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# Développement d'un procédé en aval pour la production de biodiesel en utilisant la biomasse de levure oléagineuse et la boue municipale

Par,

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# RÈSUMÈ

La production de biodiesel a attiré l'attention au cours des dernières décennies. Grâce à une réduction des émissions de gaz à effet de serre (GES), les biodiesels peuvent désormais compétitionner avec les sources de carburants à base de pétrole. Actuellement, de manière globale, les usines de production de biodiesel utilisent de l'huile végétale (comestible et non-comestible) et des graisses animales comme matière première. Par contre, ces usines cherchent des alternatives à plus faible coût pour remplacer ces matières.

La production de lipides par des microorganismes oléagineux est une approche intéressante pour les chercheurs et les producteurs de biodiésel. Par contre, la production du biodiésel à partir de lipides demeure économiquement impraticable due au coût élevé de la matière première et des différentes étapes de production telles que la récolte des microorganismes, le séchage de la biomasse, la perturbation des parois cellulaires, l'utilisation de solvants organiques pour l'extraction lipidique, la transestérification et la purification.

Dans cette étude, des matières destinées à l'enfouissement telles que de l'écume (usine d'épuration) et des boues primaires et secondaires ont été étudiées pour en extraire de l'huile. Due à la présence de seulement 30% m/m d'huile dans l'écume, les levures oléagineuses cultivées en présence de glycérol brut (*Yarrowia lipolytica et Trichosporon aleaginous*) ont été identifiées comme une meilleure alternative puisqu'elles croissent rapidement et qu'elles peuvent atteindre des concentrations de 80 g/L de biomasse avec 50 à 80% m/m de lipides. Par contre, la barrière technologique pour la production de biodiésel à partir de microorganismes oléagineux est le procédé de d'extraction et de purification. Donc, la récolte de biomasse s'est effectuée par la technologie de floculation avec l'utilisation de polymères extracellulaires (PEC) produit par fermentation des biosolides municipaux. Ensuite, une nouvelle approche de perturbation de la membrane cellulaire en milieu humide et de nouveaux solvants tels que le diésel conventionnel ont été étudiés dans le cadre de cette étude. Les lipides ainsi récupérés dans le diésel ont été trans-estérifiés en biodiésel sans évaporation du solvant.

Une recherche rigoureuse a été menée et les matières premières de 3$^{e}$ génération à haut contenu lipidique tel que les levures oléagineuses ont démontré un potentiel intéressant pour une production profitable de biodiésel. Par contre, le procédé d'extraction et de purification demeure le principal défi pour la production de biodiésel à partir de levures. Dans un premier temps, le problème de la récolte des levures a été exploré.

Dans cette recherche, un nouveau procédé de récolte de la biomasse oléagineuse (*Yarrowia lipolytica*) par floculation suivi d'une perturbation des parois cellulaires et de l'extraction des lipides avec du diésel conventionnel comme solvant a été développé. L'alun et le chlorure de calcium comme coagulants avec des PES comme floculant ont été évalués pour la précipitation de biomasse oléagineuse. Les meilleures performances de floculation avec le chlorure de calcium (36 mM) combiné avec des PES (5.85 mg EPS/g biomass) et l'alum (1.2 mM) avec des PES (18 mg PES/g biomasse) ont été de 74.3 % et 79%, avec des vitesses de décantation de 2.93 et 1.46 mm/s, respectivement. La concentration finale de biomasse obtenue dans le précipité était de 166 g/L.

Dans le cadre de cette étude, afin de réduire les coûts du précédé, la perturbation des parois cellulaires a été réalisée sur de la biomasse humide en utilisant une extraction assistée au détergent plutôt que de faire de l'extraction sur de la biomasse sèche comme le procédé conventionnel. La méthode des surfaces de réponse (MSR) a été utilisée pour évaluer l'effet de 3 principaux paramètres (concentration de N-LS, le temps et la température) sur l'efficacité d'extraction (% w/w). Les résultats obtenus à partir de l'analyse statistique indiquent que le model quadratique s'applique dans chacun des cas.

Une récupération maximum de 95.3 ± 0.3% w/w de lipides a été obtenue dans les conditions suivantes : 24.4 mg de N-LS (équivalent à 48 mg N-LS/g biomasse sèche), 8.8 min de traitement, à 30.2°C. L'étude confirme que les le traitement de la biomasse des levures oléagineuses avec le N-LS est une alternative prometteuse pour la perturbation des parois cellulaires.

Par conséquent, une étude plus approfondie a été menée et après perturbation des parois cellulaires à l'aide de N-LS, les lipides libérés en solution ont été directement convertis en biodiesel sans étape de récupération d'huile. De plus, le traitement de la biomasse suivi du traitement par des ultrasons a démontré un rendement maximum d'esters méthyliques d'acide gras (EMAGs) de 94.3 ± 1.9% w/w de lipide en présence de méthanol (ratio molaire méthanol sur lipide de 360:1) et une concentration de catalyseur de 360 mM (64 µL $H_2SO_4$/g de lipide). La durée de la réaction étant de 5 minutes. Ces résultats ont révélé que les lipides issus des levures oléagineuses peuvent être une bonne alternative pour la production de biodiésel industrielle.

Afin de réduire le coût de production du biodiésel et d'augmenter les crédits de taxes gouvernementaux, des boues municipales secondaires ont été utilisées en amont du procédé

pour la production de lipide microbiens. Dans cette étude, de la biomasse de levure oléagineuse (*Trichosporon oleaginous*) a été cultivée dans des boues municipales et la précipitation de la biomasse à l'aide de PES a été étudiée. La concentration obtenue en solides décantés était de 210 g/L. La biomasse décantée a été traitée avec un traitement séquentiel utilisant le FNA et le N-LS pour perturber la paroi cellulaire. Les lipides microbiens ont été séparés des cellules perturbées en utilisant du chloroforme et du méthanol (1 :1) ou du diésel d'origine pétrolière comme solvant. Les lipides microbiens séparés ont été transestérifiés en biodiésel en conditions ex-situ. Les efficacités maximums d'extraction des lipides de 98.1 ± 1.6 et 95.8 ± 1.1% m/m ont été obtenus en utilisant 75 mg N-LS/g de biomasse, et du chloroforme/méthanol (1 :1) et du diésel comme solvant, respectivement. Dans le cas de l'extraction de lipide assistée avec du FNA, les efficacités maximums d'extraction des lipides de 94.3 ± 1.6 et 90.7 ± 1.1% m/m ont été obtenues avec 40 mg FNA/g de biomasse, et du chloroforme/méthanol (1 :1) et du diésel comme solvant, respectivement. Par contre, durant l'extraction assistée au FNA, l'augmentation de la concentration en FNA au-delà de 10 mg FNA/g de biomasse a affecté le profil des acides gras. Par conséquent, le traitement séquentiel au FNA et au N-LS en utilisant des faibles concentrations de FNA (10 mg FNA/ g de biomasse) suivi par le N-LS (20 mg N-LS/g de biomasse) a été employé et les efficacités maxima d'extraction des lipides de 95.1 et de 92.3% m/m en utilisant l'hexane et le diésel comme solvant, respectivement, ont été obtenues, et ce, sans affecter le profil des acides gras non saturés.

Dans cette étude préliminaire, plusieurs paramètres tels que le volume de solvant, la température, l'agitation et la teneur en eau ont été optimisés pour maximiser l'efficacité d'extraction de l'huile à partir d'écume lyophilisée. L'hexane et le diésel conventionnel ont été comparés pour chaque paramètre afin de déterminer leur efficacité pour l'extraction d'huile. Les paramètres physiques optimaux identifiés pour l'extraction d'huile à partir d'écume lyophilisée sont 75 g de solide/L de solvant à 60°C, sous agitation à 300 rpm pour 30 minutes. Les maximums d'efficacité de récupération obtenus sont de 100% and 94.3% w/w avec l'hexane et le diésel, respectivement. Les paramètres optimaux pour l'écume lyophilisée ont aussi été appliqués pour séparer l'huile à partir des boues primaires et secondaires. Des efficacités de récupération de 95.2% et 94.7% w/w ont été obtenues avec l'hexane et le diésel, respectivement. De plus, la transestérification ex-situ a été réalisée et les résultats ont démontrés que l'écume contient plus de lipides neutres que les boues primaires et secondaires. En bref, l'écume est une matière première potentielle pour la production de biodiésel mais son contenu en huile est de l'ordre de 30% m/m.

Le procédé avancé d'extraction de lipides microbiens développé dans le cadre de cette recherche qui utilise les boues municipales et les levures oléagineuses (*Yarrowia lipolytica* and *Trichosporon oleaginous*) pourrait permettre d'abaisser les coûts du procédé et rendre le procédé faisable à l'échelle industrielle.

# ABSTRACT

Biodiesel production has received significant attention during the last few decades. Due to a decrease in greenhouse gases emissions (GHG), it has become an efficient approach to compete with that of petroleum-based conventional fuels. Nowadays, world-wide biodiesel industries are using vegetable oil (edible and non-edible) and animal fats for biodiesel production, but they are looking forward towards low-cost feedstocks.

The microbial lipid produced by the oleaginous microorganism is an attractive approach for researchers as well as biodiesel industries. However, biodiesel production from lipids is still not economically feasible due to high feedstock cost and multiple steps involved in downstream processing such as biomass harvesting, biomass drying, cell wall disruption, microbial lipid extraction using organic solvent, transesterification and biodiesel purification.

In this study, diverted waste feedstocks such as scum, primary and secondary sludge were investigated for oil extraction. Due to the presence of less than 30% w/w oil content in scum, an alternative feedstock such as oleaginous yeast grown in crude glycerol medium (*Yarrowia lipolytica* and *Trichosporon oleaginous*) was identified, which grow rapidly and produce up to 80g/L biomass with 50 - 80% w/w lipid content. However, the bottleneck for biodiesel production using microbial lipid bearing biomass is downstream processing. Therefore, biomass harvesting was done by flocculation technology with extra polymeric substances (EPS) produced using waste water sludge. Furthermore, a cell wall disruption approach using wet biomass for cell wall disruption and novel solvent such as petroleum diesel were investigated in this study to recover lipids. The recovered lipids present in petroleum diesel were further transesterified to biodiesel without solvent drying.

A rigorous research was conducted and high lipid containing 3$^{rd}$ generation feedstock such as microbial oil from oleaginous yeast was found to be proficient for biodiesel production. However, downstream processing is a massive challenge for oleaginous yeast biomass and the preliminary step was biomass harvesting without centrifugation.

In this research, a new process of harvesting the oleaginous yeast biomass (*Yarrowia lipolytica*) by flocculation followed by cell wall disruption and lipid extraction with petroleum diesel as a solvent was developed. Alum and calcium chloride along with EPS as a flocculant were evaluated for lipid bearing biomass settling. The maximum flocculation activity of biomass

using calcium chloride (36 mM) in combination with EPS (5.85 mg EPS/g biomass) or Alum (1.2mM) with EPS (18 mg EPS/g biomass) was 74.3 and 79 % and the settling velocity was 2.93 and 1.46 mm/s, respectively. The final settled biomass concentration obtained was 166g/L.

In this study, in order to reduce the process costs, the cell wall disruption was performed on wet biomass using detergent assisted lipids extraction as opposed to the conventional process that uses dry biomass. Response surface methodology (RSM) was used to investigate the effect of three principle parameters [N-lauroyl sarcosine (N-LS) concentration, time and temperature] on microbial lipid extraction efficiency (% w/w). The results obtained by statistical analysis showed that the quadratic model fits in all cases. Maximum lipid recovery of 95.3 ± 0.3 % w/w was obtained at the optimum level in the following conditions of process variables [N-LS concentration 24.42 mg (equal to 48 mg N-LS/g dry biomass), treatment time 8.8 min and reaction temperature 30.2 $^{o}$C. The study confirmed that oleaginous yeast biomass treatment with N-LS would be a promising approach for cell wall disruption.

Hence, further study was conducted and after N-LS assisted cell wall disruption, the lipid in solution was directly converted to biodiesel without oil recovery. Moreover, The N-LS treatment of biomass followed with ultrasonication revealed maximum Fatty acid methyl esters (FAMEs) yield of 94.3 ± 1.9 % w/w using methanol to lipid molar ratio 360:1 and catalyst concentration 360 mM (64 µL $H_2SO_4$/g lipid) within 5 minutes reaction time. These results revealed that the microbial lipid from oleaginous yeast can be good alternation for industrial biodiesel production.

To decrease the cost of biodiesel production and for tax credits, in upstream processing, municipal secondary wastewater sludge was used for microbial lipid production. In this study, oleaginous yeast biomass (*Trichosporon oleaginous*) was cultivated in a municipal wastewater sludge and develped biomass settling method usig EPS was investigated . The obtained concentration of settled solids was 210 g/L after biomass settling. The settled sludge biomass was treated with sequential treatment using (FNA) Free nitrous acid and N-LS to disrupt cell wall. The microbial lipid was separated from the disrupted cells using chloroform and methanol (1:1) or petroleum diesel as solvent. The separated microbial lipid was ex-situ trans esterified to biodiesel. The maximum lipid extraction efficiencies of 98.1± 1.6 and 95.8 ± 1.1% w/w were achieved using 75 mg N-LS/g of biomass, and chloroform/methanol (1:1) and petroleum diesel as solvent, respectively. In case of FNA assisted lipid extraction, the maximum lipid extraction efficiencies of 94.3 ± 1.6 and 90.7 ± 1.1 %w/w were obtained at 40 mg FNA/g biomass using chloroform and methanol (1:1) and petroleum diesel as a solvent, respectively. However, during

FNA assisted lipid extraction, increase in FNA concentration beyond 10 mg FNA/g biomass, affected the fatty acid profile. Therefore, sequential FNA and N-LS treatment using low concentration of FNA (10 mg FNA/g biomass) followed by N-LS (20 mg N-LS /g biomass) was employed and maximum lipid extraction efficiency of 95.1 and 92.3% w/w using hexane and petroleum diesel was obtained without any effect on unsaturated fatty acids profile.

