45	2.27	0.04	16.37	0.15
60	10.10	0.03	2.45	0.14	12.38	0.01
72	11.60	0.57	3.10	0.07	8.82	0.15
94	11.48	0.46	3.15	0.00	6.35	0.12
120	11.80	0.06	3.95	0.35	4.46	0.07

52.3g/L Glycerol

	BM avg	Bm stv	lip Avg	Lip Stv	gly avg	gly stv
0	0.60	0.07			52.78	0.58
6	1.63	0.11			51.13	0.29
9	1.88	0.04			49.48	0.29
12	2.35	0.42	0.75	0.07	47.84	0.29
18	3.45	0.28	1.07	0.11	46.08	0.73
24	3.88	0.25	0.97	0.18	43.09	0.29
30	5.48	0.46	2.23	0.11	43.40	1.31
36	6.40	0.21	2.00	0.00	42.68	0.58
42	7.30	0.14	2.13	0.11	41.44	0.87
48	10.20	0.42	2.80	0.14	40.62	0.58
60	11.65	0.14	2.57	0.04	33.30	0.44
72	15.40	0.28	3.57	0.04	26.08	0.44
94	14.60	0.28	4.10	0.14	22.58	0.44
120	16.14	0.08	3.90	0.14	18.68	0.09

112.5g/L glycerol

	BM avg	Bm stv	lip Avg	Lip Stv	gly avg	gly stv
0	0.83	0.04			111.96	0.87
6	1.63	0.18			110.72	1.46
9	1.95	0.07			110.10	0.58
12	3.03	0.04	0.97	0.11	108.66	0.87
18	4.58	0.18	1.35	0.07	107.42	0.87
24	5.33	0.04	1.60	0.14	106.39	1.75
30	5.55	0.07	2.50	0.14	104.74	1.17
36	5.85	0.14	2.57	0.11	101.44	0.58
42	5.58	0.11	2.82	0.18	99.18	0.29
48	8.10	0.14	3.83	0.18	98.14	0.58
60	9.38	0.18	4.25	0.14	92.16	0.87
72	11.33	0.46	5.20	0.21	89.28	0.87
94	12.20	0.42	6.62	0.04	80.82	1.17
120	14.84	0.23	6.14	0.20	63.30	2.62

168.2g/L glycerol

	BM avg	Bm stv	lip Avg	Lip Stv	gly avg	gly stv
0	0.80	0.07			167.22	1.46
6	1.35	0.21			164.95	0.58
9	1.75	0.07			163.71	0.58
12	1.88	0.11	0.40	0.28	162.68	0.29
18	2.90	0.14	1.10	0.21	159.38	0.87
24	3.00	0.14	1.68	0.11	158.14	0.29
30	3.58	0.04	1.92	0.04	157.32	0.29
36	4.05	0.21	2.05	0.07	155.88	1.17
42	4.15	0.07	2.87	0.04	156.49	0.87
48	6.38	0.39	4.22	0.04	154.85	1.46
60	7.45	0.07	4.55	0.07	153.81	0.58
72	9.13	0.04	5.47	0.04	152.37	0.87
94	9.48	0.04	5.78	0.25	144.12	0.87
120	11.38	0.48	5.98	0.03	134.64	1.46

Figure 13 Effect of glycerol concentration on specific growth rate

Time t	34.3g/L	52.3g/L	112.5g/L	168.2g/L
0	0	0	0	0
6	0.141	0.166	0.113	0.087
9	0.114	0.127	0.096	0.087
12	0.107	0.114	0.108	0.071
18	0.096	0.097	0.095	0.072
24	0.084	0.078	0.078	0.055
30	0.067	0.074	0.064	0.050
36	0.058	0.066	0.054	0.045
42	0.054	0.059	0.045	0.039
48	0.053	0.059	0.048	0.043
60	0.045	0.049	0.041	0.037
72	0.040	0.045	0.036	0.034
94	0.030	0.034	0.029	0.026
120	0.024	0.027	0.024	0.022

Figure 14, A variation in biomass yield (Yx/s) and B lipid yield (Yp/s) upon different glycerol concentration

A (Yx/s)

Time t	34.3g/L	52.3g/L	112.5g/L	168.2g/L
0	0.020	0.011	0.007	0.005
6	0.794	0.621	0.647	0.243
9	0.271	0.386	0.341	0.209
12	0.302	0.354	0.485	0.137
18	0.456	0.425	0.674	0.231
24	0.530	0.338	0.624	0.222
30	0.423	0.520	0.449	0.245
36	0.356	0.574	0.393	0.303
42	0.326	0.591	0.344	0.271
48	0.358	0.789	0.368	0.416
60	0.367	0.567	0.377	0.448
72	0.388	0.554	0.337	0.361
94	0.359	0.463	0.234	0.266
120	0.322	0.456	0.125	0.063

B Yp/s

Time t	34.3g/L	52.3g/L	112.5g/L	168.2g/L
0	0	0	0	0
6	0.000	0.000	0.000	0.000
9	0.000	0.000	0.000	0.000
12	0.140	0.112	0.215	0.051
18	0.136	0.111	0.243	0.121
24	0.138	0.104	0.222	0.169
30	0.161	0.220	0.238	0.170
36	0.136	0.176	0.201	0.191
42	0.103	0.175	0.204	0.232
48	0.103	0.144	0.193	0.315
60	0.095	0.096	0.187	0.306
72	0.110	0.118	0.167	0.237
94	0.105	0.120	0.136	0.177
120	0.114	0.074	0.055	0.036

Figure 15 A Biomass concentration, B Glycerol concentration, and C lipid concentration obtained on different initial C/N molar ratio

C/N 25

	Biomass Avg	Biomass stv	lipid Avg	lipid stv	glyrol avg	glycerol stv
0	0.60	0.07			102.20	0.68
6	1.10	0.00			100.08	0.52
12	3.75	0.28	1.83	0.18	79.79	0.87
18	4.53	0.04	1.85	0.21	82.64	5.60
24	5.83	0.25	2.55	0.28	85.61	7.00
36	8.53	0.04	3.28	0.18	84.00	0.52
48	9.18	0.11	3.80	0.07	77.20	1.05
60	13.38	0.25	3.73	0.11	73.98	1.05
72	15.50	0.57	4.88	0.25	69.15	1.22
96	21.05	0.85	3.68	0.25	37.36	0.35
120	22.63	0.25	4.55	0.35	22.76	0.70

C/N 50

	bm Avg	bm stv	lip Avg	lip stv	gly avg	gly stv
0	0.55	0.28			97.36	0.87
6	1.30	0.00			94.02	0.35
12	3.40	0.21	1.83	0.18	92.78	1.05
18	4.55	0.35	2.03	0.04	90.68	0.17
24	5.98	0.25	2.85	0.14	88.21	0.87
36	8.83	0.46	3.08	0.11	84.12	0.70
48	12.03	0.53	3.13	0.32	82.89	0.70
60	12.70	0.35	3.45	0.49	79.05	0.52
72	14.55	0.35	5.00	0.07	69.03	1.05
96	17.85	0.71	3.80	0.78	50.23	0.70
120	18.88	1.17	4.92	0.18	32.04	0.17

C/N 100

	bm Avg	bm stv	lip Avg	lip stv	gly avg	gly stv
0	0.375	0.035			98.103	0.525
6	1.450	0.212			96.000	0.700
12	3.475	0.035	1.400	0.071	94.268	0.350
18	3.950	0.141	1.950	0.071	93.031	0.350
24	5.475	0.247	2.725	0.035	90.186	0.875
36	9.325	0.389	3.750	0.141	87.464	0.525
48	10.350	0.141	3.850	0.141	81.031	0.525
60	10.850	0.283	5.250	0.071	73.113	0.175
72	12.650	0.354	6.225	0.035	65.072	1.050
96	13.900	0.778	6.300	0.212	52.948	0.350
120	16.375	0.106	6.660	0.141	47.629	1.225

C/N 150

	bm Avg	bm stv	lip Avg	lip stv	gly avg	gly stv
0	0.55	0.14			98.23	0.35
6	1.43	0.25			96.25	0.35
12	3.70	0.35	1.50	0.07	92.29	0.70
18	4.00	0.07	1.70	0.28	90.19	0.52
24	5.45	0.28	2.00	0.07	87.46	0.17
36	9.63	0.81	3.62	0.04	84.00	0.52
48	9.83	0.81	4.43	0.25	81.90	0.35
60	11.93	0.18	5.30	0.00	72.99	0.35
72	12.43	0.04	5.33	0.39	67.30	0.35
96	12.33	0.18	5.13	0.32	62.23	0.52
120	13.58	0.32	5.40	0.06	58.14	0.70

Figure 16. Difference between the specific growth rate obtained on different C/N molar ratio

Time t	**25**	**50**	**100**	**150**
0	0	0	0	0
6	0.101023	0.143367	0.225399	0.158668
12	0.152715	0.151801	0.185535	0.158847
18	0.112247	0.117387	0.130808	0.11023
24	0.094708	0.099393	0.111709	0.095561
36	0.073717	0.077095	0.089265	0.079506
48	0.056819	0.064267	0.069121	0.060058
60	0.051737	0.052324	0.056083	0.051275
72	0.045162	0.045492	0.048868	0.043299
96	0.03706	0.036248	0.037632	0.03239
120	0.030249	0.029464	0.031472	0.026717

Figure 17 A Biomass yield and B lipid yield at various C/N molar ratio

A Yx/s

Time t	25	50	100	150
0	0.006	0.006	0.004	0.006
6	0.236	0.225	0.511	0.442
12	0.190	0.623	0.808	0.530
18	0.223	0.599	0.705	0.429
24	0.259	0.593	0.644	0.455
36	0.317	0.625	0.841	0.638
48	0.304	0.793	0.584	0.568
60	0.387	0.664	0.419	0.451
72	0.230	0.494	0.372	0.384
96	0.257	0.367	0.300	0.327
120	0.216	0.281	0.317	0.325

Yp/s

Time t	25	50	100	150
0	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000
12	0.110	0.273	0.276	0.253
18	0.105	0.221	0.246	0.211
24	0.126	0.215	0.256	0.186
36	0.131	0.212	0.220	0.255
48	0.135	0.171	0.154	0.271
60	0.113	0.122	0.159	0.210
72	0.075	0.106	0.138	0.172
96	0.046	0.058	0.125	0.142
120	0.045	0.051	0.068	0.135

Figure 18. Variation in substrate utilization (dg/dt)

Time t	25	50	100	150
0	0.000	0.000	0.000	0.000
6	0.353	0.557	0.351	0.330
12	1.034	0.381	0.320	0.495
18	0.920	0.371	0.282	0.447
24	0.733	0.381	0.330	0.448
36	0.561	0.368	0.296	0.395
48	0.521	0.302	0.356	0.340
60	0.470	0.305	0.416	0.421
72	0.459	0.393	0.459	0.430
96	0.675	0.491	0.470	0.375
120	0.662	0.544	0.421	0.334

ANNEX 3

Harnessing the effect of pH on lipid production in batch cultures of *Yarrowia lipolytica* – SKY7

Raw data of figures

Figure 20 Variation of biomass, glycerol and lipid concentration during fermentation with and without pH control

pH controlled			
Time	Biomass g/L	Lipid g/L	Glycerol g/L
0	0.88± 0.11	0± 0	107.84± 0.87
6	1.12± 0.11	0.25± 0.02	106.61± 1.4
12	4.7± 0.08	1.41± 0.02	106.19± 1.4
18	10.42± 0.02	3.72± 0.22	95.28± 1.7
24	14.06± 0.14	4.47± 0.11	89.22± 1.7
30	15.62± 0.36	5.39± 0.39	83.33± 1.1
36	16.36± 0.22	6.08± 0.6	77.77± 1.4
42	16.96± 0.05	6.66± 0.03	72.5±0
48	16.58± 0.19	6.72± 0.05	68.83± 0.17
54	17.24± 0.16	6.89± 0.03	68.5± 0.3
60	18± 0.05	7.77± 0.03	63.93± 0.1
66	18.36± 0.16	6.61± 0.14	62.43± 0.2
72	18.84± 0.05	6.18± 0.03	55.11± 0.8
78	19.7± 0.19	6.05± 0.08	48.77± 0.3
84	20.48± 0.33	6.09± 0.03	35.33± 0.07
90	20.96± 0.11	6.6± 0.28	32.79± 0.05
96	20.98± 0.25	6.69± 0.36	31.6± 0.1
102	21.04± 0.17	6.64± 0.45	30.84± 0.1
108	20.44± 0.45	6.20± 0.16	28.59± 0.04
114	20.68± 0.22	5.61± 0.36	26.9± 0.08
120	20.58± 0.48	5.80± 0.22	26.81± 0.02

pH-un-controlled			
Time	Biomass g/L	Lipid g/L	Glycerol g/L
0	0.84± 0.05	0	97.96± 2.03
6	2.36± 0.22	1.49± 0.08	94.25± 1.4
12	7.54± 0.31	3.02± 0.08	84.36± 0.8
18	12.5± 0.31	4.37± 0.42	79.62± 0.5
24	14.74± 0.19	5.32± 0.28	71.94± 0.08
30	16.14± 0.7	4.65± 0.36	70.44± 0.05
36	17.36± 0.33	5.45± 0.36	66.63± 0.2
42	18.5± 0.25	5.76± 0.28	61.62± 0.1
48	18.52± 0.05	5.82± 0.19	59.25± 0.3
54	19.34± 0.19	5.85± 0.02	55.57± 0.1
60	19.54± 0.59	5.77± 0.19	54.99± 0.1
66	19.72± 0.39	6.11± 0.16	54.72± 0.1
72	20.42± 0.03	6.64± 0.39	52.6± 0.2
78	20.6± 0.22	6.68± 0.22	48.07± 0.2
84	20.12± 0.11	6.66± 0.25	31.18± 0.08
90	20.46± 0.48	6.29± 0.14	30.85± 0.1
96	20.18± 0.48	6.49± 0.14	29.92± 0.5
102	20.34± 0.08	6.48± 0.34	28.64± 0.1
108	19.38± 0.53	5.61± 0.31	28.28± 0.03
114	19.3± 0.53	5.81± 0.19	27.45± 0.06
120	20.48± 0.22	5.59± 0.28	25.32± 0.2

Figure 21: Variation of biomass, lipid free biomass and lipid yield in the pH controlled (2A) and pH uncontrolled (2B) fermentation

2A

Time h	Lipid (product)yield (YL/S)	Biomass yield (YX/S)	Lipid free biomass yield (Yx'/s)
0	0	0.008	-0.01618
6	0.210	0.194	0.424945
12	0.113	0.304	0.463215
18	0.200	0.512	0.46724
24	0.183	0.538	0.381056
30	0.180	0.490	0.312582
36	0.172	0.438	0.266516
42	0.171	0.412	0.23019
48	0.171	0.399	0.240462
54	0.157	0.373	0.212691
60	0.171	0.377	0.239117
66	0.126	0.332	0.223406
72	0.105	0.304	0.216003
78	0.084	0.260	0.186174
84	0.081	0.261	0.179598
90	0.087	0.263	0.175758
96	0.087	0.261	0.175577
102	0.084	0.254	0.168584
108	0.077	0.242	0.175175
114	0.069	0.244	0.171542
120	0.054	0.183	-0.01618

2B

Time h	Lipid (product)yield (YL/S)	Biomass yield (YX/S)	Lipid free biomass yield (Yx'/s
0	0	0.009	0.001
6	0.405	0.410	0.201
12	0.165	0.365	0.280
18	0.168	0.448	0.312
24	0.193	0.505	0.340
30	0.149	0.488	0.304
36	0.150	0.455	0.307
42	0.149	0.456	0.280
48	0.137	0.417	0.294
54	0.136	0.431	0.299
60	0.134	0.433	0.281
66	0.135	0.416	0.259
72	0.133	0.392	0.196
78	0.100	0.296	0.188
84	0.099	0.287	0.196
90	0.093	0.288	0.185
96	0.094	0.279	0.187
102	0.093	0.280	0.183
108	0.080	0.263	0.174
114	0.080	0.254	0.143
120	0.057	0.200	0.001

Figure 22: Variation of Specific growth rate, specific lipid production rate and point productivity in the pH uncontrolled (3A) and pH uncontrolled (3B) fermentation

pH uncontrolled (3A)

Time h	Specific growth rate (μ)	Point productivity	U
0	0	0	0
6	0.040	0.043	0.106
12	0.140	0.193	0.061
18	0.137	0.383	0.024
24	0.115	0.127	0.010
30	0.096	0.153	0.009
36	0.081	0.113	0.006
42	0.070	0.097	0.003
48	0.061	0.010	0.001
54	0.055	0.030	0.005
60	0.050	0.147	-0.001
66	0.046	-0.193	-0.007
72	0.043	-0.073	-0.002
78	0.040	-0.020	0.000
84	0.037	0.007	0.002
90	0.035	0.083	0.002
96	0.033	0.017	0.000
102	0.031	-0.010	-0.002
108	0.029	-0.073	-0.004
114	0.028	-0.097	-0.002
120	0.026	0.030	0.002

pH uncontrolled (3B)

Time h	Specific growth rate (μ)	Point productivity	Specific lipid production rate
0	0	0	0
6	0.172	0.25	0.107
12	0.183	0.252	0.032
18	0.150	0.243	0.015
24	0.119	0.222	0.002
30	0.099	0.155	0.001
36	0.084	0.152	0.005
42	0.074	0.137	0.002
48	0.064	0.121	0.000
54	0.058	0.109	0.000
60	0.052	0.096	0.001
66	0.048	0.093	0.004
72	0.044	0.092	0.002
78	0.041	0.086	0.000
84	0.038	0.079	-0.002
90	0.035	0.070	-0.001
96	0.033	0.068	0.001
102	0.031	0.064	-0.004
108	0.029	0.052	-0.003
114	0.027	0.051	0.000
120	0.027	0.047	0.002

Figure 23: Specific lipid production rate of pH controlled and uncontrolled fermentation Vs specific growth rate

	pH controlled			pH uncontrolled	
Time h	**Specific growth rate (μ**	**Specific lipid production rate**		**Specific growth rate (μ)**	**Specific lipid production rate**
0	0	0	97.96	0	0
6	0.040	0.106	94.25	0.172	0.107
12	0.140	0.061	84.37	0.183	0.032
18	0.137	0.024	79.63	0.150	0.015
24	0.115	0.010	71.95	0.119	0.002
30	0.096	0.009	70.44	0.099	0.001
36	0.081	0.006	66.63	0.084	0.005
42	0.070	0.003	61.63	0.074	0.002
48	0.061	0.001	59.26	0.064	0.000
54	0.055	0.005	55.57	0.058	0.000
60	0.050	-0.001	54.99	0.052	0.001
66	0.046	-0.007	54.73	0.048	0.004
72	0.043	-0.002	52.61	0.044	0.002
78	0.040	0.000	48.07	0.041	0.000
84	0.037	0.002	31.18	0.038	-0.002
90	0.035	0.002	30.85	0.035	-0.001
96	0.033	0.000	29.93	0.033	0.001
102	0.031	-0.002	28.64	0.031	-0.004
108	0.029	-0.004	28.28	0.029	-0.003
114	0.028	-0.002	27.46	0.027	0.000
120	0.026	0.002	25.32	0.027	0.002

Figure 24: C/N evolution upon time in pH controlled and uncontrolled experiments

pH controlled

Time	Carbon in glycerol g	Nitrogen mg	C/N ratio
0	48.4	0.372	130.1075
12	44.6	0.206	216.5049
24	41	0.098	418.3673
36	34.7	0.0779	445.4429
48	30	0.068	441.1765
60	26.5	0.091	291.2088
72	22.3	0.09	247.7778
84	21.7	0.085	255.2941
96	21	0.085	247.0588
108	21	0.085	247.0588
120	20.6	0.092	223.913

pH uncontrolled

Time	Carbon in glycerol g	Nitrogen mg	C/N ratio
0	43.5	0.33	131.8182
12	39.8	0.122	326.2295
24	32.7	0.089	367.4157
36	33.9	0.077	440.2597
48	30.1	0.074	406.7568
60	26.1	0.072	362.5
72	22.2	0.07	317.1429
84	22.3	0.07	318.5714
96	23	0.066	348.4848
108	18.4	0.053	347.1698
120	14.3	0.053	269.8113

ANNEX 4

Optimization of culture parameters for lipid production using response surface methodology and elucidating the kinetics of lipid production at lab scale fermenters of new isolate *Y.lipolytica* SKY7

Raw data of figures

Research article 4

Figure 25- Variation of Biomass, glycerol, citric acid, and lipid concentration during fermentation in 15L fermenter

Time t	Biomass (g/L)	Biomass Stv	Lipid yield (g/L)	Lipid Stv	Glycerol concentration (g/L)	Glycerol Stv	Citric acid concentration (g/L)
0	1.42	0.023	0	0	84.50	1.4	0.49
6	3.12	0.054	2.04	0.11	83.68	0.95	
12	8.65	0.58	3.24	0.89	77.50	0.84	0.58
18	13.25	0.91	4.2	0.62	59.37	1.25	
24	15.62	1.02	4.37	1.08	47.01	1.6	1.04
30	18.23	0.67	4.87	0.73	36.30	0.69	
36	21.03	0.38	4.9	0.65	28.06	1.1	1.58
42	22.63	0.14	7.64	1.2	21.47	1.92	
48	24.32	1.2	8.2	0.95	17.76	1.7	2.25
54	26.45	0.51	10.2	1.1	14.06	0.61	
60	28.98	0.36	11.08	1.3	8.70	0.78	2.36
66	29.74	0.54	12.8	0.78	5.78	0.29	
72	29.46	1.06	15.16	0.66	6.72	0.11	2.52

Figure 26- Variation of the yield of biomass (Yx/s), lipid free biomass (Yx/s"), the yield of lipid (Yp/s) and yield of lipid content per gram of dry biomass (Yp/x) with fermentation time. The yield was calculated based on the average biomass and lipid obtained on duplicates

Time t	Lipid (product)yield (Yp/s)	Biomass yield (YX/S)	Lipid free biomass yield (Yx/s")	Yp/x
0	0	0.017	-0.41268	0
6	2.476	2.063	0.569749	1.200
12	0.129	0.288	0.303636	0.448
18	0.112	0.316	0.262223	0.355
24	0.091	0.295	0.247729	0.308
30	0.086	0.298	0.260646	0.290
36	0.078	0.311	0.215302	0.250
42	0.114	0.318	0.220273	0.360
48	0.116	0.325	0.210525	0.358
54	0.135	0.330	0.21742	0.408
60	0.141	0.350	0.197147	0.402
66	0.165	0.364	0.165605	0.452
72	0.179	0.332	-0.0168	0.541

Figure 27 Variation of specific growth rate and specific lipid production rate and lipid productivity with fermentation time

Time t	Specific growth rate (U)	Point productivity	U
0	0	0	0
6	0.131	0.34	0.087
12	0.151	0.270	0.021
18	0.124	0.233	0.007
24	0.100	0.182	0.004
30	0.085	0.162	0.002
36	0.075	0.136	0.011
42	0.066	0.182	0.012
48	0.059	0.171	0.009
54	0.054	0.189	0.009
60	0.050	0.185	0.007
66	0.046	0.194	0.011
72	0.042	0.211	0.007

ANNEX 5

Replacement of commercial nutrients sources by silo liquidate for biomass and lipid production using crude glycerol as a sole carbon source by *Y.lipolytica* SKY7

Raw Data of Figures

Research article 5

Figure 28 Biomass production of *Y.lipolytica SKY7* on different nitrogen sources in crude glycerol medium

Column1	(NH4)2SO4	YE	Silos 100	Silos 200
0	0.36	0.56	1.88	1.16
12	3	3.8	4	4.48
24	4.96	5.56	4.09	5.9
36	6.12	6.68	2.92	6.32
48	7.56	7.44	3.92	7.17
60	7.92	8.64	4.64	8.68
72	9.6	10.68	5.2	8.72

Figure 29 Lipid production of *Y.lipolytica SKY7* on different nitrogen sources in crude glycerol medium

	(NH4)2SO4	YE	Silos 100	Silos 200
0				
12	0.28	0.4	0.28	0.8
24	1.24	1.48	1.48	1.36
36	2.16	3.44	1.24	2.84
48	1.84	3.6	2.96	3.08
60	1.6	4.04	1.16	3.44
72	2.44	2.02	1.16	3.6

Figure 30 Glycerol consumption of *Y.lipolytica* on different nitrogen sources

Column1	AM	YE	SL 100	SL 200
0	53.87	51.31	41.69	48.76
12	45.93	45.79	40.13	45.93
24	41.69	45.23	39.57	42.11
36	39.28	30.08	38.86	37.58
48	37.16	26.12	37.87	36.31
60	36.45	25.13	36.17	34.33
72	33.91	22.30	34.33	32.21

Figure 31 Time course analysis of Fermentation conducted on a 15L scale with 200ml/L silos liquidate supplementation. The lines represent cumulative biomass, (blue), Lipid free biomass (Violet), Glycerol (green), soap (sky blue) and lipid (red).