Low cost feedstock such as primary, secondary sludge and scum obtained from the municipal wastewater treatment plant for biodiesel production were investigated. Various parameters such as solvent volume, temperature, agitation and moisture content were optimized to maximize the oil extraction efficiency using freeze-dried scum. The oil extraction efficiency was compared for each parameter using hexane and petroleum diesel as a solvent. The optimum physical parameters for oil extraction using freeze-dried scum were 75 g solids/L solvent, temperature 60$^{o}$C, agitation 300 rpm for 30 min and maximum oil extraction efficiency of 100 and 94.3% w/w were obtained using hexane and petroleum diesel respectively. The optimized parameters for freeze-dried scum were further used to separate oil from dried primary and secondary sludge and oil extraction efficiency of 95.2 and 94.7 % w/w was obtained using hexane and petroleum diesel respectively. Furthermore, the ex-situ transesterification was performed, and results showed that scum had a higher content of neutral lipid than that of primary and secondary sludge. In brief, scum is a potential feedstock for the production of biodiesel but the oil content in scum is ≤ 30% w/w.

The advanced downstream process for lipid extraction from oleaginous biomass and municipal sludge (scum) followed by lipid conversion to biodiesel developed during this research will make biodiesel production feasible at industrial scale.

# PUBLICATION DANS CETTE THÈSE

1. **Yellapu, S. K**., Kaur, R., Kumar, L. R., Tiwari, B., Zhang, X., & Tyagi, R. D. (2018). Recent developments of downstream processing for microbial lipids and conversion to biodiesel. ***Bioresource technology***.

2. **Yellapu, S. K**., Bezawada, J., Kaur, R., Kuttiraja, M., & Tyagi, R. D. (2016). Detergent assisted lipid extraction from wet yeast biomass for biodiesel: A response surface methodology approach. ***Bioresource technology***, *218*, 667-673.

3. **Yellapu, S. K**., Kaur, R., & Tyagi, R. D. (2017). Detergent assisted ultrasonication aided in situ transesterification for biodiesel production from oleaginous yeast wet biomass. ***Bioresource technology***, *224*, 365-372.

4. **Yellapu, S. K**., Nouha, K., Kaur, R., Tyagi, R. D & Surrampali Rao (2019). Oleaginous yeast biomass flocculation using Bioflocculant produced in wastewater sludge and transesterification using petroleum diesel as a co-solvent. ***Renewable Energy***.

5. **Yellapu, S. K**., Kaur, R., & Tyagi, R. D. Oil extraction from Scum, primary and secondary sludge assisted with petroleum diesel and conversion to biodiesel. ***Biofuels*** (Submitted).

6. **Yellapu, S. K**., Kaur, R., & Tyagi. Free nitrous acid and N-lauroyl sarcosine assisted Ex-situ transesterification of municipal sludge derived lipid. ***Bioresource technology*** (Submitted).

# PUBLICATION EN DEHORS DE CETTE THÈSE

1. R. Kaur, **S. K. Yellapu,** Bharti Bhadana, L. R. Kumar, R. D. Tyagi, Rao Y. Sumrampalli, and Tian C. Zhang (2018). “Chapter 1-Biodiesel Basics”. In book: ***Biodiesel production: technologies, challenges, and future prospects***, American society of civil engineers (Accepted).

2. R. Kaur, **S. K. Yellapu,** R. D. Tyagi, (2018). “Chapter 4 - Enzyme assisted transesterification”. In book: ***Biodiesel production: technologies, challenges, and future prospects***, American society of civil engineers (Accepted).

3. L. R. Kumar, Bharti, **S. K. Yellapu,** R. D. Tyagi (2018). “Chapter 20- Purification of Biodiesel using Resins and Adsorbents”. In book: ***Biodiesel production: technologies, challenges, and future prospects***, American society of civil engineers (Accepted).

4. S K Ram; L R Kumar; **S K Yellapu;** R Kaur and R D Tyagi (2018). “Chapter 22-Economic Evaluation of Biodiesel Production Processes” In book: ***Biodiesel production: technologies, challenges, and future prospects***, American society of civil engineers (Accepted).

5. LR Kumar, R Kaur, **SK Yellapu,** X Zhang and RD Tyagi (2018). “Biodiesel production from oleaginous micro-organisms” In book: ***Biofuels***, Bioresources technology (Accepted).

6. L. R. Kumar, **S. K. Yellapu**, L. Kumar, R. D. Tyagi (2018). **“Oil extraction using wastewater sludge.”** In book: ***Biodiesel production: technologies, challenges, and future prospects***, American Society of Civil Engineers (accepted for publication).

# CONGRÈS ET CONFÈRENCES

1. **Yellapu, S. K.,** Bezawada, J., & Tyagi, R. D. (2016). Detergent assisted lipid extraction for biodiesel. Poster presentation Conference of 52nd 22nd Feb 2016, Ryerson university.
2. **Yellapu, S. K.,** Nouha Klai., Tyagi, R. D. (2016). Oleaginous yeast biomass settling using extra polymer substance (EPS) produced using wastewater sludge. 30th Eastern Canadian Symposium on Water Quality Research, May 27, 2016, University of Ottawa, Canada.
3. **Yellapu, S. K** and R.D.Tyagi (2018). Oil extraction from scum, primary and secondary sludge with petroleum diesel and conversion to biodiesel. 54th CAWQ conference 22nd Feb 2018, Toronto university.

# TABLE DES MATIÈRES

# LISTE DES TABLEAUX

## CHAPITRE I



## CHAPITRE 2, PARTIE 1



## CHAPITRE 2, PARTIE 2



## CHAPITRE 2, PARTIE 3

## CHAPITRE 2, PARTIE 4



## CHAPITRE 2, PARTIE 5



## CHAPITRE 2, PARTIE 6

# LIST DES FIGURES

## CHAPTIRE 1



## CHAPTIRE 2, PARTIE 1



## CHAPTIRE 2, PARTIE 2

## CHAPTIRE 2, PARTIE 3

## CHAPTIRE 2, PARTIE 4



## CHAPTIRE 2, PARTIE 5

## CHAPTIRE 2, PARTIE 6

# LISTE DES ABRÉVIATIONS

**ASTM** : American Society of Testing and Materials

**CN** : Cetane Number

**GHG** : Greenhouse Gas

**TAG**s : Triacyl glycerides

**FAME** : Fatty acid methyl esters

**N-LS** : N-lauroyl sarcosine

**FNA** : Free nitrous acid

**GC-FID** : Gas Chromatraphy – Flame Ion-Detector

**FT-IR** : Fourier transform – Infra red spectroscopy

**ISTE** : In-situ Transesterification

**PD** **:** Petroleum Diesel

# INTRODUCTION

Les sources d'énergie renouvelables se développent à travers le monde dû au prix élevé des combustibles fossiles et aux émissions de gaz à effet de serre. L'épuisement des réserves de pétrole connues rendent les sources d'énergie renouvelable plus attractive que jamais. Le biodiésel est renouvelable, non-toxique et biodégradable (98% de biodégradation en seulement quelques semaines). Les biodiésels contiennent aussi moins de composés sulfurés que le diésel et possède un point d'ignition élevé (>130°C). Le biodiésel est principalement produit à partir d'huiles végétales et de gras animal. Cependant, l'essor du prix de l'huile comestible rend la production de biodiésel trop couteuse. De plus, les sources actuelles de matières premières pour la production de biodiésel sont aussi limitées. Les cultures vivrières telles que le Jatropha, le colza et le canola sont utilisées pour la production de biodiésel mais elles sont grandement dépendantes des conditions de cultures (sol, température), nécessitent beaucoup de main d'œuvre et sont énergivores. L'abattage d'animaux pour la production de biodiésel est aussi une mauvaise option pour des raisons éthiques.

Par conséquent, au cours des dernières décennies, des voies alternatives ont été explorées pour la production de biodiésel. Les chercheurs ont essayés de produire du biodiésel en utilisant des lipides microbiens dérivés de microorganismes oléagineux tels que des bactéries, des levures, des moisissures et des algues. Le concept de raffinerie biologique est une approche prometteuse pour créer une industrie profitable basée sur l'exploitation de la biomasse. Plus spécifiquement, ces raffineries peuvent convertir la biomasse en produits tels que du carburant et des produits de remplacement aux produits pétrochimiques. Parmi tous les produits, la production de biodiésel est une approche efficace et renouvelable.

La production de biodiésel à partir de microorganismes oléagineux comprend quatre étapes : 1) la culture/fermentation, 2) la récolte de la biomasse, 3) la perturbation de la paroi cellulaire et l'extraction des lipides, et 4) la trans-estérification des lipides microbiens. Au cours de la dernière décennie, les chercheurs ont été plus concernés par les différentes étapes impliquées dans la production du biodiésel puisqu'elles affectent significativement le prix de production. Il y a plusieurs limitations impliquées pour chacune des étapes tel que la source de carbone utilisée pour la culture microbienne (Li *et al.*, 2008) et la récolte de la biomasse par centrifugation qui représente des coûts importants (procédé énergivore) (Uduman *et al.*, 2010). L'extraction lipidique et la transestérification des lipides utilisent des solvants organiques toxiques qui ne peuvent être tolérés dû à leurs risques environnementaux. Donc, dû à ses limitations, la

production de biodiésel à partir de microorganismes oléagineux n'est pas en mesure de compétitionner avec les carburants fossiles (Milano *et al.*, 2016).

Il est donc nécessaire de développer un procédé d'extraction des lipides efficace, modulable et économique. En comparaison à la récupération des lipides à partir de biomasse humides, l'extraction des lipides à partir de biomasse séchée est habituellement plus efficace. L'extraction des lipides à partir de biomasse séchée a largement été utilisée pour des fins d'analyses et à l'échelle laboratoire pour des fin de recherche avec des méthodes telles que l'extraction au Soxhlet, l'extraction en fluide pressurisé, l'extraction en fluide supercritique et le procédé réactif d'extraction. Malgré que l'extraction des lipides à partir de biomasse séchée est généralement favorisée dû à son haut rendement, le séchage de la biomasse avant l'extraction énergivore et économiquement prohibitif pour les applications à grande échelle.

Afin de maximiser le ratio Énergie récupérée sur Énergie investie (ER/EI), il est devenu nécessaire de développer un procédé d'extraction modulable qui peut extraire les lipides directement à partir de la biomasse humide avec une faible demande énergétique. Cependant, la présence d'eau limite l'efficacité des procédés d'extraction à base de solvant. Cette limitation est occasionnée par des facteurs tels qu'un faible transfert de masse et la formation d'émulsion. Ces facteurs n'ont pas encore été minutieusement examinés. L'extraction de lipides à partir de biomasse humide requiert des recherches intensives et du développement afin de comprendre les mécanismes complexes impliqués et établir une approche robuste et économique qui peut être implantée à l'échelle industrielle pour la production de biodiésel.

Cette thèse met en lumière les considérations importantes impliquées dans le développement d'une approche pratique d'extraction des lipides directement à partir de la biomasse microbienne humide. Cette étude investigue des matières premières à faible coûts et renouvelable telles que les boues de station d'épuration, l'écume des bassins de station d'épuration et la biomasse de levures oléagineuses pour la production de biodiésel. De plus, l'aval du procédé de production de biodiésel à partir de levures oléagineuses, qui implique la récolte, la perturbation des parois cellulaires de la biomasse humide et la récupération des lipides en utilisant le diésel comme solvant a été étudiée pour encourager l'industrie du biodiésel à utiliser des matières premières de troisième génération.

# CHAPITRE – 1

# SYNTHÈSE

# PARTIE 1: REVUE DE LITTÉRATURE

## 1.1 Le Biodiesel

Le mot «biodiesel» a été utilisé pour la première fois en 1984 Van Gerpen et al. (2004). L'Autriche était le premièr pays à instaurer une usine de fabrication de biodiesel en 1985, cependant quelques années plus tard, sa production a été bien établie en Europe où l'Allemagne est le producteur le plus actif. Aux États-Unis, le biodiesel a été commercialisé pour la première fois à Kansas City en 1991. En 1995, un biodiesel fourni par l'Université d'Idaho n'avait aucun effet néfaste sur les moteurs des vehicules du parc national de Yellowstone qui ont parcouru plusieurs kilomètres (Pahl, 2008). Les biocarburants comme le biodiesel pourraient contribuer à la réduction de la pollution de l'air, des émissions de gaz a effet de serre et de la dépendance aux combustibles fossiles. De nos jours, les gouvernements incitent à la production de biocarburants et offrent des crédits d'impôt pour leur utilisation. La production de biodiesel a connu une importante et rapide hausse entre 2008 et 2009. En effet, elle a augmenté d'environ 10% chaque année,comme le montre la Figure 1.1.

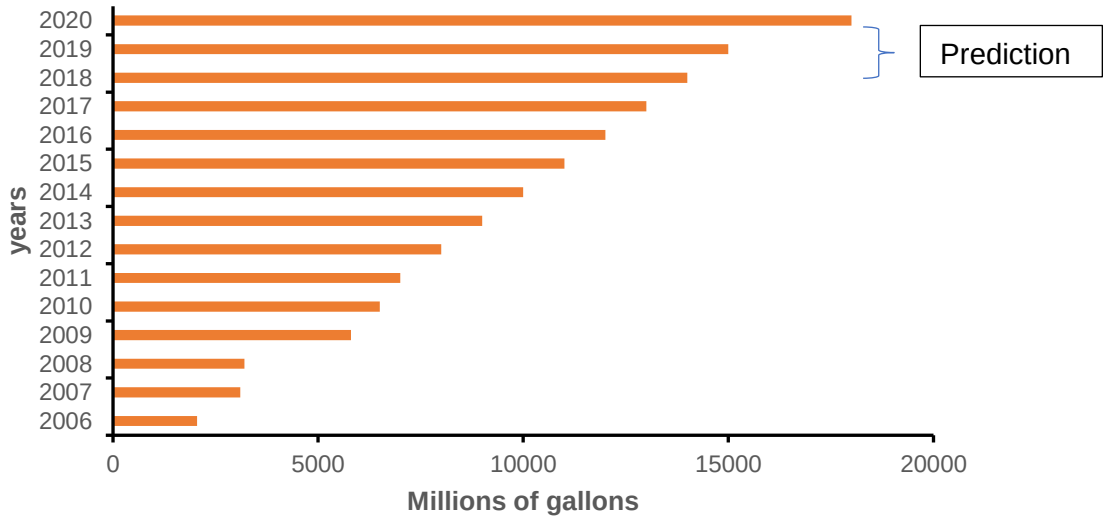


**Figure 1.1 Production annuelle mondiale de biodiesel**

La loi de 2005 sur la politique énergétique visait à réduire les besoins en combustibles fossiles importés. Le gouvernement a décidé d'acheter du carburant de remplacement pour les automobiles. Lorsque le département de l'énergie a finalisé le biodiesel comme une approche

substantielle pour l'automobile sans changer la configuration du moteur, le biodiesel est devenu une option si attrayante et alternative pour certains opérateurs.