Time	Biomass(g/L)	Lipid (g/L)	Lipid free biomass (g/L)	Glycerol (g/L)	Soap (g/L)
0	2.58	0.28	2.3	44.39588	17.53637
3	2.96	0.94	2.02	42.58144	16.81967
6	3.56	1.62	1.94	40.76701	16.10297
9	6.5	2.98	3.52	39.11753	15.45142
12	8.12	3.7	4.42	36.6433	14.4741
15	11.08	4.04	7.04	35.07629	13.85513
18	15.92	5.32	10.6	33.17938	13.10586
21	17.64	5.8	11.84	29.46804	11.63988
24	19.78	9.18	10.6	28.72577	11.34668
27	21.015	9.94	11.075	28.14845	11.11864
30	22.78	10.86	11.92	27.15876	10.72771
36	23.02	11.78	11.24	26.82887	10.5974
39	24.04	12.96	11.08	24.76701	9.782969
42	25	13.54	11.46	24.18969	9.554928
45	25.66	14.02	11.64	23.94227	9.457196
48	25.8	15	10.8	23.77732	9.392041
51	25.82	15.12	10.7	21.71546	8.577608
54	25.92	14.06	11.86	20.6433	8.154103
57	27.28	13.26	14.02	20.06598	7.926062
60	27.4	12.9	14.5	19.73608	7.795753
63	28.3	12.06	16.24	19.73608	7.795753
66	28.64	12.06	16.58	18.91134	7.469979
69	29.28	12.18	17.1	18.82887	7.437402
72	29.16	11.88	17.28	18.74639	7.404825

Figure 32 Specific growth rates

Time	Specific growth
0	0
3	-0.32253
6	-0.00337
9	0.151788
12	0.142666
15	0.142671
18	0.143932
21	0.129145
24	0.118538
27	0.107935
30	0.100189
36	0.083819
39	0.07862
42	0.074047
45	0.069755
48	0.065521
51	0.061684
54	0.058336
57	0.05626
60	0.053527
63	0.051544
66	0.0494
69	0.047604
72	0.045558

Figure 33 Lipase activities on fermentation broth

Time (h)	Optical density	Lipase activity U/mL
	0.0522	0.64227
12	0.519	9.777299
24	0.702	13.35851
36	0.837	16.00039
48	0.4915	9.239139
60	0.434	8.113894
72	0.291	5.31546

Figure 34 Yield of biomass and lipid produced and the time curse analysis of nitrogen content in the fermentation broth.

Time	Y x/s	Yp/s	Yp/x	Yxf/s (lipid free)
0				
3	17.0564	29.62427	1.736842	-12.5679
6	21.99378	30.07312	1.367347	-8.07935
9	60.48288	41.65913	0.688776	18.82375
12	58.19803	35.9273	0.617329	22.27073
15	74.27914	32.8576	0.442353	41.42154
18	96.85974	36.59468	0.377811	60.26506
21	82.16234	30.11528	0.366534	52.04706
24	89.39253	46.25544	0.517442	43.13709
27	92.40667	48.42139	0.524003	43.98527
30	95.44023	49.988	0.523762	45.45223
36	94.76057	53.31441	0.562622	41.44616
39	89.03877	52.61005	0.590867	36.42872
42	90.3641	53.4446	0.591436	36.9195
45	91.89894	54.70933	0.595321	37.18961
48	91.71674	58.14256	0.633936	33.57417
51	83.45067	53.28778	0.638554	30.16289
54	80.02667	47.24797	0.590403	32.7787
57	82.68016	43.44893	0.525506	39.23123
60	81.97038	41.67874	0.508461	40.29165
63	84.94272	38.90456	0.458009	46.03816
66	83.28031	37.64551	0.452034	45.6348
69	85.05032	37.90632	0.445693	47.144
72	84.39583	36.83189	0.436418	47.56394

Figure 35 Nitrogen concentration

Time	Nitrogen mg/L
0	662
6	557
12	319
18	245
24	214
30	197
36	187
42	177
48	177
54	175
60	171
72	184

ANNEX 6

Studies on influence of methanol in biomass and lipid production and development of a non-sterile fermentation process for lipid production from crude glycerol obtained from biodiesel industry

Raw Data of Figures

Figure 37. The evaluation of biomass and lipid obtained at 72h of fermentation and the percentages of lipid obtained per gram of dry biomass

Description	AVG BM	AVG lipid	AVG Lipid %
Sterile	16.86	7.5	44.47568
0% methanol	12.98	3.44	26.50288
1% methanol	14.1	4.9	34.75925
2% methanol	12	4.6	38.36978
3% methanol	9.88	4.88	49.39016
4% methanol	9.06	4.16	45.94608
5% methanol	4.92	2.76	56.11007

Figure 38. Time course analysis of biomass, lipid, glycerol and colony forming units of *Y.lipolytica* and the contaminant

Sample (h)	Cell Conc. (CFU/mL)		Residual glycerol (g/L)	Biomass Conc. (g/L)	Lipid Conc. (g/L)	Lipid content (%)
	YL	Contaminants				
0	2.40E+06		38.52	0.98	0.12	12.24
6	3.20E+06		35.88	1.26	0.16	12.69
12	4.40E+06		33.28	1.38	0.24	17.39
18	8.00E+06		31	1.98	0.36	18.18
24	9.00E+06	5.00E+06	29.52	2.64	0.6	22.72
30	1.00E+07	1.50E+08	28	3	0.8	26.66
36	1.50E+07	3.00E+08	22.64	3.52	1.16	32.95
42	1.90E+07	1.10E+09	17.64	4.92	1.64	33.33
48	2.10E+07	1.60E+09	14.68	5.8	2.04	35.17
54	3.00E+07	1.50E+09	13.45	6	2.16	36
60	2.00E+07	1.30E+09	13	5.42	1.88	34.68
66	1.00E+07	1.60E+09	12.7	5	1.72	34.4
72	8.00E+06	1.40E+09	11.4	4.9	1.72	35.1

Figure 39. Time course analysis of biomass, lipid, glycerol and soap determination during UV treated nan sterile fermentation supplemented with 3%v/v methanol as contamination controlling agent

Time	Biomass(g/L)	Biomass Stv	Lipid (g/L)	Lipid Stv	Glycerol concentration (g/L)	Soap concentration g/L
0	0.475	0.035355	0.36	0.042426	43.71	38
6	0.55	0.353553	0.70	0.180312	43.29	37.2
12	2.65	0.742462	1.57	0.371231	42.47	36.8
18	4.225	0.671751	2	0.424264	37.44	35.4
24	5.95	0.247487	2.65	0.070711	29.52	30.6
30	8.85	1.661701	3.47	0.477297	20.53	27.8
36	9.35	2.510229	4.5	0.954594	17.89	26
42	11.1	0.424264	5.07	0.654074	10.39	23
48	12.57	2.156676	5.95	0.919239	5.69	20.2
54	14.7	3.181981	6.45	1.025305	2.63	13.6
60	15.25	2.757716	7.15	1.202082	0	9
66	15.95	1.59099	7.8	0.848528	0	8.4
72	17.57	1.732412	8.8	0.707107	0	8

CFU Data

Sample (h)	Cell Conc. (cfu/mL)	
	YL	Contaminants
0	1.50E+05	
6	1.89E+05	
12	2.20E+06	
18	3.60E+06	
24	9.40E+06	
30	1.32E+07	
36	3.89E+07	
42	2.26E+08	1.10E+04
48	2.91E+08	8.60E+04
54	2.93E+08	1.82E+05
60	3.80E+08	2.10E+05
66	5.84E+08	2.60E+05
72	5.82E+08	4.40E+05

Figure 40. Lipase production with respect to fermentation time

Time	Optical density	Lipase activity U/ml
0	0.0577	0.749902
12	0.433	8.094325
24	0.606	11.47984
36	0.717	13.65205
48	0.525	9.894716
60	0.453	8.485714
72	0.384	7.135421

Figure 42. The point yield of biomass, lipid free biomass, lipid, and yield of lipid per gram of biomass

Time	Y x/s g/C mole	Yp/sg/C mole	Yp/x g/g	Yx/s"g/C mole
0				
6	10.19916	46.91616	4.6	-36.717
12	121.146	68.46156	0.565116	52.68441
18	48.4433	21.18587	0.437333	27.25743
24	30.44733	12.67716	0.416364	17.77017
30	29.13399	10.80385	0.370833	18.33013
36	27.51569	12.79943	0.465169	14.71625
42	25.55724	11.3414	0.443765	14.21584
48	25.37504	11.72285	0.461983	13.65219
54	26.6532	11.41076	0.42812	15.24245
60	25.52252	11.72913	0.45956	13.79339
66	26.58946	12.80425	0.481553	13.78522
72	29.35647	14.48939	0.493567	14.86708

Figure 43. Specific growth rate of *Y.lipolytica* on UV treated nonsterile fermentation with 3%v/v methanol added medium

Time	Specific growth
0	0
6	-0.43171
12	0.063789
18	0.073431
24	0.071031
30	0.070941
36	0.060724
42	0.056267
48	0.051942
54	0.049167
60	0.044882
66	0.041479
72	0.039432

Figure 44. Specific substrate utilization rate of *Y.lipolytica* on UV treated nonsterile fermentation with 3%v/v methanol added medium

Time	Specific Substrate utilization rate
0	0
6	0.508169
12	0.538044
18	0.700476
24	0.453841
30	0.190804
36	0.144176
42	0.119534
48	0.127374
54	0.112
60	0.050125
66	0.009231
72	-0.01277

ANNEX 7

A biochemical approach to improving the biomass and lipid production using biotin and intermediate supply of Diammonium sulfate

Raw Data of Figures

Figure 45 Comparision of biomass concentration at different biotin concentration

Time	Controll	10µg/L	50µg/L	100µg/L
0	3.00	0.60	0.60	0.60
6	4.00	3.50	5.86	6.05
12	7.05	5.00	7.05	7.05
18	8.95	6.95	8.25	8.65
24	8.65	8.55	9.40	11.60
30	11.15	9.40	10.10	12.95
36	14.30	12.00	11.15	15.50
42	14.55	13.05	12.15	16.45
48	14.50	13.80	13.95	16.10
60	14.60	13.15	16.70	18.55
72	14.95	14.55	16.25	18.65

Figure 46 Effect of biotin on lipid concentration

Time	Controll	10µg/L	50µg/L	100µg/L
0				
6	1.30	1.00	1.00	1.25
12	1.70	1.05	1.15	2.30
18	2.00	2.85	3.10	3.35
24	3.20	3.25	3.25	4.30
30	4.05	3.70	4.15	5.75
36	4.20	3.95	4.30	5.85
42	4.55	3.80	4.95	5.95
48	5.15	4.55	7.45	6.75
60	5.00	6.45	7.25	8.75
72	5.60	6.10	7.35	9.50

Figure 47 Time course analysis of glycerol concentration on control and biotin supplemented studies

Time	Control	10µg/L	50µg/L	100µg/L
0	44.14	46.21	48.28	46.30
12	40.01	41.60	43.99	42.50
24	35.07	40.52	46.46	35.90
36	32.63	36.07	41.92	32.11
48	33.00	32.28	39.62	27.90
60	28.55	26.09	32.77	22.13
72	21.25	17.51	16.85	16.11

Figure 48 Evolution of biomass in biotin, biotin with intermediate supplementation of diammonium salt and control studies.

	Biomass		
Time	Biotin	Biotin NH_4	Control
0	0.4	0.2	0.28
6	2.32	1.92	2.68
12	15.08	9.64	13.04
18	20.12	18.6	17.56
24	22.84	22.96	23.64
30	24.08	24.96	24.64
36	25.04	26	26.44
42	25.84	26.68	26.08
48	26.36	26.8	27.08
60	27	27.64	27.44
66	27.16	27.72	26.52
72	27.6	27.72	26.56

Figure 49 Comparison of lipid-free biomass in biotin with intermediate supplementation of diammonium salt and control studies

Lipid free Biomass			
Time	Biotin	Biotin NH_4	Control
0	0.4	0.2	0.28
6	1.24	0.8	-0.16
12	12.72	7.44	10.04
18	16.92	13.68	13.92
24	18.48	16.4	18.52
30	18.92	17.72	17.72
36	19.8	17.04	18.92
42	19.44	17.08	17.4
48	16.92	16	17.2
60	15.04	16.36	16.88
66	11.96	16.28	16.28
72	11.44	14.96	16.44

Figure 50 Effect of biotin on lipid production at reactor level

Lipid			
Time	Biotin	Biotin NH_4	Control
0	0	0	0
6	1.08	1.12	2.84
12	2.36	2.2	3
18	3.2	4.92	3.64
24	4.36	6.56	5.12
30	5.16	7.24	6.92
36	5.24	8.96	7.52
42	6.4	9.6	8.68
48	9.44	10.8	9.88
60	11.96	11.28	10.56
66	15.2	11.44	10.24
72	16.16	12.76	10.12

Figure 51 Time course analysis of nitrogen content in fermentation broth

Nitrogen concentration mg/L			
Time	Biotin	Biotin NH_4	Control
0	531	526	554
6	395	439	494
12	100	186	243
18	91	136	127
24	87	137	114
30	87	74	93
36	87	71	100
42	83	73	92
48	80	68	98
60	79	68	100
66	81	68	99
72	66	63	95