## 1.2 Production générale de biodiesel

Plusieurs produits alimentaires (huile végétale, huile végétale non comestible, huile de cuisson usagée, graisses animales, etc.) peuvent être utilisés pour produire du biodiesel en fonction de leur disponibilité et de leur coût (Knothe & Razon, 2017). Les huiles végétales contiennent généralement des acides gras libres, des phospholipides, des stérols, de l'eau, des substances odorantes et d'autres impuretés (Anitescu & Bruno, 2012).

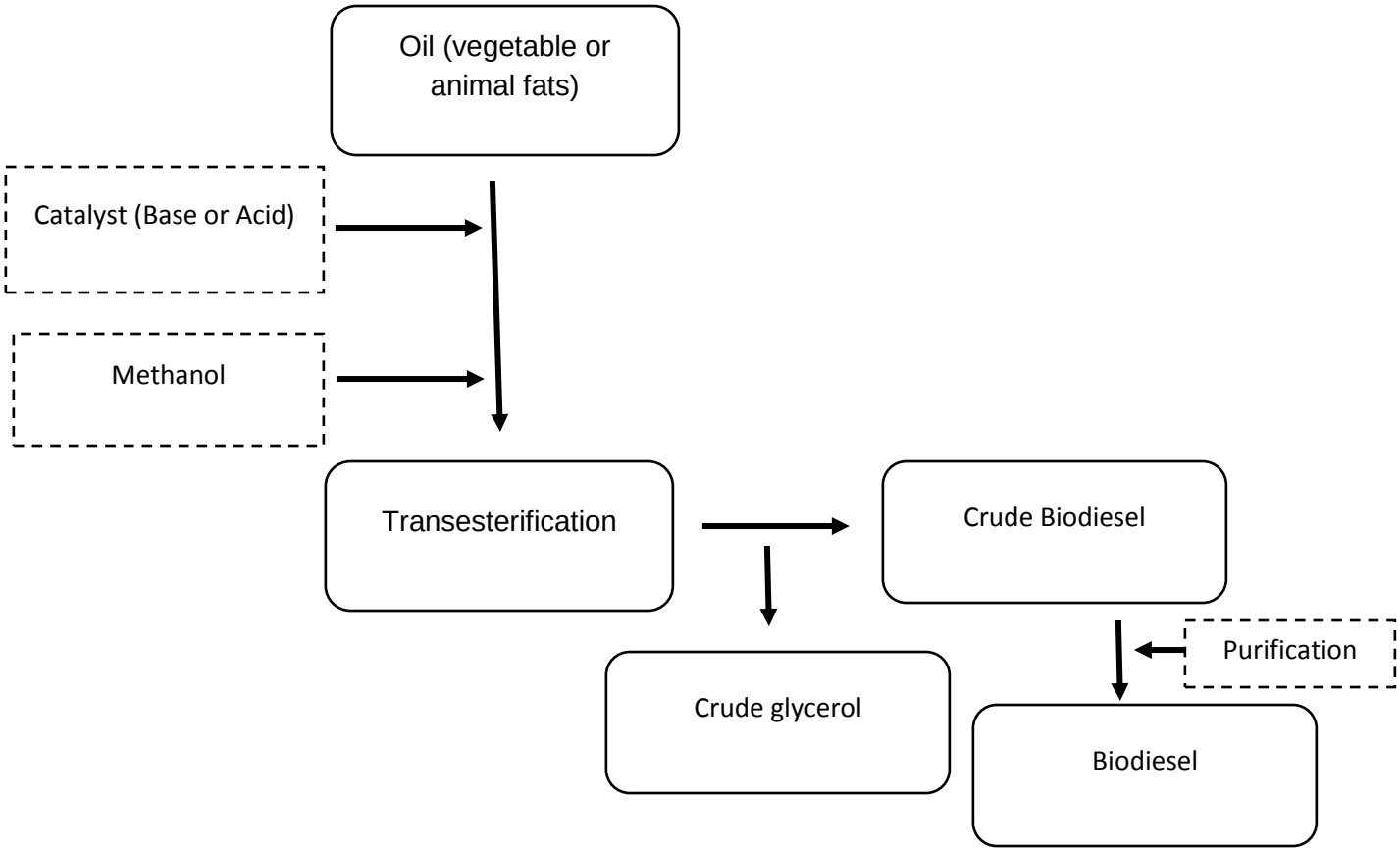


**Figure 1. 2 Production générale de biodiesel**

C’est la raison pour laquelle, l'huile ne peut pas être utilisée directement comme combustible, elle nécessite une légère modification chimique, principalement la transestérification (Figure 1.2), la pyrolyse et l'émulsification. Parmi celles-ci, la transestérification est l'étape clé et primordiale afin de produire un carburant pure et respectueux de l'environnement.

Le biodiesel est composé d'esters monoalkyliques d'acides gras à longue chaîne, dérivés de matières premières renouvelables, telles que l'huile végétale ou les graisses animales, il est destiné à être utilisé dans un moteur à allumage par compression (Figure 1.2). Le biodiesel, un potentiel substitut du carburant diesel classique, est couramment composé d'esters méthyliques d'acides gras qui peuvent être produites par transestérification avec du méthanol, à partir de triglycérides contenus dans les huiles végétales (Hincapié et al., 2011). Le biodiesel résultant est assez similaire au carburant diesel classique dans ses principales caractéristiques (Leiva-Candia et al., 2015).

## 1.3 Matières premières utilisées pour la production de biodiesel

Les huiles renouvelables sont dérivées de graines de cultures largement disponibles en fonction de la région agro-climatique; le colza (canola) en Europe du Nord, le soja aux États-Unis, l'huile de canola au Canada, l'huile de palme, la noix de coco et le tournesol dans les régions tropicales; L'Irlande utilise de l'huile de friture et des graisses animales. Parmi les graisses animales, les graisses bovines, les huiles de poisson, le gras de porc, le saindoux et le suif de canard et de bœuf sont considérés comme des huiles renouvelables. La matière première utilisée pour la production de biodiesel comprend a) matières premières de première génération b) matières premières de 2ème génération et c) matières premières de 3ème génération. Les matières premières de première génération sont comestibles et sont utilisées pour la consommation humaine. Les matières premières de seconde génération sont non comestibles comme les huiles végétales (non comestibles) ou les huiles animales et les huiles de cuisson usagées (OMD), alors que les matières premières de troisième génération sont des huiles microbiennes (levures, champignons et bactéries).

### 1.3.1 Huile végétale (comestibles et non comestibles)

Les huiles végétales comestibles obtenues à partir de colza, de tournesol, de soja, d'arachide, de palme, etc. sont les principales sources de production de biodiesel dans le monde (Kaya et al., 2009, Leiva-Candia et al., 2015, Saka & Kusdiana, 2001). Diverses huiles non comestibles se sont également avérées prometteuses comme sources d'alimentation pouvant remplacer les huiles comestibles. Celles-ci comprennent le jatropha, le neem, le mahua, le karanja, le ricin, le lin (Adebayo et Ameen, 2017, Alameldin et al., 2017, Karmakar et Mukherjee, 2017, Takase et al., 2015). Une recherche intense est toujours en cours pour découvrir d'autres huiles végétales qi peuvent être utilisées en tant que matières premières supplémentaires. Néanmoins, l'utilisation

des huiles végétales soulève beaucoup de problématiques (a) l'expansion de la base de matières premières pour potentiellement augmenter l'offre de biodiesel; b) la production de biodiesel à partir d'huiles végétales qui sont utilisées pour des fins nutritionnelles pourrait affecter leurs prix; c) l'affectation des terres, c'est-à-dire l'utilisation de terres agricoles pour «cultiver du combustible» au lieu de nourriture ou pour changer l'utilisation de terres vierges à des fins agricoles à des fins de «croissance du combustible»; et d) l'utilisation des pesticides et des engrais nécessaires à la croissance de la matière première qui peuvent s'infiltrer dans les eaux souterraines ou les plans d'eau. Par conséquent, les sources «alternatives» de biodiesel sont souvent des sources d'huiles usagées.

### 1.3.2 Graisses animales

Les matières grasses animales (matière première de seconde génération) sont des déchets plus disponibles et moins couteuses que les huiles de cuisson usagées. L'utilisation des graisses animales dans la production de biodiesel pourrait certainement diminuer l'épuisement du pétrole pendant quelques décennies. De nombreux chercheurs ont étudié différentes graisses animales y compris la graisse de poulet, la graisse de mouton, le suif de boeuf, le suif de canard, la peau de porc et le lard de porc. da Cunha et al. (2009) ont évalué la production à l'échelle pilote de biodiesel à partir de suif de bœuf et ont obtenu l'approbation de l'Agence nationale du pétrole (ANP) pour vérifier la qualité du carburant. Soldi et al. (2009) ont étudié le suif de boeuf et l'huile de soja contenant 53 mg de KOH / g d'indice d'acide pour la conversion du biodiesel en utilisant du polystyrène sulfoné catalytique hétérogène dérivé du polystyrène linéaire. Nelson et Schrock (2006) ont analysé la disponibilité des matières premières, l'efficacité énergétique et les facteurs économiques pour convertir le suif de bœuf en biodiesel. Chakraborty et Sahu (2014) ont examiné le suif de chèvre en utilisant un réacteur à rayonnement infrarouge (IRAR) pour la production de biodiesel et ont souligné qu'il y avait une réduction du temps de réaction par rapport au réacteur discontinu conventionnel. Yahyaee et al. (2013) ont examiné l'huile de poisson comme matière première potentielle pour la conversion de l'ester méthylique en concevant une machine d'extraction séparée, et comparé les propriétés de l'huile de colza et l'huile de cuisson usagée. Mata et al. (2011) ont évalué les méthodes de purification du biodiesel produit à partir de la graisse de poulet, du suif de boeuf et du lard de porc. Ils ont conclu que les graisses animales pourraient être efficacement utilisées pour la production de biodiesel en tant que source de matière première viable non comestible. Il faut noter qu'il est manifeste que la graisse de poulet en termes de disponibilité et de faible coût par rapport aux huiles végétales vierges peut présenter une matière première potentielle pour la production de biodiesel de haute qualité.

Toutefois, la haute teneur en eau et en acides gras libres limite les possibilités production de biodiesel à partir des huiles prévenant de graisses animales. Les esters méthyliques de l'huile animale contiennent environ 20% d'esters méthyliques en C6-C12, ce qui conduit à des résultats médiocres au point d'éclair et à une faible stabilité d'oxydation. En raison des inconvénients que présentent les carburants de première et de deuxième génération, les chercheurs se concentrent plutôt sur l'huile microbienne accumulée par les microalgues, les levures, les champignons et les bactéries pour la production du biodiesel.

### 1.3.3 Lipides microbiens Oleagineux

Le biodiesel est le plus souvent extrait des Triacylglycérols (TAG) stockés dans les plantes ou les graisses animales. Toutefois, les procaryotes peuvent également accumuler des lipides de stockage et synthétiser les TAG en utilisant des composés organiques (Knothe & Razon, 2017). Historiquement, le recours aux huiles microbiennes présentait une alternative aux huiles végétales et aux acides gras polyinsaturés spécifiques pour la consommation humaine (Wynn et Ratledge, 2005).

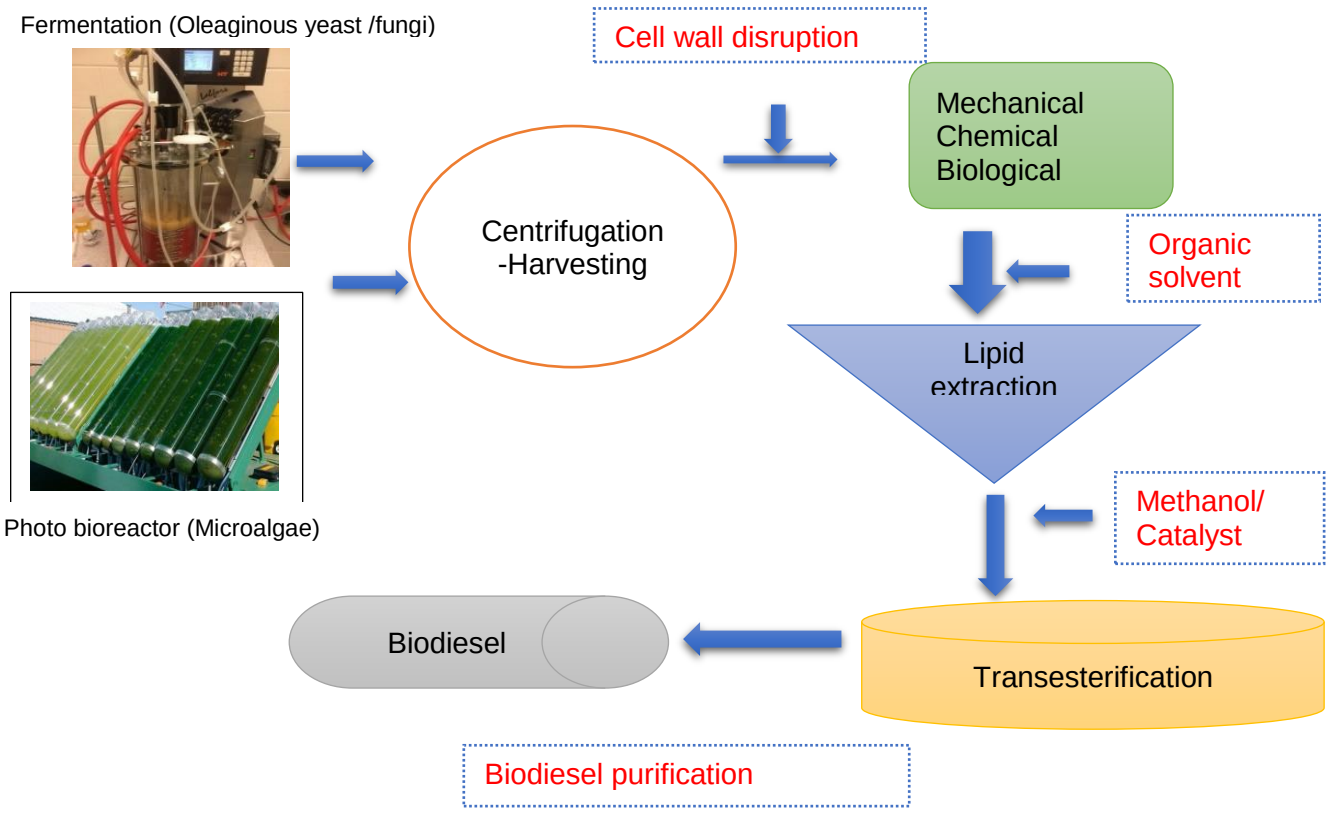


**Figure 1. 3 Production de biodiesel à partir de lipides microbiens oléagineux**

Le cout de la plupart des huiles microbiennes a affecté la faisabilité de leur production industrielle, mais avec l'avancement des techniqes de fermentation, l'huile microbienne (principalement de champignons et de levures) peut être produite en quantités équivalentes à des acres de terres agricoles (Alvarez & Steinbüchel, 2002; & Razon, 2017, Wynn et Ratledge, 2005). Les huiles microbiennes offrent l'avantage ultime (Ochsenreither et al., 2016) d'être cultivées dans un environnement contrôlé et par conséquent, la cohérence et la reproductibilité de la production de biodiesel (Rawat et al., 2013).