Figure 52 production of citric acid in different cultivation conditions

Citric acid g/L			
Time	Biotin	Biotin NH_4	Control
0	0.25	0.25	0.50
6	0.60	0.45	0.51
12	0.75	0.60	0.91
18	1.34	1.13	2.62
24	1.12	2.83	5.04
30	2.17	2.62	5.64
36	2.16	3.51	6.96
42	2.59	3.88	7.46
48	2.96	3.26	7.96
60	3.72	2.62	8.04
66	3.58	2.23	7.46
72	0.67	1.45	7.18

Figure 53 Lipase production in different cultivation condition

	Time	Optical density	Lipase activity U/mL
Biotin 15L	0	0.214	3.808611
	6	0.147	2.497456
	12	0.868	16.60705
	18	0.494	9.288063
	24	0.541	10.20783
	30	0.46	8.622701
	36	0.466	8.740117
	42	0.452	8.466145
	48	0.395	7.350685
	60	0.361	6.685323
	66	0.323	5.941683
	72	0.304	5.569863
Biotin +NH_4	0		-0.37926
	6	0.142	2.399609
	12	0.158	2.71272
	18	0.873	16.70489
	24	0.314	5.765558
	30	0.362	6.704892
	36	0.23	4.121722
	42	0.226	4.043444
	48	0.219	3.906458
	60	0.151	2.575734
	66	0.15	2.556164
	72	0.156	2.673581
Control	0	0.124	2.047358
	6	0.39	7.252838
	12	0.376	6.978865
	18	0.492	9.248924
	24	0.38	7.057143
	30	0.225	4.023875
	36	0.146	2.477886
	42	0.142	2.399609
	48	0.134	2.243053
	60	0.131	2.184344
	66	0.141	2.380039
	72	0.135	2.262622

Figure 54. The yield of cumulative biomass at different time intervals

Yield of biomass g/moles of carbon			
Time	Biotin	Biotin NH_4	Control
0			
6	56.24722	64.88663	241.0611
12	55.07628	59.08365	63.96726
18	51.73289	64.26808	45.05788
24	46.5354	46.84389	49.50477
30	47.18606	44.40835	46.84058
36	45.69893	44.60348	47.22676
42	47.02474	43.76617	45.01979
48	47.2576	43.96451	46.52597
60	48.25063	44.74322	45.26936
66	46.91582	44.30464	42.71749
72	47.16123	44.27899	42.88715

Figure 55 Yield of lipid-free biomass at different time points

Yield of lipid-free biomass g/moles of carbon			
Time	Biotin	Biotin NH_4	Control
0			
6	24.60816	22.63487	-9.45338
12	46.22205	45.31416	48.58273
18	43.3381	47.08335	35.48224
24	37.49376	33.34231	38.32279
30	36.90397	31.42303	33.51204
36	35.98049	29.11328	33.32803
42	35.19461	27.89928	30.11506
48	30.07302	26.11426	30.076
60	26.55599	26.35024	27.52855
66	20.26707	25.8873	25.87081
72	19.14191	23.74847	25.91507

Figure 56 Yield of lipid in biotin and biotin with intermediate diammonium salt added and control experiments at different time scale

Yield of lipid-free biomass g/moles of carbon			
Time	Biotin	Biotin NH_4	Control
0			
6	31.63906	42.25176	250.5145
12	8.854224	13.7695	15.38453
18	8.394789	17.18473	9.575639
24	9.041637	13.50158	11.18197
30	10.2821	12.98532	13.32854
36	9.718442	15.4902	13.89873
42	11.83012	15.86689	14.90473
48	17.18458	17.85025	16.44998
60	21.69464	18.39299	17.74081
66	26.64875	18.41733	16.84668
72	28.01932	20.53052	16.97208

Figure 57 Evolution of lipid content in cell on different cultivation conditions

Yield of lipid-free biomass g/moles of carbon			
Time	Biotin	Biotin NH_4	Control
0			
6	0.5625	0.651163	0.785714
12	0.160763	0.233051	0.23511
18	0.162272	0.267391	0.210648
24	0.194296	0.288225	0.219178
30	0.217905	0.292407	0.284072
36	0.212662	0.347287	0.287462
42	0.251572	0.362538	0.336434
48	0.363636	0.406015	0.368657
60	0.449624	0.408683	0.392012
66	0.568012	0.411079	0.388807
72	0.594118	0.463663	0.390244

ANNEX 8

Diversity and Composition of Bacterial Communities Vary in Sludge of Different Pulp and Paper Industrial Wastewater

Raw Data of Figures

Figure 59 and Figure 60-Dendrogram showing the similarity of microbiome in different samples were prepared using the following script in R software

R version 3.1.1 was used for this script.

```
# The aim of this analysis is to classify the isolates according to their
# molecule signature. Strains will be classified into clusters and these
# cluster will be visualized in a Principal Component Analysis. This will allow
# us to interpret the cluster and relate them to patient or "small colony variant".
# ************ Cluster analysis **************
# Load required libraries
library(ade4)
library (vegan)  # should be loaded after ade4 to avoid some conflicts!
library (gclus)
library(cluster)
library(RColorBrewer)
library(labdsv)
library(mvpart)
library(MVPARTwrap)
library(ape)
library(clustsig)
# Specification of the location of the data files
setwd("C:\\RBook\\")
# Import the data from CSV files
env <- read.csv("OTU_table_U_results_rarefied7378_filtered_time_zero.csv", row.names=1)
#############################################################################
# STEP 1: We need to identify the best model fitting to our dataset.
```

```
#############################################################################
# Ribotyping data: compute Euclidean distance matrix of standardized
# absolute abundance of ribotypes See the paper by Legendre & Gallagher, 2001 for
# more details - we have selected Hellinger transformation due to several zero values in our
# dataset.
# ************************************************************
spe.norm <- decostand(env, "hellinger")
spe.ch <- dist(spe.norm, method = "euclidean")
# Compute single-linkage agglomerative clustering
spe.ch.single <- hclust(spe.ch, method="single")
# Plot a dendrogram using the default options
windows(title="Biolog - Standardized - Single linkage",12,8)
plot(spe.ch.single)
# Compute complete-linkage agglomerative clustering
# ************************************************
spe.ch.complete <- hclust(spe.ch, method="complete")
windows(title="Biolog - Standardized - Complete linkage",12,8)
plot(spe.ch.complete)
# Compute UPGMA agglomerative clustering. This is the most common approach in literature.
# If another model is selected (e.g. Single-linkage), make sure you explain to the reader
# the reason why you choose it.
# ************************************
spe.ch.UPGMA <- hclust(spe.ch, method="average")
windows(title="Biolog - Standardized - UPGMA",12,8)
plt(spe.ch.UPGMA)
```

```
# Compute Ward's minimum variance clustering
# ****************************************
spe.ch.ward <- hclust(spe.ch, method="ward")
windows(title="Biolog - Standardized - Ward",12,8)
plot(spe.ch.ward)
# The next command will produce data more comparable to the other model (the
# scale of the distances is adjusted by the square root).
windows(title="Biolog - Standardized - sqrt(Ward)",12,8)
spe.ch.ward$height <- sqrt(spe.ch.ward$height)
plot(spe.ch.ward)
# Cophenetic correlation. This will allow us to determine which model is the
# best in explaining our dataset (the ramifications represented by the models
# are compared to the real distance among the different samples). Correlations
# are then calculated between modelled and real distances. The higher is the
# correlation score, the better is the model(THIS IS AN ASSUMPTION as it is not
# possible to calculate a significance level to the correlation scores). In the
# utilized in the following command, Pearson correlations are specified since
# the argument "Method" of the command "cor" is not specified. This imply that
# normal distribution is assumed for the individual variables.
# **********************
# Single linkage clustering
spe.ch.single.coph <- cophenetic(spe.ch.single)
cor(spe.ch, spe.ch.single.coph)
# Complete linkage clustering
spe.ch.comp.coph <- cophenetic(spe.ch.complete)
```

```
cor(spe.ch, spe.ch.comp.coph)
# Average clustering
spe.ch.UPGMA.coph <- cophenetic(spe.ch.UPGMA)
cor(spe.ch, spe.ch.UPGMA.coph)
# Ward clustering
spe.ch.ward.coph <- cophenetic(spe.ch.ward)
cor(spe.ch, spe.ch.ward.coph)
# These correlations could also being computed using Spearman correlation. This
# method does not assume normal distribution of the variables.
cor(spe.ch, spe.ch.single.coph, method="spearman")
cor(spe.ch, spe.ch.comp.coph, method="spearman")
cor(spe.ch, spe.ch.UPGMA.coph, method="spearman")
cor(spe.ch, spe.ch.ward.coph, method="spearman")
# Shepard-like diagrams. This will give us an overview of the calculated
# correlations.
windows(title="Cophenetic correlation",8,9)
par(mfrow=c(2,2))
plot(spe.ch, spe.ch.single.coph, xlab="Standardized distance",
ylab="Cophenetic distance", asp=1, xlim=c(0,12), ylim=c(0,12),
main=c("Single linkage",paste("Cophenetic correlation ",
round(cor(spe.ch, spe.ch.single.coph),3))))
abline(0,1)
lines(lowess(spe.ch, spe.ch.single.coph), col="red")
plot(spe.ch, spe.ch.comp.coph, xlab="Standardized distance",
ylab="Cophenetic distance", asp=1, xlim=c(0,12), ylim=c(0,12),
```

```
main=c("Complete linkage", paste("Cophenetic correlation ",
round(cor(spe.ch, spe.ch.comp.coph),3))))
abline(0,1)
lines(lowess(spe.ch, spe.ch.comp.coph), col="red")
plot(spe.ch, spe.ch.UPGMA.coph, xlab="Standardized distance",
ylab="Cophenetic distance", asp=1, xlim=c(0,12), ylim=c(0,12),
main=c("UPGMA", paste("Cophenetic correlation ",
round(cor(spe.ch, spe.ch.UPGMA.coph),3))))
abline(0,1)
lines(lowess(spe.ch, spe.ch.UPGMA.coph), col="red")
plot(spe.ch, spe.ch.ward.coph, xlab="Standardized distance",
ylab="Cophenetic distance", asp=1, xlim=c(0,12),
ylim=c(0,max(spe.ch.ward$height)),
main=c("Ward clustering", paste("Cophenetic correlation ",
round(cor(spe.ch, spe.ch.ward.coph),3))))
abline(0,1)
lines(lowess(spe.ch, spe.ch.ward.coph), col="red")
#########################################################################
# STEP 2: We need to identify how many clusters we should define in the selected
# dendrogram.
#########################################################################
# Graphs of fusion level values. Fusion level value represents the distance between
# a pair of samples bridged by a node. The dendrogram possess a maximum of N cluster
# where N represent the number of sample. The graph of fusion level will show the
# weight of each cluster in term of represented distance. At some point, addition of
```

```
# new cluster will not bring ecologically-relevant differences among site (topology
# impossible to interprete).
# ****************************
windows(title="Fusion levels",12,8)
par(mfrow=c(2,2))
# Plot the fusion level values of the single linkage clustering
summary(spe.ch.single)  # List of available results
plot(spe.ch.single$height, nrow(env):2, type="S",
main="Fusion levels - Standardized - Single",
ylab="k (number of clusters)", xlab="h (node height)", col="grey")
text(spe.ch.single$height, nrow(env):2, nrow(env):2, col="red", cex=0.8)
# Plot the fusion level values of the complete linkage clustering
plot(spe.ch.complete$height, nrow(env):2, type="S",
main="Fusion levels - Standardized - Complete",
ylab="k (number of clusters)", xlab="h (node height)", col="grey")
textspe.ch.complete$height, nrow(env):2, nrow(env):2, col="red", cex=0.8)
# Plot the fusion level values of the UPGMA clustering
plot(spe.ch.UPGMA$height, nrow(env):2, type="S",
main="Fusion levels - Standardized - UPGMA",
ylab="k (number of clusters)", xlab="h (node height)", col="grey")
text(spe.ch.UPGMA$height, nrow(env):2, nrow(env):2, col="red", cex=0.8)
# Plot the fusion level values of the Ward clustering
plot(spe.ch.ward$height, nrow(env):2, type="S",
main="Fusion levels - Standardized - Ward",
ylab="k (number of clusters)", xlab="h (node height)", col="grey")
```

```
text(spe.ch.ward$height, nrow(env):2, nrow(env):2, col="red", cex=0.8)
# Optimal number of clusters according to Mantel statistic (Pearson)
# ************************************************************************
# Function to compute a binary distance matrix from groups
grpdist <- function(X)
{
require(cluster)
gr <- as.data.frame(as.factor(X))
distgr <- daisy(gr, "gower")
distgr
}
# Run based on the UPGMA clustering
kt <- data.frame(k=1:nrow(env), r=0)
for (i in 2:(nrow(env)-1)) {
gr <- cutree(spe.ch.UPGMA, i)
distgr <- grpdist(gr)
mt <- cor(spe.ch, distgr, method="pearson")
kt i,2 <- mt
}
kt
k.best <- which.max(kt$r)
# The plot is produced by function plot.silhouette {cluster}
windows(title="Optimal number of clusters - Mantel")
plot(kt$k, kt$r, type="h", main="Mantel-optimal number of clusters - UPGMA",
xlab="k (number of groups)", ylab="Pearson's correlation")
```

```
axis(1, k.best, paste("optimum", k.best, sep="\n"), col="red", font=2,
col.axis="red")
points(k.best, max(kt$r), pch=16, col="red", cex=1.5)
# Silhouette plot of the final partition. This will help us to figure out if
# grouped samples really belong to the identified clusters (they must be all
# positive or negative in the cluster - samples with different magnitude are
# considered not so well classified. Again, this is not a statistical test (no
# significance level is calculated).
# ************************************
# Choose the number of clusters (we select according to the Mantel statistic AND
# OUR OWN INTERPRETATION OF THE CLUSTERS).
k <- 5
# Silhouette plot
cutg <- cutree(spe.ch.UPGMA, k=k)
sil <- silhouette(cutg, spe.ch)
silo <- sortSilhouette(sil)
rownames(silo) <- row.names(env)attr(silo,"iOrd")
windows(title="Silhouette plot - UPGMA - K=9")
plot(silo, main="Silhouette plot - Srandardized - UPGMA",
cex.names=0.8, col=cutg+1, nmax.lab=100)
##########################################################################
# STEP 3: We combine clustering and ordination results.
##########################################################################
#Cut the dendrogram to yield 5 groups
gr <- cutree(spe.ch.UPGMA, k=5)
```

```
grl <- levels(factor(gr))

spe.chwo <- reorder.hclust(spe.ch.UPGMA, spe.ch)

plot(spe.chwo, hang=-1, xlab="5 groups", sub="", ylab="Sites", main="UPGMA",
labels=cutree(spe.chwo, k=5))

rect.hclust(spe.chwo, k=5)

source("hcoplot.R")

hcoplot(spe.ch.UPGMA, spe.ch, k=5)

##############################################################################

# STEP 4: Number of significant clusters

##############################################################################

sim <- simprof(data=spe.norm, num.expected=1000, num.simulated=999,
method.cluster="average", method.distance="euclidean",

method.transform="identity", alpha=0.05, sample.orientation="row", const=0, silent=TRUE,
increment=100, undef.zero=TRUE, warn.braycurtis=TRUE)

##############################################################################

# ANNEX : We will calculate diversity indexes

##############################################################################

# We start with Shannon diversity Index

H <- diversity(env)

summary(H)

H

# We then calculate the Simpson diversity Index

simp <- diversity(env, "simpson")

summary(simp)

simp
```

Figures 61Heat map of OTUS

OUT	A87_ Kraft	B88_ Kraft	A89_Kraft WAS	A84_ CTMP	B90_ CTMP	A91_Dolbeau	B92_Dolbeau	A93_Dolbeau	B94_Dolbeau	C86_ CTMP
OUT_94_Spirosoma	81	44	1	0	0	0	0	0	0	0
OUT_59_Terrimonas	82	45	3	0	0	0	0	0	0	0
OUT_119_Hydrocarboniphaga	28	70	2	0	0	0	0	0	0	0
OUT_182_Ilumatobacter	15	11	1	0	0	0	0	0	0	0
OUT_472_Microbacterium	5	1	1	0	0	0	0	0	0	0
OUT_358_Caloramator	5	1	1	0	0	0	0	0	0	0
OUT_20_Bacillus	233	87	8	0	12	0	0	0	0	0
OUT_10_Hippea	406	170	84	0	25	0	0	1	0	0
OUT_206_Aciditerrimonas	16	7	1	0	1	0	0	0	0	0
OUT_112_Ferrimicrobium	48	19	6	0	4	0	0	0	0	0
OUT_192_Terrimonas	14	9	2	0	0	3	0	0	0	0
OUT_230_Thauera	3	2	12	0	5	0	0	0	0	0
OUT_224_Ralstonia	4	1	6	0	4	0	0	0	0	0
OUT_465_Ethanoligenens	0	0	0	3	1	0	0	0	0	1
OUT_133_Sediminibacterium	0	1	0	15	2	0	0	0	0	9
OUT_173_Geosporobacter	0	0	1	11	1	0	0	0	0	8
OUT_239_Zoogloea	0	1	0	12	1	3	0	0	0	21
OUT_265_Zoogloea	0	2	2	19	4	3	0	0	0	18
OUT_222_Ottowia	0	3	2	20	11	2	0	0	0	11
OUT_443_Vasilyevaea	0	0	0	1	1	2	0	0	0	3
OUT_161_Propionicimonas	4	27	1	147	5	6	0	0	0	82
OUT_380_Sarci	1	166	2	12	1	6	0	0	0	24

na										
OUT_1047_Bacillus	1	9	1	15	1	2	0	0	0	19
OUT_307_Opitutus	6	4	1	5	4	3	0	0	0	6
OUT_141_Anaerolinea	7	18	1	1	3	4	0	0	0	1

Figure 62 The difference in relative abundance of CTMP sludge

Figure 62 (A)

#OTU ID	**A84_0H**
OTU_4_Acidisphaera	733
OTU_13_Curvibacter	449
OTU_2_Parabacteriodes	1327
OTU_67_Amphitea	95
OTU_25_Nitrosococcus	553
OTU_108_Microbacterium	151
OTU_53_Paludibacter	252
OTU_46_Methylobacillus	301
OTU_35_Methylocaldum	285
OTU_27_Ramlibacter	85
OTU_152_Pelotomaculum	84
OTU_89_Propionicimonas	243
OTU_11_Algoriphagus	335
OTU_161_Propionicimonas	147
OTU_33_Ignavibacterium	87
OTU_73_Paludibacter	175
OTU_100_Micropruina	99
OTU_158_Sporocytophaga	60
OTU_66_Sulfurospirillum	100
OTU_12_Bacteroides	62
OTU_147_Sulfurimonas	85
OTU_118_Proteiniclasticum	54

Figure 62(B)

#OTU ID	B94_0H
OTU_2_Parabacteriodes	620
OTU_82_Paludibacter	50
OTU_3_Arcobacter	4246
OTU_9_Janthinobacterium	639
OTU_748_Arcobacter	666
OTU_52_Arcobacter	113
OTU_96_Arcobacter	47
OTU_68_Paludibacter	98
OTU_15_Meniscus	58
OTU_31_Olivibacter	43
OTU_201_Salinimicrobium	56

Figure 62(C)

#OTU ID	B90_0H
OTU_13_Curvibacter	84
OTU_2_Parabacteriodes	2255
OTU_1148_Anaerobacter	290
OTU_35_Methylocaldum	64
OTU_72_Dechloromonas	40
OTU_27_Ramlibacter	49
OTU_39_Eubacterium	63
OTU_1_Cloacibacterium	760
OTU_110_Cloacibacterium	45
OTU_36_Wautersiella	173
OTU_195_Xanthomonas	82
OTU_687_Cloacibacterium	989
OTU_102_Herbaspirillum	466
OTU_115_Undibacterium	286
OTU_26_Acinetobacter	47
OTU_103_Wautersiella	321
OTU_41_Chryseobacterium	116
OTU_24_Acinetobacter	97
OTU_164_Chryseobacterium	218
OTU_325_Chryseobacterium	68
OTU_168_Chryseobacterium	64

Figure 63 Difference in relative abundance of OTUs in kraft sludge

Figure 63(A)

#OTU ID	A87_0H
OTU_4_Acidisphaera	403
OTU_1148_Anaerobacter	106
OTU_85_Rubrivivax	42
OTU_3_Arcobacter	56
OTU_232_Proteiniclasticum	60
OTU_9_Janthinobacterium	1505
OTU_29_Sulfuricurvum	598
OTU_7_Sulfuricurvum	774
OTU_32_Giesbergeria	1081
OTU_10_Hippea	406
OTU_699_Paludibacter	266
OTU_94_Spirosoma	81
OTU_748_Arcobacter	172
OTU_59_Terrimonas	82
OTU_20_Bacillus	233
OTU_64_Paludibacter	99
OTU_609_Sulfuricurvum	298
OTU_956_Sulfuricurvum	84
OTU_75_Selenomonas	47
OTU_112_Ferrimicrobium	48

Figure 63 (B)

#OTU ID	B88_0H
OTU_22_Anaerobacter	123
OTU_4_Acidisphaera	501
OTU_5_prevotella	81
OTU_13_Curvibacter	139
OTU_2_Parabacteriodes	865
OTU_177_Anaerobacter	104
OTU_380_Sarcina	166
OTU_25_Nitrosococcus	65
OTU_1148_Anaerobacter	62
OTU_53_Paludibacter	122
OTU_35_Methylocaldum	211
OTU_74_Novosphingobium	50
OTU_39_Eubacterium	1215
OTU_34_Flavisolibacter	44
OTU_73_Paludibacter	36
OTU_60_Acidaminococcus	38
OTU_196_Anaerobacter	53
OTU_100_Micropruina	44
OTU_77_Sulfurospirillum	74
OTU_10_Hippea	170
OTU_94_Spirosoma	44
OTU_59_Terrimonas	45
OTU_20_Bacillus	87
OTU_119_Hydrocarboniphaga	70
OTU_44_Pseudomonas	1391
OTU_107_Sarcina	176
OTU_171_Sarcina	126
OTU_227_Iodobacter	49
OTU_57_Sulfurospirillum	60
OTU_43_Paludibacter	172

Figure 63 (C)

#OTU ID	A89_0H
OTU_4_Acidisphaera	56
OTU_13_Curvibacter	44
OTU_2_Parabacteriodes	498
OTU_1148_Anaerobacter	100
OTU_35_Methylocaldum	47
OTU_27_Ramlibacter	76
OTU_1_Cloacibacterium	2297
OTU_110_Cloacibacterium	55
OTU_36_Wautersiella	54
OTU_687_Cloacibacterium	2763
OTU_10_Hippea	84
OTU_102_Herbaspirillum	107
OTU_115_Undibacterium	98
OTU_103_Wautersiella	145
OTU_41_Chryseobacterium	162
OTU_93_Acinetobacter	56
OTU_24_Acinetobacter	42
OTU_164_Chryseobacterium	52

Figure 64 the relative abundance of OTUs in TMP (Dolbeau) Sludge

Figure 64(A)

#OTU ID	A91_0H
OTU_22_Anaerobacter	206
OTU_4_Acidisphaera	127
OTU_13_Curvibacter	192
OTU_177_Anaerobacter	168
OTU_25_Nitrosococcus	84
OTU_1148_Anaerobacter	1437
OTU_51_Geosporobacter	62
OTU_139_Levilinea	42
OTU_72_Dechloromonas	61
OTU_74_Novosphingobium	55
OTU_27_Ramlibacter	441
OTU_47_Kitasatospora	117
OTU_33_Ignavibacterium	52
OTU_55_Campylobacter	40
OTU_34_Flavisolibacter	61
OTU_38_Adhaeribacter	301
OTU_403_Anaerobacter	48
OTU_267_Proteiniclasticum	205
OTU_169_Levilinea	54
OTU_45_Paludibacter	186
OTU_83_Terrimonas	164
OTU_6_Arthrobacter	374
OTU_118_Proteiniclasticum	686
OTU_223_Hydrogenoanaerobacterium	67
OTU_65_Ethanoligenens	1134
OTU_1142_Ethanoligenens	83

Figure 64(B)

#OTU ID	B92_0H
OTU_22_Anaerobacter	69
OTU_1_Cloacibacterium	5216
OTU_687_Cloacibacterium	151
OTU_24_Acinetobacter	41
OTU_16_Comamonas	254
OTU_17_Flavobacterium	515
OTU_70_Flavobacterium	55
OTU_49_Chryseobacterium	187
OTU_76_Comamonas	46
OTU_71_Flavobacterium	194
OTU_228_Bacteriovorax	55

Figure 64(C)

#OTU ID	A93_0H
OTU_22_Anaerobacter	78
OTU_2_Parabacteriodes	46
OTU_1_Cloacibacterium	4873
OTU_687_Cloacibacterium	324
OTU_103_Wautersiella	109
OTU_93_Acinetobacter	37
OTU_24_Acinetobacter	94
OTU_16_Comamonas	251
OTU_17_Flavobacterium	296
OTU_37_Aquaspirillum	87
OTU_14_Flavobacterium	534

Figure 64(D)

#OTU ID	B94_0H
OTU_2_Parabacteriodes	620
OTU_82_Paludibacter	50
OTU_3_Arcobacter	4246
OTU_9_Janthinobacterium	639
OTU_748_Arcobacter	666
OTU_52_Arcobacter	113
OTU_96_Arcobacter	47
OTU_68_Paludibacter	98
OTU_15_Meniscus	58
OTU_31_Olivibacter	43
OTU_201_Salinimicrobium	56