Les micro-organismes oléagineux ont la capacité de stocker des lipides à plus de 20% de leur masse sèche cellulaire totale (Tableau 1.1)**.** Les lipides microbiens, également appelés huiles unicellulaires (SCO), sont produits par des micro-organismes oléagineux, y compris les bactéries, les champignons, les algues et les levures (Liang et Jiang, 2013). Les bactéries procaryotes peuvent synthétiser des types spécifiques de lipides et les microorganismes eucaryotes, y compris les champignons, les algues et les levures peuvent synthétiser ou accumuler des triacylglycérols (TAG), qui sont les principaux composants nécessaires à la production de biodiesel (Figure 1.3).

Les bactéries oléagineuses peuvent accumuler des lipides dans des conditions particulières et la composition de ces lipides produits diffère des autres sortes d'huiles microbiennes (Li et al., 2008a). Seules quelques bactéries, y compris *Mycobacterium*, *Nocardia*, *Streptomyces* et *Rhodococcus*, *Gordonia*, pourraient produire des lipides similaires à ceux produits par les micro-organismes oléagineux (Miller, 2012). Il a également été rapporté que *Rhodococcus* et *Gordonia* pouvaient accumuler jusqu'à 80% de lipides dans des conditions particulières - forte teneur en carbone, faible concentration en azote et très faible concentration de biomasse (Gouda et al., 2008). Les micro-algues oléagineuses accumulent normalement une teneur en lipides variant de 20 à 80% (Ochsenreither et al., 2016). *Chlorella, Neochloris, Nannochloropsis, Scenedesmus* sont les souches de micro-algues les plus courantes qui produisent 40 à 60% de leur masse sèche cellulaire (Islam et al., 2017; Zhang et al., 2012). Bien que les recherches ont principalement montré l'efficacité des microalgues pour la production de lipides en utilisant des photo-bioréacteurs fermés, ces derniers présentent certains inconvénients. Par exemple, le passage à des niveaux pilotes est considérablement compliqué. De plus, la fixation des cellules sur les parois du tube du réacteur peut également empêcher la pénétration de la lumière. De plus, malgré que les systèmes fermés puissent améliorer la concentration de la biomasse, la croissance des micro-algues est toujours sous-optimale en raison des variations de température et d'intensité lumineuse (Reijnders, 2018).

Les levures oléagineuses peuvent atteindre rapidement une densité élevée dans des conditions contrôlées dans un bioréacteur et peuvent accumuler jusqu'à 70% de leur masse sèche cellulaire en lipides. Les levures oléagineuses les plus courantes qui ont été signalées comprennent *Lipomyces starkeyi, Lipomyces lipofer*, *Rhodosporidium tortuloides, Yarrowia lipolytica, Cryptococcus albidus*, *Cryptococcus albidun*, *Trichosporon pullulans* et *Rhodotorula glutinis* (Tang et al., 2007; Alvarez et Steinbüchel, 2002; al., 2008; Zhao et al., 2008).

**Tableau 1. 1 Micro-organismes oléagineux ayant un potentiel de production de lipides> 40% de leur poids sec cellulaire**

| **Micro-organismes** | **Lipid content (% cell dry weight)** | **Références** |
|---|---|---|
| **Bacteria** | | |
| *Nocardia globerula* 432 | >49.7% | (Alvarez & Steinbüchel, 2002) |
| *Rhodococcus opacus* PD630 | >50% | (Miller, 2012) |
| *Streptomyces coelicolor* TR0958 | 83% | (Arabolaza *et al.*, 2008) |
| *Streptomyces coelicolor* TR0123 | 64% | (Arabolaza *et al.*, 2008) |
| **Yeast** | | |
| *Lipomyces starkeyi* | 61% | (Zhao *et al.*, 2008) |
| *Rhodosporidium toruloides Y4 (Batch culture)* | 48% | (Papanikolaou & Aggelis, 2011) |
| *Rhodosporidium tortuloides* AS 2.1389 | 66% | (Xu *et al.*, 2016) |
| *Candida sp.* LEB-M3 | 50% | (Duarte *et al.*, 2013) |
| *Rhodosporidium toruloides Y4* (Fed batch culture) | 67.5% | (Li *et al.*, 2008a) |
| *Kodamaea Ohmeri* BY4-523 | 53% | (Kitcha & Cheirsilp, 2013) |
| *Trichosporon fermentans* | 62.4% | (Zhu *et al.*, 2008) |
| *Rhodosporidium diobovatum* | 50% | (Munch *et al.*, 2015) |
| **Fungal species** | | |
| *Mortierella isabellina* | 50-55% | (Papanikolaou *et al.*, 2004) |
| *Mortierella isabellina* | 65.5 % | (Chatzifragkou *et al.*, 2010) |
| *Colletotrichum* | 73% | (Dey *et al.*, 2014) |
| *Fusarium oxysporum* | 43% | (Matsakas *et al.*, 2017) |

La teneur en lipides des souches variait avec le changement des conditions de culture. Certaines des espèces fongiques *Mortierella isabellina, Aspergillus terreus et Motierella alpina* produisent également de 50 à 80% en poids de lipides (Gardeli et al., 2017; Shafiq, 2017). Par rapport aux microalgues, les levures et les champignons poussent plus rapidement et nécessitent moins de

terre. Comme représenté sur la Figure 1.3, les micro-organismes oléagineux ont été cultivés par fermentation pour accumuler les lipides et ensuite différentes étapes du procédé suivent comme la récolte de la biomasse, la rupture de la paroi cellulaire, l'extraction des lipides microbiens en utilisant un solvant organique.

## 1.4 Traitement en aval de la biomasse de levure contenant des lipides

L'accumulation de lipides présentée sur la Figure 1.3, est suivie de la récolte qui est l'étape préliminaire pour le traitement de la biomasse lipidique. La phase de séparation est faite par centrifugation qui compte 20-30% du coût de production total du biodiesel (Yuan et al., 2015). L'utilisation de la centrifugation est dûe au fait que la sédimentation par gravité est lente. La filtration tangentielle est également coûteuse et ne peut pas être utilisée en raison de sa faible valeur marchande (Hsu & Hsiau, 2015). Jusqu'à présent il n'existe aucune meilleure technologie de récolte conçue pour les industries (Uduman et al., 2010).

### 1.4.1 Stratégies de récolte des lipides contenus dans la biomasse

**Centrifugation :** La centrifugation est la méthode de récolte de base utilisée pour la séparation des produits extracellulaires et l'eau. Le principe de base de la centrifugation implique une force centrifuge, une accélération de la séparation basée sur la masse des particules, et ensuite la biomasse et l'eau sont séparées en drainant l'excès. La centrifugation peut également être suivie d'une sédimentation pour séparer le surnageant (Harun et al., 2010). La centrifugation est efficace pour récupérer plus de 90% de la biomasse du bouillon fermenté (Dong et al., 2016). Récemment, plusieurs centrifugeuses nouvellement conçues ont été utilisées pour la récolte de la biomasse dans le domaine de la production du biodiesel. Néanmoins, ces centrifugeuses nécessitent toujours un investissement et des coûts d'exploitation élevés par rapport à d'autres approches. La consommation d'énergie pour le fonctionnement de la centrifugation calculée approximativement est de 13,8 MjkgDW-1 (Japar et al., 2017).

**Flocculation :** La floculation est un processus dans lequel les particules de soluté dans une solution se rejoignent pour former des agrégats appelés flocs (Uduman et al., 2010). La sédimentation de ces flocs est par la suite facilité grace à la force de pesanteur (Chen et al., 2011). La floculation conventionnelle fonctionne par un mécanisme de dispersion de charge (Rawat et al., 2011). La biomasse porte généralement une charge négative (Klis et al., 2006, Klis

et al., 2002). En conséquence, les particules de la biomasse ne peuvent pas s'agréger à l'intérieur d'une suspension (Salim et al., 2011). Les produits chimiques appelés floculants aident à contrer cette charge négative à la surface de la levure (Calderón et Cerbón, 1992). Les forces qui influencent la liaison entre deux cellules peuvent également inclure des interactions électrostatiques (Caridi, 2006). La floculation n'est pas adaptée à la biomasse de champignons qui contient une combinaison de parties hautement structurées sous forme de mycélium et un certain degré de colloïdes difficiles à éliminer par filtration. Cependant, dans certains cas l'extraction des protéines par fragmentation intracellulaire et perturbation avant la floculation peut rendre la longueur les hyphes 70% plus inférieure à celle d'origine ce qui favorisera l'agglomération (Knudsen et al., 2005). La floculation améliore le taux de sédimentation des micro-algues en agrégeant les particules dispersées en plus gros flocs et par la suite la récupération de la biomasse est plus importante (Salim et al., 2011). La charge de surface négative sur le micro algues peut également être neutralisée ou réduite par l'ajout de coagulants à base de fer ou d'aluminium (Chen et al., 2011). Bio floculant comme la substance extra-polymère (EPS) sont une alternative respectueuse de l'environnement aux sels inorganiques et polymères organiques synthétiques en raison de leur biodégradabilité et ils sont renouvelables. De plus, il est connu que les micro-organismes produisent des polymères naturels qui aident à l'agrégation de la matière organique et des cellules. Plusieurs chercheurs ont exploré l'extraction de bio floculants à partir de souches microbiennes (More et al., 2014). La littérature suggère que les macromolécules, mélanges principalement de polysaccharides et de protéines, sont responsables des propriétés de floculation des bio floculants rapportés (Nouha et al., 2017). L'amélioration de l'agrégation naturelle et de la bio floculation des cellules de levure pour une simple sédimentation par gravité pourrait s'avérer une méthode prometteuse en termes de qualité des effluents et d'économie.

### 1.4.2 Séchage de la biomasse

Le séchage de la biomasse est une autre étape qui doit être prise en considération. Le séchage au soleil est probablement la méthode la moins chère souvent utilisée pour le traitement de la biomasse (Li et al., 2008b). Cependant, cette méthode nécessite de larges laps de temps, une grande surface de séchage et risque la perte de certains produits bio-réactifs. La méthode de séchage la moins couteuse qui suit le séchage au soleil serait le séchage sur étagère à basse pression (Verma et al., 2017). Il est néanmoins de faible efficacité.

Les techniques de séchage les plus efficaces mais les plus coûteuses ayant été étudiées pour le séchage de la biomasse oléagineuse comprennent le séchage au tambour, le séchage par pulvérisation, le séchage en lit fluidisé, le séchage libre et la déshydratation par fenêtre réfractaire (Brennan et Owende, 2010). Il est important de trouver l'équilibre entre l'efficacité du séchage et le rapport coût-efficacité afin de maximiser la production d'énergie nette des carburants issus de la méthode de la biomasse oléagineuse.

### 1.4.3 Perturbation de la paroi cellulaire et extraction d'huile

La rupture de la paroi cellulaire est un phénomène fondamental pour récupérer les produis intracellulaires contenus dans du micro-organisme. La paroi cellulaire varie en fonction du type de micro-organisme : procaryote ou eucaryote. La paroi cellulaire d'un microorganisme eucaryote tel que de la levure et des champignons, est constituée de différentes concentrations de mannoprotéines, de β1,6-glucane, de β1,3-glucane et de chitine. Tandis que les bactéries sont constituées de N-acétyl glucosamine [NAG] et d'acide N-acétyl muramique [NAM] liées par des liaisons β-glycosidiques. Au cours des trois dernières décennies, plusieurs technologies de perturbation des parois cellulaires ont été développées sur la base du type de paroi cellulaire, du coût du produit et de l'efficacité du produit. Comme le montre la Figure 1.3, les méthodes de désintégration des parois cellulaires peuvent généralement être classées en trois types: 1) mécanique, 2) chimique et 3) biologique. Récemment, la perturbation des parois cellulaires de la biomasse sèche et humide a été étudiée en utilisant diverses méthodes mécaniques, notamment le traitement par des ultrasons. Le battement des billes, les micro-ondes et l'électroporation (D'Hondt et al., 2014b). Les méthodes chimiques utilisent des acides, des bases et des solvants organiques, alors que les méthodes biologiques impliquant principalement l'utilisation d'enzymes.

**Perturbation mécanique des parois cellulaires:** La biomasse oléagineuse obtenue après la récolte de la biomasse est lyophilisée pour éliminer l'humidité et désactiver la biomasse microbienne contenant des lipides (Guldhe et al., 2014). Les produits extracellulaires sont faciles à séparer des cellules par filtration ou centrifugation. Les produits intracellulaires soit résident dans le cytoplasme, soit existent dans la cellule en tant que corps d'inclusion tels que les lipides. Le lipide se présente principalement sous forme de gouttelettes lipidiques. Afin d'extraire les produits intracellulaires désirés, une rupture de cellule doit être effectuée pour libérer ces produits en vue de séparation supplémentaire. Par conséquent, la rupture des cellules est une étape critique de la séparation des lipides des cellules.

L'homogénéisation des lames, le broyage des billes, l'homogénéisation des liquides, lles ultrasonset le gel / dégel sont les approches physiques les plus utilisées (Dhanani et al., 2013, Prabakaran et Ravindran, 2011, Zhang et al., 2014a). Dans ces approches ultrasonication a été largement utilisé au niveau industriel pour l'extraction des protéines, la synthèse chimique, la désinfection, et la rupture des cellules avec une addition chimique réduite. Une étude a été réalisée en utilisant le traitement par les ultrasons pour l'extraction des lipides de *Nannochloropsis oculata* (Adam et al., 2012). Le rendement lipidique le plus élevé était de 0,21% en poids / poids, ce qui est inférieur au rendement d'extraction du solvant (chloroforme et méthanol) (5,47% en poids). Dans une autre étude de Zhang et al. (2014a), les ultrasons(50 Hz, 2800 W) ont été appliqués pour l'extraction des lipides à partir de *Trichosporon* oléagineux et de SKF-5 (une souche fongique oléagineuse). L'étude a comparé l'efficacité de l'eau, du méthanol, de l'hexane et du chloroforme / méthanol 1: 1 v / v sous ultrasons. Dans le cas de *Trichosporon oléagineux*, la récupération des lipides était de 43,2%, 10,2%, 75,7% et 100% en poids en utilisant l'hexane, l'eau, le méthanol et le chloroforme / méthanol, respectivement. De même, pour SKF-5, la récupération de lipides était de 100% p / p en utilisant le chloroforme / méthanol à la fréquence des ultrasons de 50 Hz avec une puissance de 2800 W pendant 15 min. Il faut indiquer que l'eau dans ce scenario a donné 9,3% (p/p), le méthanol 65,1% ( p / p) et l'hexane 33,2% (p / p). En somme. La récupération des lipides avec des ultrasons devrait davantage être étudiée pour améliorer son rendement.