Supplimenry data

Complete OUT table

#OTU ID	A87_Kraft	B88_Kraft	A89_KraftWAS	A84_CTMP	B90_CTMP	A91_Dolbeau	B92_Dolbeau	A93_Dolbeau	B94_Dolbeau	C86_CTMP
OTU_22	0	123	26	2	26	206	69	78	0	1
OTU_4	403	501	56	733	37	127	0	2	0	2576
OTU_5	0	81	0	22	0	0	0	0	0	3
OTU_30	0	8	0	0	4	0	0	0	0	0
OTU_13	1	139	44	449	84	192	0	3	1	286
OTU_2	2	865	498	1327	2255	7	23	46	620	746
OTU_177	5	104	8	47	15	168	2	7	0	6
OTU_67	0	13	4	95	2	22	0	0	0	103
OTU_380	1	166	2	12	1	6	0	0	0	24
OTU_565	0	2	0	1	0	0	0	0	0	0
OTU_25	1	65	13	553	24	84	0	0	1	434
OTU_1047	1	9	1	15	1	2	0	0	0	19
OTU_108	0	17	1	151	6	2	0	0	0	43
OTU_134	0	5	5	15	0	1	0	0	0	24
OTU_868	0	9	0	10	1	0	0	0	0	24
OTU_1148	106	62	100	22	290	1437	4	10	3	20
OTU_851	0	0	0	1	0	0	0	0	0	6
OTU_85	42	20	17	38	2	19	0	1	0	21
OTU_51	0	8	0	45	0	62	0	0	0	57
OTU_53	0	122	0	252	2	0	0	0	0	90
OTU_500	18	1	0	0	0	18	0	0	0	2
OTU_46	0	35	2	301	1	13	0	0	0	187
OTU_69	0	4	1	28	0	1	3	0	0	70
OTU_35	0	211	47	285	64	0	0	0	0	86
OTU_139	0	1	2	15	2	42	0	0	0	28
OTU_72	0	19	12	45	40	61	0	0	0	13
OTU_74	22	50	1	39	2	55	0	0	0	173
OTU_27	21	27	76	85	49	441	13	16	11	50
OTU_302	1	2	0	4	0	1	0	0	0	7
OTU_221	0	7	1	25	3	0	0	0	0	7
OTU_39	0	1215	20	0	63	0	5	1	0	0
OTU_152	0	5	2	84	3	1	0	0	0	18
OTU_544	0	12	0	15	0	0	0	0	0	0
OTU_117	0	0	0	0	0	0	0	0	0	0

OTU_445	0	0	0	2	0	0	0	0	0	6
OTU_527	3	10	1	0	1	10	0	2	1	4
OTU_47	0	2	0	46	2	117	0	0	0	23
OTU_1	1	5	2297	3	760	1	5216	4873	2	3
OTU_89_	2	21	5	243	0	6	1	0	1	140
OTU_11_	1	28	4	335	1	36	0	0	7	252
OTU_295_	0	1	0	4	0	0	0	0	0	4
OTU_311_	6	1	0	5	0	3	0	0	0	13
OTU_333_	0	1	0	44	0	0	0	0	0	9
OTU_258_	0	0	0	11	0	0	0	0	0	5
OTU_203_	0	0	0	13	0	4	0	0	0	4
OTU_242_	3	4	1	7	0	14	1	0	0	9
OTU_161_	4	27	1	147	5	6	0	0	0	82
OTU_33_	0	12	0	87	1	52	0	0	0	20
OTU_222_	0	3	2	20	11	2	0	0	0	11
OTU_275_	0	3	0	6	2	1	0	1	0	2
OTU_151_	7	2	0	3	0	1	0	0	16	0
OTU_133_	0	1	0	15	2	0	0	0	0	9
OTU_54_	0	0	0	11	0	0	0	0	0	17
OTU_613_	0	1	0	2	0	0	0	0	0	1
OTU_447_	0	0	0	1	0	1	0	0	0	1
OTU_55_	0	5	3	32	4	40	1	2	3	33
OTU_80_	0	0	0	11	0	11	0	1	0	10
OTU_479_	0	4	2	1	2	0	0	1	0	0
OTU_110_	1	2	55	15	45	1	16	33	0	8
OTU_741	0	1	1	6	1	1	0	0	0	3
OTU_153_	0	0	0	0	0	0	1	1	5	0
OTU_265	0	2	2	19	4	3	0	0	0	18
OTU_34	28	44	2	4	0	61	0	1	1	11
OTU_284	0	1	0	3	0	9	0	0	0	2
OTU_294	0	1	0	1	0	0	0	0	0	10
OTU_239	0	1	0	12	1	3	0	0	0	21
OTU_193	0	0	0	2	11	16	0	0	0	1
OTU_656	0	0	0	4	0	0	0	0	0	1
OTU_184	0	0	0	0	0	0	0	0	0	0
OTU_36	0	0	54	15	173	0	0	0	0	7
OTU_537	1	1	0	1	0	1	0	0	0	0
OTU_73	0	36	4	175	7	0	0	0	0	96
OTU_38	0	2	0	7	0	301	0	2	0	3
OTU_99	19	7	3	10	0	10	0	0	0	26
OTU_403	0	1	0	0	0	48	1	3	0	1
OTU_60	0	38	5	26	2	3	0	0	0	299
OTU_259	0	2	0	35	0	0	0	0	0	85

OTU_149	0	9	2	22	1	0	0	0	0	10
OTU_196	13	53	1	1	0	15	0	0	0	1
OTU_174	0	2	0	4	0	0	0	0	0	16
OTU_631	0	0	0	10	1	0	0	0	0	0
OTU_340	0	0	0	3	0	4	0	0	0	2
OTU_82	0	1	0	16	0	0	0	0	50	4
OTU_84	0	7	1	1	0	27	3	1	0	16
OTU_278	0	0	0	0	0	0	0	0	0	0
OTU_113	2	0	0	3	1	1	2	2	7	4
OTU_711	0	0	0	1	0	0	0	0	0	0
OTU_61	0	1	1	8	5	12	0	1	0	27
OTU_267	0	0	0	7	0	205	0	0	0	0
OTU_178	0	3	0	2	2	8	0	0	0	4
OTU_638	0	9	0	2	1	0	0	4	0	2
OTU_195	0	0	35	0	82	0	0	0	0	0
OTU_307	6	4	1	5	4	3	0	0	0	6
OTU_398	0	1	0	5	0	0	0	0	0	3
OTU_276	0	0	0	0	0	11	0	0	0	1
OTU_326	0	0	0	0	0	0	0	0	0	0
OTU_846	0	1	0	4	0	1	0	0	0	9
OTU_173	0	0	1	11	1	0	0	0	0	8
OTU_487	2	5	0	0	0	1	0	0	0	0
OTU_159	0	1	1	25	0	0	0	0	0	5
OTU_229	0	0	0	1	3	15	0	0	0	2
OTU_395	0	0	0	0	0	15	0	0	0	0
OTU_121	0	1	0	1	1	0	2	6	14	0
OTU_129	0	2	0	35	0	0	0	0	0	32
OTU_169	0	1	0	8	0	54	0	0	0	6
OTU_785	0	0	0	5	0	0	0	0	0	1
OTU_362	4	0	0	5	0	4	0	0	0	7
OTU_138	11	2	1	3	0	26	0	0	0	6
OTU_79	2	0	0	5	0	4	0	0	0	12
OTU_297	0	2	0	18	0	1	0	0	0	8
OTU_320	0	1	0	0	0	0	0	0	0	0
OTU_245	0	0	0	6	0	0	0	0	0	0
OTU_274	0	0	0	6	0	2	0	0	0	7
OTU_252	4	2	0	4	4	8	0	0	1	0
OTU_497	0	0	0	17	0	0	0	0	0	24
OTU_45	0	3	0	19	0	186	0	2	0	19
OTU_355	0	2	0	8	0	0	0	0	0	0
OTU_405	0	1	0	3	1	0	0	0	0	1
OTU_293	0	0	0	2	0	0	0	0	0	2
OTU_379	0	0	0	2	0	0	0	0	0	4

OTU_563	0	1	1	7	0	1	0	0	0	6
OTU_373	0	1	0	3	0	0	0	0	0	0
OTU_476	0	1	0	1	0	4	0	0	0	0
OTU_235	0	3	2	11	1	1	0	0	0	3
OTU_212	23	11	0	0	0	3	0	0	0	3
OTU_268	0	0	0	0	0	0	0	0	0	1
OTU_689	0	0	0	0	1	1	1	0	13	0
OTU_434	0	0	0	0	0	0	0	0	0	0
OTU_517	0	4	4	17	13	28	0	0	0	0
OTU_111	0	1	0	5	0	2	0	0	0	11
OTU_619	0	0	0	1	0	2	0	0	0	1
OTU_648	0	1	0	4	0	0	0	0	0	7
OTU_202	0	0	0	6	0	17	0	0	0	3
OTU_428	0	0	0	10	0	0	0	0	0	7
OTU_58	0	0	0	1	0	30	0	1	0	1
OTU_270	0	0	0	11	0	3	0	0	0	10
OTU_560	6	3	0	0	0	0	0	0	0	0
OTU_271	0	0	0	0	0	0	0	0	0	0
OTU_100	0	44	3	99	5	2	1	0	0	78
OTU_351	0	1	0	6	0	0	0	0	0	4
OTU_443	0	0	0	1	1	2	0	0	0	3
OTU_158	0	12	0	60	0	0	0	0	0	7
OTU_3	56	9	5	5	1	0	8	5	4246	67
OTU_244	1	1	0	6	0	6	0	0	0	10
OTU_551	0	4	0	10	1	0	0	0	0	2
OTU_238	0	4	0	3	0	8	0	0	0	7
OTU_926	0	0	0	7	0	0	0	0	0	0
OTU_461	0	0	0	0	0	9	0	0	0	0
OTU_315	0	29	1	0	1	0	0	3	0	0
OTU_526	1	1	0	5	0	1	0	0	0	15
OTU_179	0	0	0	0	0	16	0	0	0	0
OTU_150	0	5	1	14	0	5	0	0	0	12
OTU_83	0	0	0	1	0	164	0	2	0	0
OTU_784	0	2	0	9	0	0	0	0	0	2
OTU_346	1	0	4	4	5	0	1	0	0	2
OTU_510	0	0	0	6	0	0	0	0	0	5
OTU_6	0	0	1	0	0	374	0	4	0	1
OTU_66	0	0	3	100	0	1	0	0	2	86
OTU_232	60	0	0	1	0	4	0	0	5	0
OTU_1136	12	3	0	0	0	0	0	0	0	0
OTU_109	0	0	0	0	0	1	15	22	0	1
OTU_77	0	74	13	3	30	1	0	0	1	7
OTU_607	0	0	0	0	0	2	0	0	0	0

OTU_87	0	0	0	0	0	0	1	1	2	0
OTU_9	1505	10	0	11	6	16	4	2	639	15
OTU_687	2	3	2763	0	989	0	151	324	1	1
OTU_359	0	1	0	3	0	10	0	0	0	0
OTU_247	0	0	0	4	0	3	0	0	0	2
OTU_142	0	0	0	0	0	0	0	0	0	0
OTU_29	598	0	0	1	0	0	0	0	0	0
OTU_7	774	0	0	2	0	0	0	0	0	2
OTU_32	1081	0	0	0	0	0	0	1	27	0
OTU_914	37	0	0	0	0	0	0	0	0	0
OTU_10	406	170	84	0	25	0	0	1	0	0
OTU_699	266	0	0	0	0	0	0	0	0	0
OTU_94	81	44	1	0	0	0	0	0	0	0
OTU_748	172	0	7	3	12	1	17	0	666	11
OTU_59	82	45	3	0	0	0	0	0	0	0
OTU_286	13	0	0	0	0	0	0	0	0	0
OTU_192	14	9	2	0	0	3	0	0	0	0
OTU_20	233	87	8	0	12	0	0	0	0	0
OTU_64	99	0	0	0	0	0	0	0	0	0
OTU_609	298	0	0	0	0	0	0	0	0	0
OTU_956	84	0	0	0	0	1	0	0	1	0
OTU_106	33	11	26	1	21	0	3	1	12	0
OTU_290	31	0	0	0	0	0	0	0	0	0
OTU_438	6	2	0	0	0	0	0	0	0	0
OTU_254	13	3	0	0	0	0	0	0	0	0
OTU_309	6	4	0	0	0	0	0	0	0	0
OTU_75	47	9	0	0	0	0	0	0	0	0
OTU_141	7	18	1	1	3	4	0	0	0	1
OTU_78	41	23	0	0	0	0	0	0	0	0
OTU_206	16	7	1	0	1	0	0	0	0	0
OTU_241	29	1	0	0	1	0	0	0	0	0
OTU_209	5	0	0	0	0	12	0	0	0	0
OTU_119	28	70	2	0	0	0	0	0	0	0
OTU_264	12	1	0	0	0	0	0	0	0	0
OTU_182	15	11	1	0	0	0	0	0	0	0
OTU_345	1	17	0	0	0	0	0	0	0	0
OTU_502	3	3	0	0	0	0	0	0	0	0
OTU_733	10	1	0	0	0	0	0	0	0	0
OTU_535	6	0	0	0	0	0	0	0	1	0
OTU_191	33	10	0	0	0	0	0	0	0	0
OTU_122	3	0	8	0	13	0	0	0	6	0