Plusieurs méthodes de perturbation cellulaires sont utilisées dans les laboratoires ou les industries à grande échelle. Cependant, l'énergie consommée via ces techniques est généralement plus élevée que l'énergie de combustion de la biomasse (Lee et al., 2013). Une étude des techniques de perturbation cellulaire suggère des besoins énergétiques variant entre 3.3 × 107 J / kg pour la perturbation de la biomasse sèche par cavitation hydrodynamique et 5.3 × 108 J / kg pour les homogénéisateurs à haute pression (HPH) (Lee et al., 2012). Il faut noter que la chaleur de combustion de la biomasse n'est que de 2,7 × 107 J / kg (Lee et al., 2012), ce qui entraîne généralement un bilan énergétique net négatif dans les le domaine de production de biocarburants. Cependant, l'utilisation de l'énergie varie en fonction du dispositif utilisé pour la perturbation cellulaire. Par exemple, lorsque les algues sont perturbées par une pointe de microscopie à force atomique (AFM), l'énergie spécifique de rupture n'est que de 6,73 × 102 J / kg, ce qui est environ 105 fois moins que la cavitation hydrodynamique actuelle (Lee et al., 2013). Les estimations théoriques basées sur les propriétés individuelles des cellules suggèrent également une énergie de perturbation spécifique significativement plus faible que les processus

actuels. Une estimation fondée sur la résistance à la traction des parois cellulaires suggère une énergie de rupture cellulaire de 2,26 × $10^2$ J / kg de biomasse sèche (Lee et al., 2012). Une estimation similaire basée sur l'énergie de liaison anticipée dans les parois cellulaires serait de 3,32 × $10^2$ J / kg (Lee et al., 2012). L'énergie nécessaire à la perturbation cellulaire en utilisant la tension critique pour rompre une membrane lipidique suggère seulement 1,3 × 10-1 J / kg de biomasse cellulaire (Krehbiel, 2014). Zhang et al. (2016) ont rapporté que le broyage des billes a nécessité une énergie de 1894,7 GJ pour perturber la paroi cellulaire, ce qui équivalant à l'énergie de stérilisation requise avant toute fermentation. Par conséquent, les technologies de perturbation des parois cellulaires à faible énergie doivent être identifiées pour des produits comme le biodiesel.

**Méthodes chimiques de désintégration des parois cellulaires:** La perméabilité des cellules peut être augmentée par divers produits chimiques, tels que la polymyxine, les polymères de lysine, la protamine, les peptides polycationiques et les détergents cationiques. Si la perméabilité dépasse une certaine limite, cela peut causer la rupture des cellules. Les acides et les alcalis induisent l'hydrolyse de l'enveloppe cellulaire. L'enveloppe cellulaire peut également être affaiblie par chauffage, ce qui peut entraîner une hydrolyse, et une dénaturation des protéines. Le traitement de S. obliqus sec avec de l'acide sulfurique 2 N'a augmenté le rendement de fermentation de l'éthanol à 95,6% du rendement des cellules témoins soumises à une hydrolyse quantitative acide rigoureuse avec 76% d'acide sulfurique (Miranda et al., 2012). Cette méthode de perturbation de la biomasse humide (teneur en eau de 80%) a atteint 60% de performance de celle obtenue avec une biomasse sèche. En outre, une acidité relativement bégnine peut traiter adéquatement la biomasse des microalgues. Sathish et Sims (2012) ont effectué une extraction par étapes utilisant des acides et des alcalis pour la perturbation de la biomasse humide composée principalement de *Chlorella sp. Et Scenedesmus sp*. Les enveloppes cellulaires ont été hydrolysées par acide sulfurique 1 M et hydroxyde de sodium 5 M à 90 ° C pendant 30 minutes chacune. La précipitation les acides gras libres, a été réalisée en ajoutant de l'acide sulfurique 0,5 M pour dissoudre la chlorophylle. Par la suite, une récupération de 60% des lipides totaux a été obtenue en utilisant de l'hexane. En résumé, malgré que cette étude ait nécessité plusieurs étapes avec centrifugation, elle s'avère intéressante du fait qu'une séparation des lipides et la chlorophylle qui est un sous-produit de l'extraction classique des lipides, a été testée. Un traitement chimique a également été appliqué pour extraire l'astaxanthine, un supplément antioxydant à haute valeur économique. Parmi divers produits chimiques, y compris l'acétone, le méthanol, le diméthylsulfoxyde (DMSO), l'acide chlorhydrique (HCl) et les acides organiques, le

4N HCl était le meilleur réactif chimique de perturbation cellulaire à pouvoir permettre de récupérer 94% de l'astaxanthine des corps cellulaires. La performance de récupération de 4 N HCl était beaucoup plus élevée que celle du DMSO (67%) et du méthanol (19%). Malgré les performances élevées des produits chimiques en termes de perturbation des cellules, les méthodes chimiques présentent certains inconvénients : 1) Ils peuvent corroder la surface des réacteurs à force d'alimentation, 2) La neutralisation des acides et des alcalis dilue la biomasse et double le coût, 3) les produits chimiques peuvent réagir avec les produits cibles. Pour ces raisons, diverses approches synergiques avec des méthodes mécaniques devraient être étudiées pour réduire l'utilisation de produits chimiques.

L'une de ces approches serait la perméabilisation qui est normalement utilisé pour augmenter l'accessibilité des enzymes intracellulaires. Les cellules perméabilisées pourraient être utilisées comme source d'enzymes intracellulaires réagissant en tant que biocatalyseurs de biotransformation d'un substrat en divers produits. De nombreux produits chimiques et détergents (éthanol, isopropanol, n-butanol, n-propanol, toluène, benzène, chloroforme, Triton X-100, dodécylsulfate de sodium (SDS), digitonine et bromure de cétyltriméthylammonium (CTAB)) sont utilisés pour la perméabilisation des cellules de levure. Cependant, la plupart sont toxiques et non biodégradables, ce qui entrave leur application en particulier dans l'industrie alimentaire.

Il existe un détergent dérivé des acides aminés appelé N-lauroyl sarcosine (N-LS), (un détergent anionique composé d'acide aminé sarcosine et d'acide gras) qui est non toxique, biodégradable et complètement métabolisable dans le corps humain en sarcosine et en acides gras. Ainsi, il peut être utilisé en toute sécurité pour perméabiliser les cellules de levure afin d'exploiter diverses activités enzymatiques intracellulaires. (Yadav et al., 2014)

Des études récentes ont montré que l'acide nitreux libre (FNA), qui est la forme protonée du nitrite, peut détruire la cellule et causer des effets d'interférence enzymatique dans plusieurs microorganismes (Jiang et al., 2011). Le FNA, considéré comme un réactif biochimique, et dérivés tels que le radical oxyde nitrique (NO) et l'anhydride nitreux ($N_2O_3$) ont un effet sur la dégradation des protéines et des polysaccharides (Dedon et Tannenbaum, 2004). Il a donc été appliqué dans l'industrie de l'eau et des eaux usées pour le traitement des boues et le contrôle des biofilms. Cependant, il n'y a eu aucun rapport sur l'effet de FNA sur la biomasse oléagineuse. Notre hypothèse est que le prétraitement par FNA pourrait aider à perturber l'enveloppe cellulaire des algues, augmentant ainsi le taux de transfert de masse lipidique des cellules algales dans un solvant organique.

**Autres méthodes d'extraction:** Il existe plusieurs autres méthodes d'extraction telles que la méthode biologique (enzymes). Si les enzymes sont choisies avec soin, la rupture des cellules enzymatiques est efficace. Cependant, le coût élevé des enzymes présente un inconvénient majeur. Les autres technologies d'extraction regroupent le choc osmotique,la rupture de paroi cellulaire sous haute pression, l'électroporation, le CO2 supercritique, les micro-ondes et le liquide ionique. Toutes ces technologies de perturbation des parois cellulaires sont à l'échelle du laboratoire et l'efficacité de l'extraction des lipides varie selon les technologies et le type de biomasse. Par conséquent, une technologie en une seule étape doit être développée pour la production de biodiesel à base de lipides microbiens à l'échelle industrielle.

### 1.4.4 Extraction des lipides en utilisant des solvants organiques

Les solvants les plus utilisés pour l'extraction des lipides après la rupture de la paroi cellulaire comprennent les alcools (se référant principalement au méthanol), le chloroforme, l'hexane, l'éther de pétrole et l'éther diéthylique. Le choix des solvants est essentiel car il influence l'efficacité de l'extraction, les propriétés physiques des lipides et la récupération des solvants. Pour extraire les lipides des tissus, il est nécessaire de créer suffisamment de force pour rompre la membrane cellulaire et la lipoprotéine pour libérer les lipides. Cependant, il est primordial que les solvants ne réagissent pas chimiquement avec les lipides (Dufreche et al., 2007, Ferraz et al., 2004).

La membrane cellulaire a une double couche lipidique. Chaque couche lipidique est composée d'une tête polaire et d'une queue non polaire. La queue est orientée vers l'intérieur et la tête vers l'extérieur (vers le cytosol aqueux de la cellule ou de l'environnement extérieur). Ces queues ou têtes se regroupent pour former la bicouche. La structure de la membrane cellulaire est fait d'une manière que le solvant non polaire est incapable de l'extraction du fait qu'il ne peut ni approcher ni extraire les lipides de la membrane cellulaire, et par conséquent ni rompre la membrane. Cependant, si la rupture des cellules est effectuée avant l'ajout du solvant non polaire, l'extraction sera possible. Le solvant polaire (sous forme d'eau) peut s'approcher de la membrane, cepandant si sa polarité est inférieure, il ne peut pas extrairer le lipide car les queues sont étroitement liées (interaction hydrophobe). Par conséquent, un mélange de solvants polaires et non polaires est requis. Le solvant polaire interagit et détache la membrane cellulaire, alors que le solvant non polaire excède la queue non polaire et dissout le lipide. Jusqu'à présent, de nombreuses techniques d'extraction (mécanique et chimique) par solvants (polaires et non

polaires) ont été utilisées (Tableau 1.2). L'utilisation du mélange de solvants polaires et non polaires augmente l'efficacité de l'extraction à environ 95% à 25 ° C.

**Tableau 1. 2 Extraction lipidique avec différents solvants organiques**

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

Les gouttelettes lipidiques, également appelées corps lipidiques, contenues les tissus contenant de l'huile sont principalement des triglycérides (TAG), qui sont des substances non polaires solubles dans l'hexane, le cyclohexane, l'éther diéthylique et le chloroforme. Par conséquent, l'extraction des TAG requière un solvant non polaire. Cependant, il est nécessaire de rompre la cellule en premier afin de permettre l'accès du solvant non-polaire aux TAG. Malgré la faisabilité de l'utilisation des solvants organiques pour la production de biodiesel, ces substances présentent plusieurs limitations telles que: 1) coûteuses, 2) non sécuritaires et 3) solubilisent les contaminants pendant le processus d'extraction de l'huile. Par conséquent, de plus amples recherches doivent être effectuées afin de pouvoir séparer l'huile des boues ou des cellules perturbées tout en égard à la sécurité environnementale et industrielle.

### 1.4.5 Transestérification

La conversion du lipide microbien en biodiesel est connue sous le nom de transestérification comme représenté sur la Figure 1.3. Cette méthode Pour réduire le coût du biodiesel, le processus de transestérification in situ a été développé à partir d'un procédé conventionnel. Un processus de transestérification classique implique les étapes suivantes: la récolte de la biomasse, la rupture de la paroi cellulaire, la séparation des lipides microbiens en utilisant la biomasse sèche, la transestérification des lipides pour produire les FAMES (biodiesel) et la purification du biodiesel. La transestérification in situ est basée sur l'utilisation directe de la biomasse riche en lipides sans extraction préalable, permettant à la réaction de transestérification d'avoir lieu au sein de la matrice solide. Durant la transestérification, l'extraction des lipides et / ou la formation de produits se produisent simultanément, ce qui peut techniquement être appelé une extraction réactive.

Dans certains cas, la réaction d'extraction et de transestérification peut effectivement se produire de manière séquentielle. En raison de l'impossibilité de déchiffrer comment le processus se déroule dans le champ de vision de la microscopie, on parle alors de transestérification in-situ tant que la cellule entière est utilisée pour la production de biodiesel. La transestérification in situ (ISTE) présente quelques points faibles incluant: a) un rapport molaire élevé de méthanol/lipides est exigé, b) l'efficacité de conversion de lipide à biodiesel est inférieure à 95%, c) temps de réaction plus long (pour la chaleur ISTE assistée).

Zhang *et al.* (2014b) ont effectué une perturbation simultanée de la paroi cellulaire et une transestérification (in situ) d'une biomasse sèche en utilisant l'ultra-sonication. Ils ont rapporté 92,1% en poids de conversion lipidique (par rapport aux lipides totaux) en FAMEs avec un rapport

molaire méthanol / lipide de 60: 1 et NaOH à 1% (p / p de lipide) en 20 minutes de temps de réaction. Alors que pour ISTE (60$^{o}$C), un rapport molaire méthanol / lipide élevé de 360: 1 était nécessaire pour la réaction. En effet, la transestérification (in situ) couplée avec l'ultra-sonication demande moins de méthanol comparé à l'ISTE thermique; cela est dû au fait que l'ultra-sonication aide à la dégradation de la paroi cellulaire et le méthanol peut donc réagir directement avec les lipides. Dans une autre étude rapportée par (Hincapié et al., 2017), la transestérification in situ a été réalisée sur une biomasse de levure séchée en utilisant l'hexane comme co-solvant couplé avec ultra-sonication suivie d'une réaction avec 1,0 mL de méthanol / g de lipide pour atteindre une conversion maximale de 88% (p / p de lipides) de FAMEs en 8 h de temps de réaction. La forte teneur en humidité explique le rapport molaire élevé méthanol / lipide.

La transestérification (in situ) présentent aussi quelques inconvénients tels que : l'allongement du temps de réaction fait que les ultrasons peuvent peut décomposer les débris cellulaires en raison des forces mécaniques de cisaillement et la solubilisation des contaminants. Pour cette raison, davantage de recherches doit être concentré sur le développement d'un procédé utilisant la biomasse humide contenant du pétrole comme matière première pour la production de biodiesel avec une efficacité de transestérification accrue en utilisant une faible concentration de méthanol et un temps de réaction court.

# PARTIE 2: PROBLEMES

Les problèmes subséquents suivants ont été mis en évidence

## 2.1 Problèmes associés aux matières organiques du biodiesel

Le cout des matières premières présente 40 à 60% du cout global de la production de biodiesel. Selon leur disponibilité, plusieurs matières premières telles que l'huile végétale comestible à base de maïs, de tournesol, de palme et d'huile végétale non comestible de jatropha sont utilisées. Cependant, la grande valeur économique et les préoccupations concernant la crise alimentaire par rapport à la crise du carburant, limitent leur applicabilité à la production de biodiesel. Les matières premières de deuxième génération telles que les graisses animales ont leurs propres inconvénients en raison de la teneur élevée en eau et en acides gras libres dans l'huile. Les matières premières de troisième génération telles que l'huile microbienne, présentent une option durable pour les chercheurs, mais le défi majeur rencontré serait le traitement en aval.

## 2.2 Consommation d'énergie élevée pendant la récolte et le séchage de la biomasse

La matière première de troisième génération telle que l'huile microbienne peut être prometteuse cependant, après la fermentation, le traitement en aval pour obtenir des lipides microbiens implique de multiples étapes. L'étape préliminaire de la récolte de biomasse dépend des caractéristiques des espèces microbiennes et du type de produit désiré. La séparation de la biomasse par centrifugation nécessite un investissement et des coûts d'exploitation élevés. Par conséquent, il est nécessaire de développer un procédé économiquement faisable et capable de réaliser une séparation complète des cellules à partir du bouillon fermenté. La lyophilisation de la biomasse est également un processus couteux en énergie et en temps.

## 2.3 Extraction et récupération des lipides : étapes importantes dans un processus industriellement réalisable

Après la récolte de la biomasse à partir de bouillon fermenté, l'extraction des lipides est un processus typique dans la production de biodiesel par des hétérotrophes oléagineux (levure). L'efficacité de l'extraction des lipides dépend principalement du processus utilisé pour la rupture de la paroi cellulaire, de la perméabilité de la paroi cellulaire, du type du micro-organisme et du

solvant utilisé. Les procédés classiques d'extraction de lipides utilisant des solvants organiques tels que le chloroforme et le méthanol ne sont pas préférés en raison de leur coût élevé et de leur toxicité.

Les procédés mécaniques (par exemple, le broyage de billes, les micro-ondes, le traitement par les ultrasons) d'extraction des lipides ne sont pas non plus industriellement réalisables en raison de la consommation élevée d'énergie et de temps. La perturbation des parois cellulaires par produits chimiques génère également des problèmes spécifiques comme la corrosion des surfaces des réacteurs et la formation du savon, de plus, la neutralisation de la biomasse après traitement est coûteuse. Il est donc nécessaire de développer un procédé d'extraction efficace pour la biomasse humide pouvant réduire les multiples étapes du traitement en aval.

## 2.4 Problèmes associés à la transestérification des lipides en biodiesel

La conversion des lipides en biodiesel par un procédé de transestérification classique implique deux étapes: 1) étape d'extraction et 2) étape de transestérification des lipides extraits. Le procédé de transestérification classique est lent (12h) et nécessite une quantité élevée de solvant organique. Alors qu'en cas de transestérification in situ, le temps de réaction et le volume de solvant sont élevés et peuvent entraîner une perte d'énergie et de solvant. Par conséquent, il est nécessaire de développer une transestérification in situ ou une transestérification directe sans séchage au solvant pour réduire le temps de réaction et des étapes de traitement en vue de conversion des lipides en biodiesel avec un rendement élevé en FAMEs.

# PARTIE 3: HYPOTHÈSE ET OBJECTIFS DE LA RECHERCHE

Dans le but d'étudier le développement d'un processus économique en aval de perturbation des parois cellulaires lipidiques, d'extraction lipidique, et de transestérification pour la production de biodiesel en utilisant un bouillon fermenté de levure oléagineuse obtenu en utilisant du glycérol brut ou des boues municipales enrichies de glycérol brut comme substrat, l'hypothèse suivante est proposée:

1. A) En raison de l'urbanisation croissante, les déchets huileux des cuisines des maisons et des restaurants peuvent être séparés des eaux usées aux stations d'épuration sous forme d'écume ou contenues dans les boues (primaires et secondaires). L'utilisation de l'huile présente dans les boues comme matière première peut réduire le coût final du biodiesel. B)

En raison de la croissance rapide des microbes hétérotrophes oléagineux les produits lipidiques microbiens peuvent être une alternative à la production de biodiesel.

2. Après la fermentation, la récolte de biomasse peut être faite par floculation en utilisant des biofloculants qui peuvent être moins énergivores par rapport à la centrifugation. Les biofloculants comme l'extrapolymère (EPS) peuvent efficacement floculer des particules chargées. La levure oléagineuse (et d'autres micro-organismes oléagineux) porte une charge négative élevée sur sa surface cellulaire. En conséquence, les cellules adsorbent différents ions provenant de la matière organique et dissocient ou ionisent les groupes fonctionnels de surface et créent ainsi des agrégats. Ensuite, un biopolymère chargé négativement peut se lier à ces agglomérats pour former des particules plus grosses et produire de gros flocs. Ainsi, la récolte (décantation) de la biomasse de levure oléagineuse par floculation à l'aide de biofloculants pourrait réduire le coût final du produit.

3. Après la récolte, la lyophilisation de la biomasse est l'un des principaux processus consommateurs d'énergie et de temps. Par conséquent, la biomasse humide devrait être directement utilisée pour l'extraction des lipides en développant des techniques de désintégration de la biomasse humide avec de l'eau présente dans la biomasse de levure (sans sécher la biomasse). L'humidité dans la biomasse peut agir comme un solvant hydrophile pour une séparation facile des lipides en phase solvant.

4. Les traitements chimiques peuvent affaiblir la paroi cellulaire en hydrolysant les liaisons interconnectées entre mannoprotéine, glucane et chitine et par la suite libérer des produits intracellulaires (lipides dans notre cas): a) La perméabilisation des parois cellulaires en utilisant des surfactants biodégradables comme la N-lauroylsarcosine est plus avantageuse que celle avec des acides et des bases. Les surfactants biodégradables sont efficaces, respectueux de l'environnement et sains. Ainsi, l'extraction des lipides en utilisant N-LS pourrait rendre le processus d'extraction vert, sain et économiquement viable. b) L'acide nitreux libre (FNA, c'est-à-dire $HN0_2$) est un acide faible et monobasique sous la forme de sels de nitrite et il a été considéré comme un réactif biochimique. Le FNA et ses dérivés tels que le radical oxyde nitrique (NO) et l'anhydride nitreux ($N_2O_3$) affectent la dégradation des protéines et des polysaccharides et pourraient être utilisés pour la perturbation de la paroi cellulaire dans une biomasse humide.

5. Le diesel petrolier peut être utilisé à la place des solvants organiques pour séparer l'huile ou les lipides microbiens. Le principal avantage de l'utilisation du diesel pétrolier comme solvant est: a) miscible avec les lipides b) faible coût c) transestérification ex-situ des lipides extraits en biodiesel sans aucun séchage du solvant

6. La transestérification in situ présente des avantages par rapport à la transestérification classique, par exemple le gain de temps et de volume de solvant requis et la non modification du profil lipidique. La transestérification in-situ de la biomasse microbienne en utilisant N-LS pourrait rendre le processus efficace et efficient avec un rendement élevé en FAMEs.

## 3.1 Objectifs de recherche

L'objectif principal de ce travail de recherche était de développer un processus en aval de production de biodiesel en utilisant de la biomasse humide de levure oléagineuse et de la boue municipale.

Les objectifs spécifiques sont:

1) Réaliser des tests de floculation de la biomasse de levures oléagineuses à l'aide de bio-floculants (substances polymériques extracellulaires) en présence de coagulants inorganiques ($Al_2(SO_4)_3$ ou $CaCl_2$).

2) Effectuer l'extraction lipidique microbienne à partir de la biomasse de levure oléagineuse en utilisant le détergent (N-lauroylsarcosine).

3) Appliquer les ultrasons assistés par le détergent (N-Lauroyl sarcosine) pour favoriser la transestérification in situ en vue de la production de biodiesel à partir de biomasse humide de levure oléagineuse.

4) Réaliser la transestérification ex-situ en utilisant l'acide nitreux libre et la N-lauroyl sarcosine.

5) Extraire de l'huile microbienne à partir de différentes matrices (ex. écumes) et ce, en présence de gasoil de pétrole, suivi de la conversion de ces huiles en biodiésel.

## 3.2 Originalité

L'originalité de ce travail de recherche réside dans le développement d'un procédé de production de biodiesel utilisant un bouillon fermenté de levure oléagineux décanté avec des substances polymères extracellulaires (EPS) produites à partir de boues d'eaux usées et de glycérol brut comme substrat. Après la récolte, la matière en suspension de la biomasse décantée a été utilisée pour la perturbation de la paroi cellulaire en utilisant la N-lauroylsarcosine ou l'acide nitreux libre. La matière en suspension est extraite en utilisant du pétrodiesel comme solvant ce qui présente une approche innovatrice pour la production de biodiesel. De plus, le travail présente une nouvelle méthode de séparation du pétrole des résidus et des boues en utilisant du pétrodiesel pour ensuite le convertir en biodiesel.

# PARTIE 4: METHODOLOGIE REPRESENTATION SCHEMATIQUE

**Objective 1:** Réaliser des tests de floculation de la biomasse de levures oléagineuses à l'aide de bio-floculants (substances polymériques extracellulaires) en présence de coagulants inorganiques ($Al_2(SO_4)_3$ ou $CaCl_2$) **(<u>Chapitre II, Partie II</u>)**

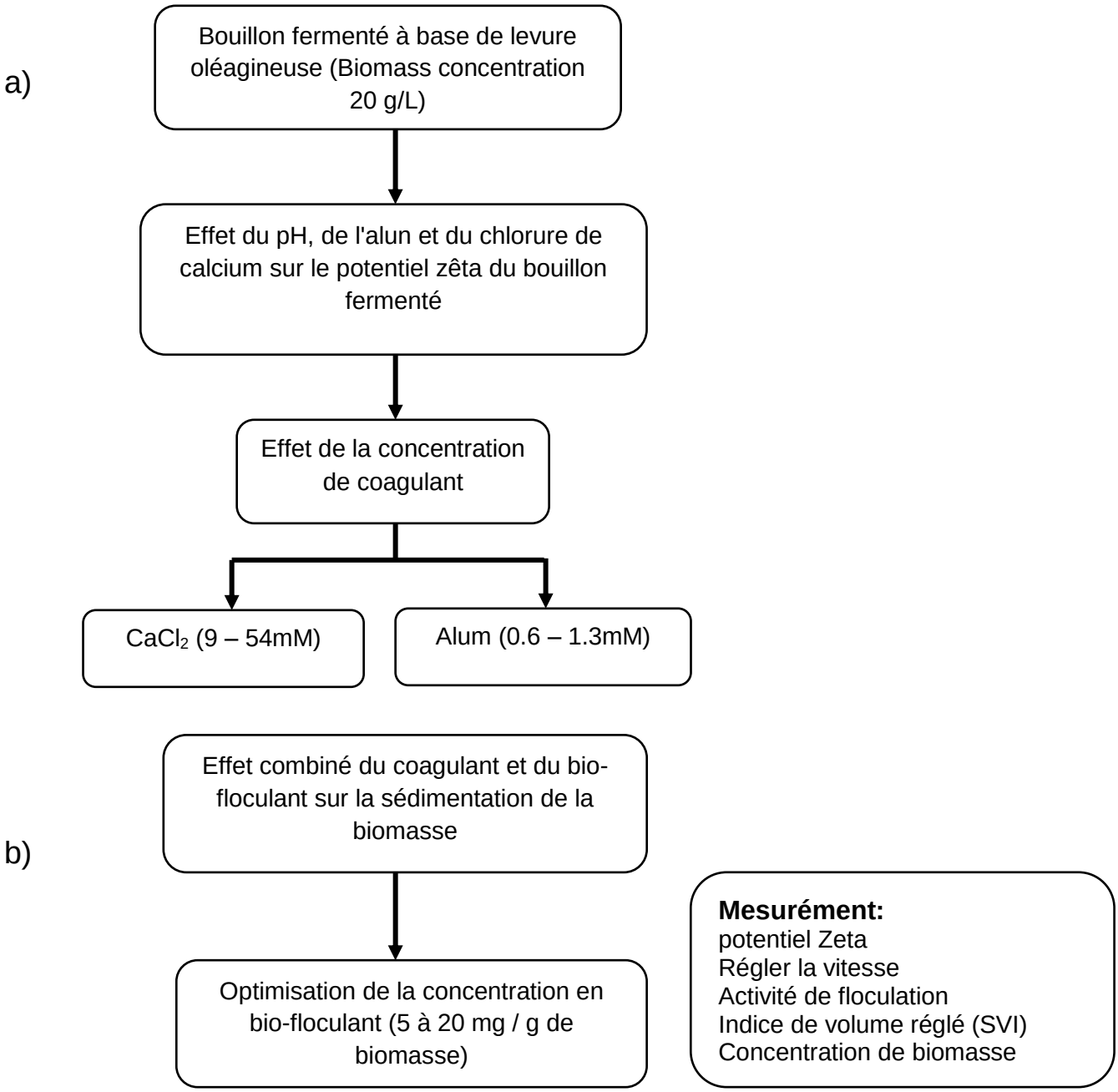


**Figure 1. 4 Optimisation de coagulant inorganique ($CaCl_2$ ou Alum) avec combinaison de biofloculant pour la récolte de la biomasse**

**Objective 2:** Effectuer l'extraction lipidique microbienne à partir de la biomasse de levure oléagineuse en utilisant le détergent (N-lauroylsarcosine) **(<u>Chapitre II, Partie III</u>)**