OTU_112	48	19	6	0	4	0	0	0	0	0
OTU_240	0	2	0	0	0	3	0	0	1	0
OTU_44	11	1391	3	8	10	5	2	3	15	6
OTU_533	4	0	0	0	0	0	0	0	0	0
OTU_940	16	8	0	0	0	0	0	0	0	0
OTU_250	24	10	0	0	0	0	0	0	0	0
OTU_292	6	4	0	0	0	0	0	0	0	0
OTU_56	31	16	0	0	0	37	0	0	0	0
OTU_331	1	1	0	0	0	0	0	0	0	0
OTU_62	15	12	0	1	0	2	2	0	21	0
OTU_207	21	25	0	0	0	0	0	0	0	0
OTU_98	0	3	16	0	15	0	3	0	0	0
OTU_472	5	1	1	0	0	0	0	0	0	0
OTU_415	4	3	0	0	0	0	0	0	0	0
OTU_393	6	7	0	0	0	0	0	0	0	0
OTU_729	1	1	0	1	0	0	0	0	0	5
OTU_50	2	1	0	1	0	0	9	9	15	0
OTU_287	8	1	0	0	1	0	0	0	0	0
OTU_230	3	2	12	0	5	0	0	0	0	0
OTU_256	11	2	0	0	0	0	0	0	0	0
OTU_200	1	1	0	0	0	0	0	0	3	0
OTU_166	3	1	4	0	7	0	0	0	0	0
OTU_469	2	0	0	0	0	0	0	0	0	0
OTU_406	5	1	0	0	0	0	0	0	0	0
OTU_408	2	22	0	0	0	10	0	0	0	0
OTU_452	2	0	0	0	0	0	0	0	0	0
OTU_710	1	0	0	0	0	0	0	0	0	0
OTU_358	5	1	1	0	0	0	0	0	0	0
OTU_180	10	3	0	0	1	0	0	0	0	0
OTU_224	4	1	6	0	4	0	0	0	0	0
OTU_701	1	0	1	2	0	0	0	0	0	3
OTU_277	7	0	0	0	0	0	0	0	1	0
OTU_313	0	32	0	0	0	0	2	0	0	0
OTU_602	0	0	0	0	0	0	0	0	0	0
OTU_610	1	0	0	0	0	16	0	0	0	0
OTU_143	0	0	2	0	0	0	1	3	3	0
OTU_377	1	0	0	0	0	0	0	0	0	0
OTU_233	1	0	0	0	0	0	0	0	7	0
OTU_102	1	0	107	0	466	0	4	1	18	0
OTU_1025	0	0	0	0	1	2	0	0	0	0
OTU_314	0	0	0	0	0	0	0	0	0	0
OTU_386	0	0	0	0	0	4	0	0	0	0

OTU_385	1	0	0	0	0	0	0	0	0	0
OTU_666	1	0	0	0	0	0	0	0	0	0
OTU_378	0	0	0	0	0	0	0	0	0	0
OTU_213	0	0	0	0	0	0	0	0	0	0
OTU_627	0	0	0	0	0	0	0	0	0	0
OTU_321	1	0	0	0	0	0	0	0	4	0
OTU_181	0	0	0	0	0	0	0	0	0	0
OTU_144	1	0	0	0	0	0	0	0	0	0
OTU_126	0	0	0	0	0	0	0	0	0	0
OTU_21	0	0	0	1	0	0	0	0	0	0
OTU_12	0	1	0	62	0	0	4	0	0	0
OTU_400	0	0	13	0	18	0	0	0	0	0
OTU_147	0	1	0	85	0	0	0	0	0	94
OTU_423	0	0	0	1	0	2	0	0	0	2
OTU_1155	0	0	0	0	0	0	0	0	0	0
OTU_115	0	0	98	0	286	0	5	1	0	0
OTU_146	0	0	0	0	0	0	0	0	1	0
OTU_8	0	0	0	0	0	0	0	0	0	0
OTU_255	0	0	0	0	0	0	0	0	0	0
OTU_167	0	0	0	0	0	0	0	0	1	0
OTU_199	0	0	0	0	0	0	0	0	0	0
OTU_283	0	0	0	0	0	0	0	0	0	0
OTU_160	0	0	0	0	0	0	0	0	0	0
OTU_217	0	0	0	0	0	0	0	0	0	0
OTU_384	0	0	0	0	0	0	0	0	0	0
OTU_214	0	2	0	2	0	34	0	1	0	0
OTU_249	0	0	0	0	0	0	0	0	0	0
OTU_857	0	0	0	0	0	0	0	0	0	0
OTU_342	0	0	0	0	0	0	0	0	0	0
OTU_542	0	0	0	0	0	0	0	0	0	0
OTU_205	0	0	0	1	0	6	0	0	0	0
OTU_262	0	0	0	0	0	0	0	0	0	0
OTU_538	0	0	0	0	0	0	0	0	0	0
OTU_877	0	0	0	0	0	0	0	0	0	0
OTU_464	0	0	0	0	0	5	0	0	0	0
OTU_215	0	0	1	45	0	0	0	0	4	17
OTU_369	0	0	0	0	0	0	0	0	0	0
OTU_432	0	0	0	0	0	0	0	0	0	0
OTU_583	0	0	1	0	1	0	0	0	0	0
OTU_490	0	0	0	0	0	0	0	0	0	0
OTU_647	0	0	0	0	0	0	0	0	0	0
OTU_617	0	0	0	0	0	0	0	0	0	0
OTU_840	0	0	0	0	0	0	0	0	0	0

OTU_269	0	0	0	1	0	0	0	0	2	0
OTU_211	0	0	0	0	0	0	11	16	0	0
OTU_417	0	0	0	0	0	0	0	0	1	0
OTU_474	0	0	0	0	0	0	0	0	0	0
OTU_1146	0	0	0	0	0	0	0	0	4	0
OTU_137	0	0	0	0	0	0	0	2	0	0
OTU_641	0	0	0	0	0	0	0	0	0	0
OTU_368	0	0	0	42	0	0	0	0	0	0
OTU_339	0	0	0	48	0	0	0	0	0	0
OTU_719	0	0	0	0	0	0	0	0	0	0
OTU_480	0	1	0	1	0	20	0	0	0	42
OTU_26	0	0	18	0	47	0	1	0	0	0
OTU_52	0	0	0	7	0	0	0	0	113	0
OTU_118	0	1	1	54	0	686	1	1	0	10
OTU_433	0	0	0	0	0	0	0	0	0	2
OTU_103	0	0	145	0	321	0	22	109	0	0
OTU_253	0	0	0	0	0	0	2	0	3	0
OTU_922	0	0	0	0	0	0	0	0	0	0
OTU_41	0	3	162	0	116	0	0	1	0	1
OTU_734	0	0	0	0	0	0	0	0	0	0
OTU_465	0	0	0	3	1	0	0	0	0	1
OTU_194	0	0	0	0	0	0	2	0	0	1
OTU_485	0	0	0	0	0	0	1	0	0	0
OTU_107	0	176	0	0	0	0	0	20	0	0
OTU_171	0	126	0	0	0	0	0	0	0	0
OTU_227	0	49	0	0	0	0	0	0	0	0
OTU_57	0	60	11	3	21	1	0	0	10	1
OTU_367	0	3	0	0	0	0	0	0	0	3
OTU_43	0	172	7	0	28	0	1	0	0	0
OTU_327	0	15	0	0	0	0	0	0	0	0
OTU_397	0	5	0	3	0	0	0	0	0	0
OTU_1080	0	19	0	0	0	0	0	0	0	0
OTU_319	0	0	0	27	0	1	0	0	0	0
OTU_130	0	22	8	0	14	0	0	0	0	0
OTU_431	0	0	0	0	0	2	0	0	0	0
OTU_495	0	2	0	5	0	0	0	0	0	2
OTU_402	0	1	0	0	0	0	0	0	0	1
OTU_665	0	3	0	4	0	2	0	0	0	3
OTU_183	0	0	0	9	0	26	0	0	0	90
OTU_690	0	0	1	3	0	1	0	0	0	0
OTU_592	0	1	0	1	0	2	0	0	0	1
OTU_504	0	0	4	0	4	0	0	0	0	0
OTU_296	0	0	0	0	2	13	0	0	0	0

OTU_343	0	0	0	0	0	0	3	0	2	0
OTU_483	0	1	14	0	27	0	0	0	0	0
OTU_481	0	0	0	0	0	0	0	0	4	1
OTU_299	0	0	0	0	1	0	2	4	0	0
OTU_713	0	0	0	3	0	0	0	0	0	0
OTU_93	0	0	56	0	16	0	7	37	0	0
OTU_105	0	0	30	0	20	0	0	2	0	0
OTU_24	0	0	42	0	97	0	41	94	0	0
OTU_786	0	0	0	0	0	0	0	0	0	0
OTU_366	0	0	19	0	2	0	0	1	0	0
OTU_101	0	0	24	1	9	0	33	34	0	0
OTU_303	0	0	0	0	2	0	0	0	0	0
OTU_1144	0	0	2	0	7	0	2	0	0	0
OTU_1050	0	0	1	0	0	0	6	14	0	0
OTU_261	0	0	0	0	0	0	4	2	0	0
OTU_615	0	0	0	0	0	0	0	0	0	0
OTU_1007	0	0	5	0	25	0	0	0	0	0
OTU_413	0	0	0	0	0	0	0	0	0	0
OTU_468	0	0	0	0	0	0	3	3	0	0
OTU_679	0	0	0	0	0	0	14	16	0	0
OTU_581	0	0	1	0	0	0	0	3	0	0
OTU_16	0	0	0	0	2	0	254	251	2	0
OTU_553	0	0	0	0	1	0	0	3	0	0
OTU_164	0	0	52	0	218	0	0	0	0	0
OTU_652	0	0	1	0	9	0	0	0	0	0
OTU_325	0	0	19	0	68	0	1	0	0	0
OTU_584	0	0	11	0	6	0	0	0	0	0
OTU_446	0	0	8	0	23	0	0	1	0	0
OTU_738	0	0	2	0	1	0	1	2	0	0
OTU_243	0	0	4	0	13	0	7	2	0	0
OTU_411	0	0	12	0	39	0	0	0	0	1
OTU_637	0	0	1	0	4	0	0	2	0	0
OTU_341	0	0	14	0	27	0	2	10	0	0
OTU_896	0	0	3	0	15	0	0	0	0	0
OTU_306	0	0	0	19	0	0	0	0	0	8
OTU_620	0	0	1	0	2	0	5	7	0	0
OTU_168	0	0	25	0	64	0	11	29	0	0
OTU_937	0	0	1	10	0	0	0	0	0	9
OTU_163	0	0	1	0	3	0	3	22	0	0
OTU_335	0	0	0	0	0	0	4	5	0	0
OTU_329	0	0	0	6	0	0	0	0	22	0
OTU_223	0	0	0	1	0	67	0	1	0	0
OTU_419	0	0	0	0	0	0	0	0	0	0

OTU_651	0	0	0	1	0	2	0	0	0	0
OTU_387	0	0	0	1	0	2	0	0	0	0
OTU_186	0	0	0	0	0	0	0	0	0	3
OTU_557	0	0	0	0	0	4	0	0	0	2
OTU_96	0	0	0	0	0	0	1	0	47	0
OTU_317	0	0	0	0	0	0	0	0	0	0
OTU_618	0	0	0	0	0	0	0	0	0	0
OTU_466	0	0	0	0	0	0	22	4	0	0
OTU_17	0	0	0	0	0	0	515	296	1	0
OTU_70	0	0	0	0	0	0	55	6	0	0
OTU_120	0	0	0	0	0	0	0	0	0	0
OTU_135	0	0	0	0	0	26	0	2	0	0
OTU_611	0	0	0	0	0	3	0	0	0	0
OTU_350	0	0	0	0	0	3	0	0	0	0
OTU_643	0	0	0	0	0	1	0	0	0	0
OTU_422	0	0	0	0	0	0	0	0	0	0
OTU_246	0	0	0	0	0	5	0	0	0	0
OTU_322	0	0	0	0	0	0	0	0	0	0
OTU_448	0	0	0	0	0	4	0	0	0	0
OTU_68	0	0	0	0	0	0	0	0	98	0
OTU_157	0	0	0	0	0	0	0	0	0	0
OTU_404	0	0	0	0	0	11	0	0	0	0
OTU_478	0	0	0	0	0	0	0	0	0	0
OTU_279	0	0	0	0	0	0	0	0	0	0
OTU_170	0	0	0	0	0	0	24	6	0	0
OTU_37	0	0	0	0	0	0	16	87	0	0
OTU_15	0	0	0	0	0	0	0	0	58	0
OTU_86	0	0	0	0	0	0	2	2	1	0
OTU_310	0	0	0	0	0	1	0	0	0	0
OTU_63	0	0	0	0	0	0	2	3	30	0
OTU_496	0	0	0	0	0	0	0	0	1	0
OTU_136	0	0	0	0	0	0	0	0	9	0
OTU_65	0	0	0	0	0	1134	1	0	0	0
OTU_456	0	0	0	0	0	26	0	0	0	0
OTU_1142	0	0	0	0	0	83	0	0	0	0
OTU_349	0	0	0	0	0	0	0	0	0	0
OTU_14	0	0	0	0	0	0	18	534	5	0
OTU_1153	0	0	0	0	0	0	3	1	0	0
OTU_18	0	0	0	0	0	0	30	21	7	0
OTU_31	0	0	0	0	0	0	0	0	43	0
OTU_189	0	0	0	0	0	0	0	0	1	0
OTU_237	0	0	0	0	0	0	5	1	2	0
OTU_219	0	0	0	0	0	0	0	0	2	0

OTU_172	0	0	0	0	0	0	0	1	6	0
OTU_40	0	0	0	0	0	0	6	32	29	0
OTU_132	0	0	0	0	0	0	0	0	10	0
OTU_127	0	0	0	0	0	0	3	0	1	0
OTU_81	0	0	0	0	0	0	1	1	8	0
OTU_28	0	0	0	0	0	0	0	0	4	0
OTU_128	0	0	0	0	0	0	0	0	12	0
OTU_90	0	0	0	0	0	0	3	3	9	0
OTU_188	0	0	0	0	0	0	1	0	4	0
OTU_208	0	0	0	0	0	0	0	0	6	0
OTU_95	0	0	0	0	0	0	0	0	3	0
OTU_23	0	0	0	0	0	0	1	0	20	0
OTU_91	0	0	0	0	0	0	1	0	1	0
OTU_263	0	0	0	0	0	0	0	0	5	0
OTU_440	0	0	0	0	0	0	0	0	0	0
OTU_371	0	0	0	0	0	0	0	1	1	0
OTU_162	0	0	0	0	0	0	1	0	2	0
OTU_114	0	0	0	0	0	0	0	0	1	0
OTU_201	0	0	0	0	0	0	0	0	56	0
OTU_231	0	0	0	0	0	0	0	0	1	0
OTU_484	0	0	0	0	0	0	0	0	0	0
OTU_154	0	0	0	0	0	0	0	0	1	0
OTU_92	0	0	0	0	0	0	4	6	5	0
OTU_48	0	0	0	0	0	0	1	2	7	0
OTU_210	0	0	0	0	0	0	0	1	4	0
OTU_42	0	0	0	0	0	0	0	1	10	0
OTU_266	0	0	0	0	0	0	0	0	0	0
OTU_19	0	0	0	0	0	0	12	3	5	0
OTU_337	0	0	0	0	0	0	0	0	13	0
OTU_236	0	0	0	0	0	0	0	0	3	0
OTU_388	0	0	0	0	0	0	0	0	0	0
OTU_410	0	0	0	0	0	0	0	0	8	0
OTU_187	0	0	0	0	0	0	0	1	1	0
OTU_430	0	0	0	0	0	0	0	0	0	0
OTU_370	0	0	0	0	0	0	2	0	2	0
OTU_298	0	0	0	0	0	0	0	0	2	0
OTU_125	0	0	0	0	0	0	1	1	3	0
OTU_165	0	0	0	0	0	0	3	0	2	0
OTU_304	0	0	0	0	0	0	0	0	0	0
OTU_97	0	0	0	0	0	0	1	2	3	0
OTU_257	0	0	0	0	0	0	0	0	3	0
OTU_323	0	0	0	0	0	0	0	0	0	0
OTU_140	0	0	0	0	0	0	1	0	1	0

OTU_116	0	0	0	0	0	0	0	0	3	0
OTU_334	0	0	0	0	0	0	0	0	1	0
OTU_175	0	0	0	0	0	0	2	3	0	0
OTU_234	0	0	0	0	0	0	0	0	5	0
OTU_225	0	0	0	0	0	0	1	1	2	0
OTU_382	0	0	0	0	0	0	0	0	2	0
OTU_401	0	0	0	0	0	0	3	1	2	0
OTU_272	0	0	0	0	0	0	0	0	3	0
OTU_216	0	0	0	0	0	0	0	0	0	0
OTU_281	0	0	0	0	0	0	0	0	1	0
OTU_301	0	0	0	0	0	0	0	0	0	0
OTU_280	0	0	0	0	0	0	0	0	4	0
OTU_439	0	0	0	0	0	0	0	0	0	0
OTU_518	0	0	0	0	0	0	1	0	0	0
OTU_595	0	0	0	0	0	0	0	0	1	0
OTU_260	0	0	0	0	0	0	0	0	0	0
OTU_683	0	0	0	0	0	0	0	1	1	0
OTU_332	0	0	0	0	0	0	0	0	1	0
OTU_123	0	0	0	0	0	0	1	7	2	0
OTU_519	0	0	0	0	0	0	2	0	0	0
OTU_543	0	0	0	0	0	0	0	0	0	0
OTU_420	0	0	0	0	0	0	0	0	1	0
OTU_486	0	0	0	0	0	0	0	0	0	0
OTU_1033	0	0	0	0	0	0	0	0	0	0
OTU_455	0	0	0	0	0	0	0	0	0	0
OTU_545	0	0	0	0	0	0	0	0	0	0
OTU_418	0	0	0	0	0	0	0	0	0	0
OTU_494	0	0	0	0	0	0	0	0	0	0
OTU_363	0	0	0	0	0	0	0	0	0	0
OTU_218	0	0	0	0	0	0	4	0	8	0
OTU_148	0	0	0	0	0	0	0	0	0	0
OTU_364	0	0	0	0	0	0	0	0	0	0
OTU_324	0	0	0	0	0	0	0	0	0	0
OTU_381	0	0	0	0	0	0	1	0	2	0
OTU_372	0	0	0	0	0	0	0	0	5	0
OTU_556	0	0	0	0	0	0	0	0	1	0
OTU_285	0	0	0	0	0	0	0	0	0	0
OTU_589	0	0	0	0	0	0	0	0	0	0
OTU_523	0	0	0	0	0	0	1	0	0	0
OTU_273	0	0	0	0	0	0	0	0	6	0
OTU_360	0	0	0	0	0	0	0	0	0	0
OTU_347	0	0	0	0	0	0	0	0	0	0
OTU_198	0	0	0	0	0	0	0	1	3	0

OTU_524	0	0	0	0	0	0	0	0	1	0
OTU_1081	0	0	0	0	0	0	1	0	0	0
OTU_352	0	0	0	0	0	0	0	0	0	0
OTU_383	0	0	0	0	0	0	0	0	0	0
OTU_548	0	0	0	0	0	0	0	0	0	0
OTU_282	0	0	0	0	0	0	1	0	3	0
OTU_394	0	0	0	0	0	0	0	0	1	0
OTU_88	0	0	0	0	0	0	0	1	3	0
OTU_441	0	0	0	0	0	0	0	0	0	0
OTU_409	0	0	0	0	0	0	1	0	0	0
OTU_344	0	0	0	0	0	0	0	0	1	0
OTU_967	0	0	0	0	0	0	0	0	1	0
OTU_389	0	0	0	0	0	0	0	0	3	0
OTU_353	0	0	0	0	0	0	0	0	2	0
OTU_579	0	0	0	0	0	0	0	1	1	0
OTU_328	0	0	0	0	0	0	0	0	1	0
OTU_599	0	0	0	0	0	0	0	0	0	0
OTU_516	0	0	0	0	0	0	0	0	0	0
OTU_204	0	0	0	0	0	0	2	0	0	0
OTU_156	0	0	0	0	0	0	0	0	1	0
OTU_435_	0	0	0	0	0	0	1	1	0	0
OTU_145_	0	0	0	0	0	0	2	1	3	0
OTU_336_	0	0	0	0	0	0	0	0	0	0
OTU_291_	0	0	0	0	0	0	0	0	0	0
OTU_559_	0	0	0	0	0	0	0	0	0	0
OTU_644_	0	0	0	0	0	0	0	0	0	0
OTU_316_	0	0	0	0	0	0	1	0	0	0
OTU_226_	0	0	0	0	0	0	0	1	1	0
OTU_885_	0	0	0	0	0	0	0	0	0	0
OTU_450_	0	0	0	0	0	0	0	0	0	0
OTU_376_	0	0	0	0	0	0	0	0	0	0
OTU_681_	0	0	0	0	0	0	0	0	0	0
OTU_312	0	0	0	0	0	0	0	0	0	0
OTU_721	0	0	0	0	0	0	0	0	0	0
OTU_338	0	0	0	0	0	0	0	0	0	0
OTU_365	0	0	0	0	0	0	0	0	1	0
OTU_493_	0	0	0	0	0	0	0	0	0	0
OTU_104_	0	0	0	0	0	0	17	18	0	0
OTU_881	0	0	0	0	0	0	0	0	0	0
OTU_488_	0	0	0	0	0	0	0	0	4	0
OTU_451_	0	0	0	0	0	0	0	0	0	0
OTU_550_	0	0	0	0	0	0	0	0	2	0
OTU_124_	0	0	0	0	0	0	0	0	1	0

OTU_220_	0	0	0	0	0	0	0	0	0	0
OTU_308_	0	0	0	0	0	0	0	0	0	0
OTU_197_	0	0	0	0	0	0	0	0	0	0
OTU_305	0	0	0	0	0	0	0	0	0	0
OTU_475_	0	0	0	0	0	0	0	0	0	0
OTU_330_	0	0	0	0	0	0	0	0	0	0
OTU_318_	0	0	0	0	0	0	0	0	0	0
OTU_426_	0	0	0	0	0	0	0	0	0	0
OTU_640	0	0	0	0	0	0	0	0	1	0
OTU_300	0	0	0	0	0	0	1	0	2	0
OTU_520_	0	0	0	0	0	0	0	0	0	0
OTU_354_	0	0	0	0	0	0	0	0	0	0
OTU_374_	0	0	0	0	0	0	0	1	0	0
OTU_190_	0	0	0	0	0	0	0	0	0	0
OTU_457_	0	0	0	0	0	0	0	0	0	0
OTU_289_	0	0	0	0	0	0	0	0	1	0
OTU_176_	0	0	0	0	0	0	0	0	0	0
OTU_49_	0	0	0	0	0	0	187	17	0	0
OTU_436_	0	0	0	0	0	0	0	0	1	0
OTU_743_	0	0	0	0	0	0	0	0	0	0
OTU_76_	0	0	0	0	0	0	46	35	0	0
OTU_155_	0	0	0	0	0	0	1	0	1	0
OTU_982_	0	0	0	0	0	0	0	0	0	0
OTU_808_	0	0	0	0	0	0	1	5	0	0
OTU_399	0	0	0	0	0	0	8	3	0	0
OTU_832_	0	0	0	0	0	0	0	0	0	0
OTU_396_	0	0	0	0	0	0	1	0	0	0
OTU_463	0	0	0	0	0	0	0	0	7	0
OTU_288_	0	0	0	0	0	0	0	0	1	0
OTU_1051_	0	0	0	0	0	0	0	1	0	0
OTU_348_	0	0	0	0	0	0	3	5	0	0
OTU_1031_	0	0	0	0	0	0	0	0	0	0
OTU_834_	0	0	0	0	0	0	0	0	0	0
OTU_71_	0	0	0	0	0	0	194	19	1	0
OTU_573_	0	0	0	0	0	0	0	0	0	0
OTU_251_	0	0	0	0	0	0	0	0	0	0
OTU_762_	0	0	0	0	0	0	0	0	0	0
OTU_248_	0	0	0	0	0	0	19	4	0	0
OTU_185_	0	0	0	0	0	0	18	15	0	0
OTU_357_	0	0	0	0	0	0	0	15	0	0
OTU_228_	0	0	0	0	0	0	55	8	0	0
OTU_498_	0	0	0	0	0	0	0	0	0	0
OTU_424_	0	0	0	0	0	0	0	0	1	0

OTU_131_	0	0	0	0	0	0	0	0	0	0
OTU_718_	0	0	0	0	0	0	0	0	0	0
OTU_482_	0	0	0	0	0	0	0	0	21	0
OTU_1052_	0	0	0	0	0	0	0	0	3	0
OTU_458_	0	0	0	0	0	0	0	0	5	0
OTU_746_	0	0	0	0	0	0	0	0	12	0
OTU_816_	0	0	0	0	0	0	0	0	0	0
OTU_454_	0	0	0	0	0	0	0	0	0	0