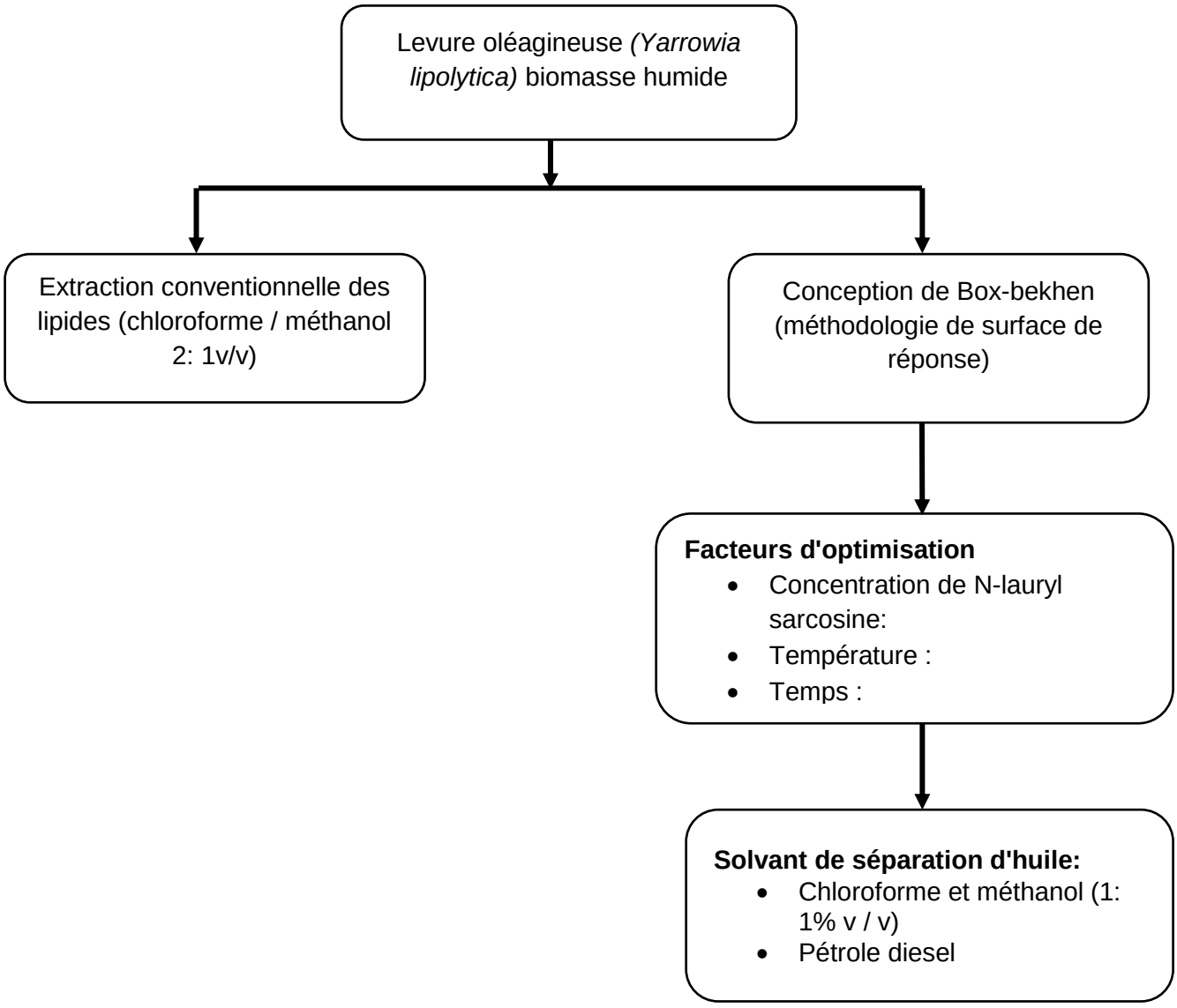


**Figure 1. 5 Optimisation de la concentration en détergent et des paramètres physiques pour la perturbation de la biomasse humide en utilisant la méthodologie de surface de réponse**

**Objective 3:** Appliquer les ultrasons assistés par le détergent (N-Lauroyl sarcosine) pour favoriser la transestérification in situ en vue de la production de biodiesel à partir de biomasse humide de levure oléagineuse. **(Chapitre II, Partie IV)**

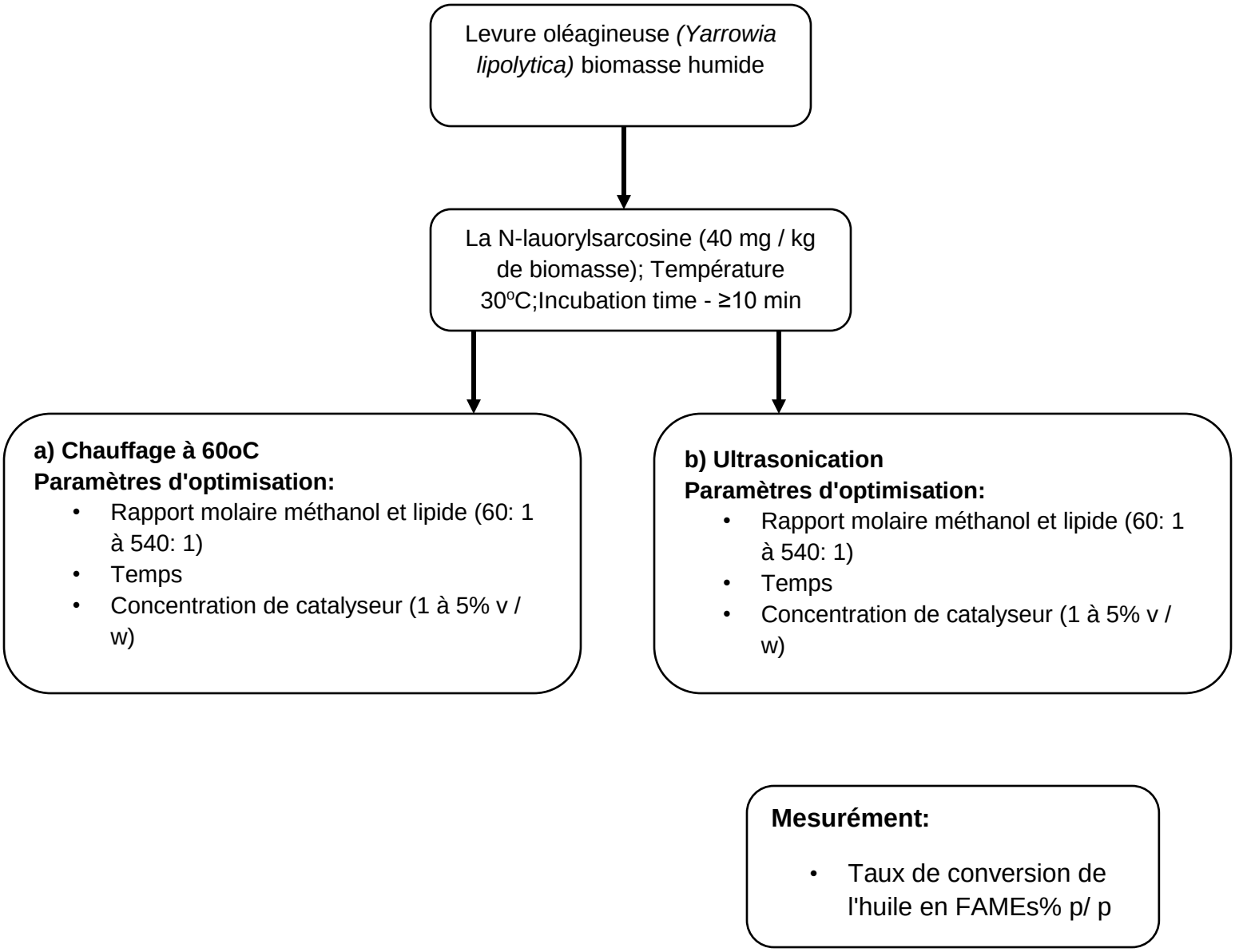


**Figure 1. 6 Méthodologie schématique de la perturbation de la biomasse humide assistée par la N-lauroyl sarcosine suivie d'une ultrasonication pour la transestérification in situ.**

**Objective 4:** Réaliser la transestérification ex-situ en utilisant l'acide nitreux libre et la N-lauroyl sarcosine **(Chapitre II, Partie V)**

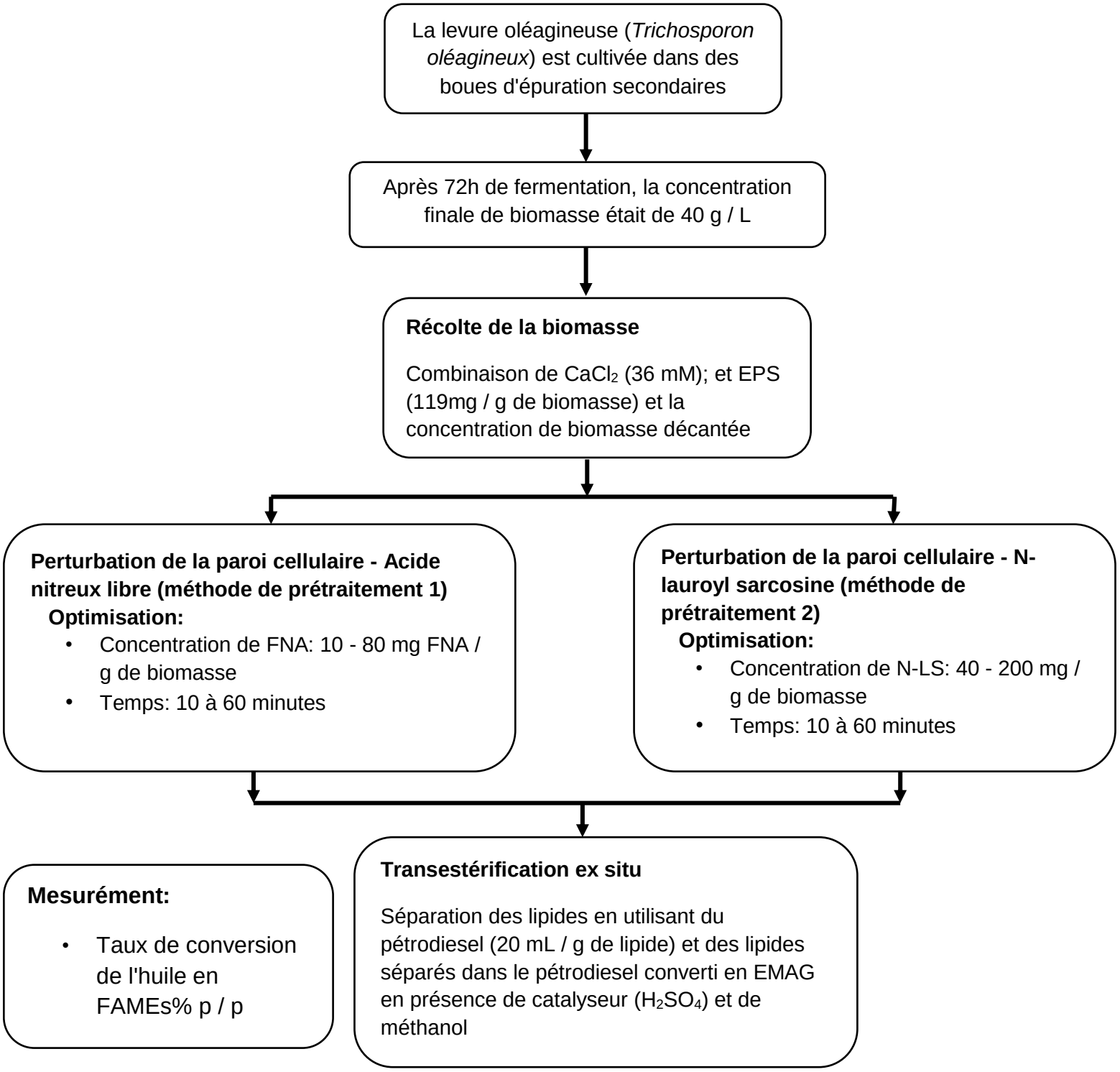


**Figure 1. 7 Méthodologie schématique de la perturbation de la biomasse humide assistée par l'acide nitreux et de la N-lauroylsarcosine et de la transestérification ex-situ pour le biodiesel**

**Objective 5:** Extraire de l'huile microbienne à partir de différentes matrices (ex. écumes) et ce, en présence de gasoil de pétrole, suivi de la conversion de ces huiles en biodiésel **(<u>Chapitre II, Partie VI</u>)**

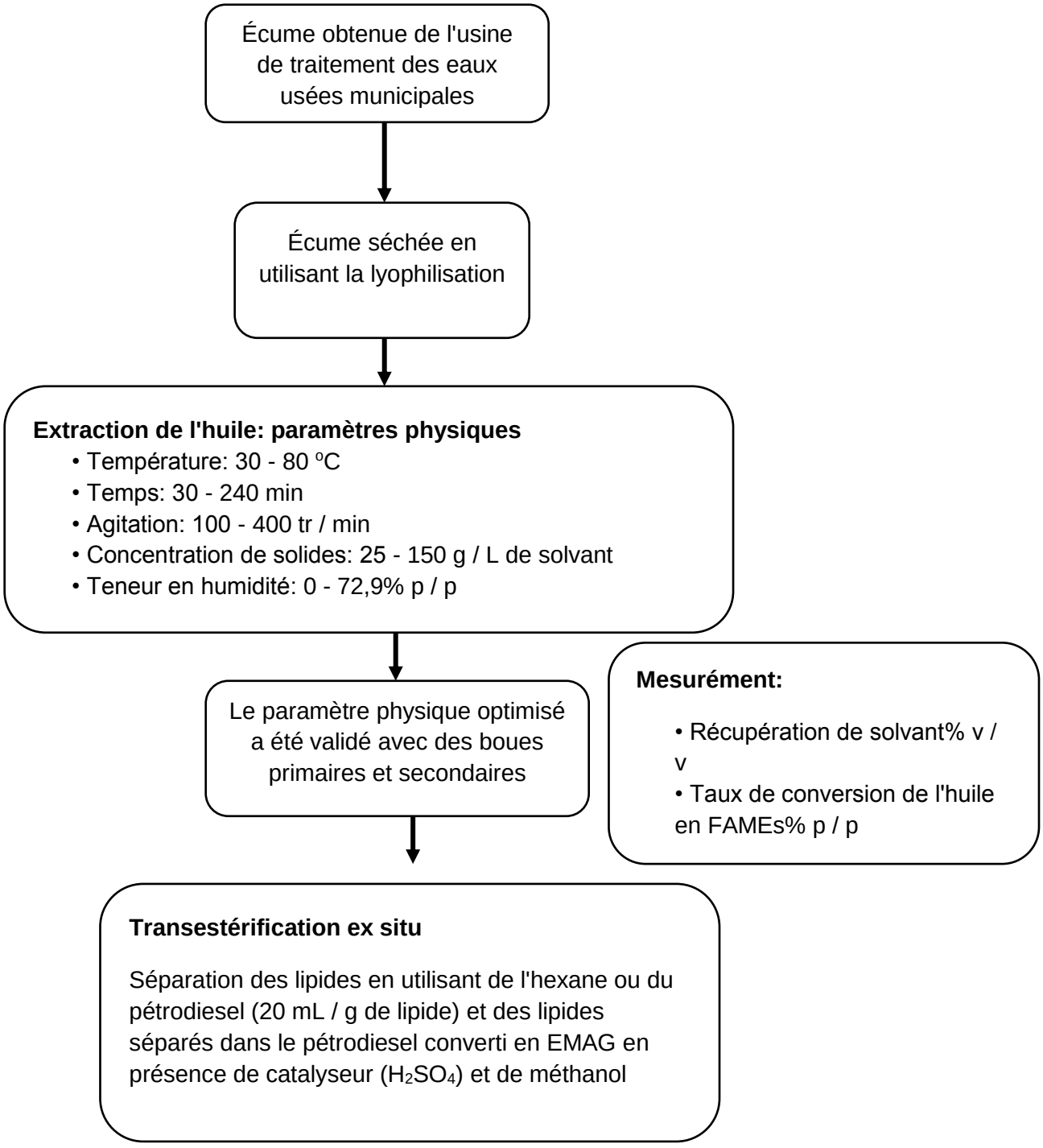


**Figure 1. 8 Méthodologie schématique de l'extraction de l'huile de l'écume, des boues primaires et secondaires et de la conversion en biodiesel**

# PARTIE 5: RÉSULTATS ET DISCUSSIONS

Comme mentionné précédemment, le principal problème dans la production de biodiesel était la disponibilité des matières premières. La levure oléagineuse dont les fortes capacités d'accumulation de lipides ont fait de la souche une matière première alternative pour cette étude. Cependant, l'obstacle majeur pour la production de biodiesel utilisant l'huile microbienne comme charge d'alimentation était un processus en aval en raison de multiples étapes telles que la récolte de la biomasse, la rupture de la paroi cellulaire et la transestérification. En outre, il est nécessaire de comprendre l'effet du taux de récupération du solvant à partir de biomasse humide et sec. Par conséquent, la recherche a été menée sur les problèmes ci-dessus et les résultats de cette étude ont été discutés en quatre parties: 1) l'extraction pétrolière d'écume, boues primaires et secondaires à l'aide du diesel de pétrole comme solvant et la conversion au biodiesel 2) La floculation de la biomasse de la levure oléagineuse utilisant les bio polymères produits dans les boues secondaires (les substances polymères extracellulaires 3) L'extraction de lipides microbiens à base de biomasse de la levure oléagineuse avec un détergent (sarcosine N-lauroyl) pour la disrution de la paroi cellulaire. 4) Le détergent (N-lauroyl sarcosine) d'ultrasonication assistée aidé dans la transestérification in situ pour la production de biodiesel à partir de la biomasse de la levure oléagineuse. 5) L'acide nitreux libre et sarcosine N-lauroyl assisté dans la transestérification ex situ des lipides tirés des boues municipales.

## 5.1 Réaliser des tests de floculation de la biomasse de levures oléagineuses à l'aide de bio-floculants (substances polymériques extracellulaires) en présence de coagulants inorganiques ($Al_2(SO_4)_3$ ou $CaCl_2$) (Chapitre II, Partie 2)

### 5.1.1 la sédimentation de la biomasse de levure oléagineuse avec différents coagulants : l'alun ou le chlorure de calcium

Le bouillon de fermentation de levure oléagineuse (produit en utilisant du glycérol brut comme substrat de carbone) avec une concentration de biomasse initiale de 20 g / L a été utilisé pour toutes les expériences de décantation de la biomasse. Les coagulants inorganiques, l'alun et le chlorure de calcium, ont été utilisés pour la coagulation du bouillon fermenté. Le potentiel zêta a augmenté de -42,3 à -22,5 en ascendant la concentration en chlorure de calcium de 9 à 54 mM à pH 9, jusqu'à ce qu'est devenu constant avec une concentration de 36 mm. Par

conséquent, la concentration optimale pour le chlorure de calcium a été déterminée à 36mm. En outre, en augmentation la concentration de l'alun de 0,6 à 1,3 mM à pH 5, le potentiel zêta a augmenté de -38,2 à -20,2 mV, et la concentration optimale a été définie à 1,2 mM. Ces résultats montrent clairement l'effet des ions d'alun et de calcium sur le potentiel zêta de lipides extraites de la biomasse traitée thermiquement.

### 5.1.2 Optimisation de la concentration du floculant biologique (les substances polymériques extracellulaires) utilisé pour la décantation de la biomasse de la levure oléagineuse

Les biopolymères (S-EPS) produits par *Cloacibacterium normanense* ont été utilisés en combinaison avec les coagulants (chlorure de calcium ou de l'alun) pour améliorer la décantation de la biomasse contenant des lipides. Les vitesses de décantation (1000 mL éprouvette) pour une combinaison de chlorure de calcium et S EPS se situaient entre 0,75 -2,93 (mm / s), alors que dans le cas de l'alun et S-EPS, la vitesse de sédimentation était comprise entre 0,56 à 1,46 (mm / s). La vitesse de sédimentation était maximale (2,93 mm / s) utilisant le chlorure de calcium et S-EPS (biomasse de 5,85 mg / g). Ainsi, une vitesse de décantation maximale a été observée avec du chlorure de calcium (36mm) et S-EPS (EPS 5.85mg / g de biomasse) et la concentration de la biomasse finale obtenue dans la suspension est réglée de 166,7 g / L pendant 5 min. Dans le cas de l'utilisation d'alun et S-EPS, une concentration de biomasse proche de 166. 7 g / L a été obtenue en 10 min avec une dose de S-EPS (biomasse de 18 mg / g). L'activité de floculation a été calculée pour différentes doses de S-EPS variant de 5,85 à 7,15 mg / g de biomasse EPS et 7,8 à 20,2 mg / g de biomasse EPS dans le cas du chlorure de calcium et de l'alun en tant que coagulant. L'activité de floculation maximale (79%) a été obtenue en utilisant 1,2 mM d'alun et de 18 mg de S-EPS / g de biomasse, alors que 74% d'activité de floculation a été observée en utilisant 36mm de chlorure de calcium et 5,85 mg de S-EPS / g de biomasse. On a remarqué qu'en cas de chlorure de calcium, plus de 3 fois la dose plus élevée de S-EPS a été nécessaire pour la floculation, presque similaire à celle obtenue en utilisant l'alun comme coagulant.

Le dosage de S-EPS 5,85, 39,9 et 119 mg EPS / g de biomasse a été respectivement utilisé pour 20, 40 et 60 g / L de concentration de la biomasse. Après décantation, la concentration finale en biomasse obtenue pendant 10 et 20 min, est de 177 et 210 g / L pour une concentration de biomasse initiale de 40 et 60 g / L, respectivement. Une sédimentation lente se produit à une concentration plus élevée de la biomasse de 60 g / L. Pour 20 g / L de concentration de la biomasse, la biomasse totale a été décanté à moins de 5 min, mais dans le cas de 40 et 60 g / L,

la biomasse est sédimentée en 10 et 20 min et la vitesse de décantation était de 1,25 et 0,64 mm/s, respectivement.

Après la récolte de la biomasse, la destruction de la paroi cellulaire est utilisée comme prochaine étape de traitement. Selon la littérature, la plupart des chercheurs ont étudié la biomasse sèche, cependant, le processus de lyophilisation pour sécher la biomasse peut augmenter le coût de production de biodiesel. Par conséquent, des études plus poussées ont été effectuées sur la rupture de la paroi cellulaire en utilisant le N-lauroyl sarcosine avec la biomasse oléagineuse humide. Le taux d'extraction lipidique a été ainsi calculé en comparaison à la méthode classique d 'extraction des lipides.

## 5.2 Effectuer l'extraction lipidique microbienne à partir de la biomasse de levure oléagineuse en utilisant le détergent (N-lauroylsarcosine) (Chapitre II, Partie 3)

Dans cette étude, les lipides sont extraits à partir de la biomasse humide (contenant 83,8% de teneur en humidité) afin d'obtenir une destruction complète des cellules et une meilleure efficacité d'extraction, utilisant la méthode de surface de réponse (BBD). Un test d'ajustement pour déterminer le manque de concordance a été considéré pour compare l'erreur résiduelle à l'erreur pure des points répliquées de design. La valeur F 6,28 n'est pas significative car la valeur P est inférieur à 0,05. Le manque d'ajustement non significatif a montré que le modèle était valable pour la présente étude. La concentration optimale de N-LS requis pour la destruction des cellules, la température et le temps peut être exprimée en utilisant l'équation suivante :

Efficacité d'extraction% = 99,62 + 2,41 × A + 1,41 × B + 1,90 × C + 0,12 × AB - 1,20 × AC - 1,00 × BC - 19,50 × A2 - 16,35 × B2 - 12,92 × C2 avec : A (concentration N-LS), B (température) et C (temps) sont des conditions de réaction

L'impact des variables de la réaction, y compris la concentration de N-LS, de la température et du temps sur l'efficacité d'extraction. L'efficacité de l'extraction des lipides a augmenté jusqu'à une concentration de N-LS 23,12 mg (correspondant à 46,2 mg de biomasse N-LS / g sec), de la température de 30 ° C et le temps > 8 min. Une prédiction statistique des paramètres de processus optimaux a été effectuée. La variable expérimentale prédite varie comme suit: la concentration N-LS 24,42 mg (ce qui est équivalent à 48 mg de biomasse N-LS / g sec), la température de 30,2 ° C et le temps de 8,8 min. la valeur prédite souhaitable ou

désirable est de 0,9. Pour vérifier les termes du modèle prédit de la valeur optimisée de l'extraction des lipides, une expérience indépendante en triple exemplaire a été réalisée et l'efficacité d'extraction a été jugée 95,43%, ce qui est à peu près égale à l'efficacité de l'extraction prévue 95,8% avec ± 0,4% erreur.

A grande échelle, la biomasse humide 124, 248 et 372 ± 2,5 g (correspondant à 20, 40 et 60 g de biomasse sèche) obtenue à partir de 1, 2 et 3 L du bouillon fermenté traitée pour 12,5 minutes avec 80 ml d'une solution contenant 0,976 g de N- LS (ce qui équivaut à 46,25, 23,15, 15,41 mg biomasse N-LS / g sec), a donné 96,9 ± 1,1, 73,8 ± 0,6 et 42,9 ± 0,3 %p/p efficacité de récupération des lipides. Cela suggère que la concentration de détergent doit augmenter proportionnellement à la concentration de la biomasse et la concentration optimale doit être utilisée [biomasse humide 124 g, solution de N-LS 80 mL avec 0.976g concentration de N-LS à 30 ± 0,1 ° C]. Dans le scénario actuel, l'efficacité d'extraction des lipides est de 1,6% en poids / poids inférieur à celui du procédé classique, ce qui est non significatif. Ainsi, l'efficacité de conversion des lipides à FAME est de 94,3% (p / p) de lipides totaux. Les lipides contiennent principalement C16, C18 et C23 des esters méthyliques d'acides gras, qui sont connus comme les principaux acides gras constituant le biodiesel (Giroud et al., 2013). La membrane cellulaire et les corps lipidiques sont recouverts de phospholipides. Le mélange de chloroforme et de methanol interagit avec les phospholipides (qui se compose d'une queue hydrophobe et une tête hydrophile) déchirant la membrane et libère ainsi des lipides neutres, contenant principalement des C16: 0, C18: 0, C 18: 1, C 18: 2 des acides gras. Dans notre cas, le taux de saturation est d'environ 42,5% p / p des lipides totaux, ce qui indique que le biodiesel aura une grande stabilité d'oxydation que le biodiesel de jatropha (qui est inférieure à 30% en poids de lipides saturés / poids des lipides totaux). En conclusion, il n'y a aucun changement significatif dans le profil d'acide gras à l'aide de N-lauroyl sarcosine assistée d'extraction de lipides à partir *de Yarrowia lipolytica* SKY-7 et les acides gras sont appropriés pour la production de biodiesel.

Selon les résultats observés, N-LS aidé à la rupture de la paroi cellulaire réussit à briser la membrane cellulaire et phospholipidique qui entourent les corps lipidiques, Par conséquent, une étude plus approfondie a été utilisée pour la transestérification sans séparation des lipides microbiens. En plus, afin d'améliorer l'efficacité de la transestérification, la combinaison des ultrasons avec N-LS a également été étudiée.

## 5.3 Appliquer les ultrasons assistés par le détergent (N-Lauroyl sarcosine) pour favoriser la transestérification in situ en vue de la production de biodiesel à partir de biomasse humide de levure oléagineuse (Chapitre II, Partie 4)

Le rendement des FAMEs obtenu à partir d'un procédé de transestérification en deux étapes (extraction de lipides suivie d'une transestérification) est de 94,6 ± 1,5% (poids/poids). La biomasse (*Yarrowia lipolytica* cultivé dans une solution de glycérol brut) humide utilisée dans ces expériences consistait en 83,8% d'humidité.

Un traitement par N-LS suivi d'un traitement par ultrasons conduit à un rendement élevé en FAMEs de 94,3 ± 1,9% poids/poids avec un rapport molaire méthanol/lipide de 360:1 et ceci en utilisant une concentration catalyse de 360 mM dans un temps de réaction de 5 minutes. Cependant, dans des conditions similaires, le rendement des FAMEs de 82,9 ± 1,8% poids/poids a été obtenu dans un temps de réaction de 25 min en utilisant la biomasse traitée aux ultrasons (sans N-LS, le détergent). Selon la réaction d'équilibre, le besoin en méthanol est de 0,08 ml de méthanol/g de lipide, mais dans cette étude, une concentration de 6,4 ml/g de méthanol a donné le rendement maximal en FAMEs. Le coût du procédé peut être réduit si le méthanol résiduel avec la solution de glycérol sont réutilisés pour la production de lipides.

Dans la présente étude, le rendement maximal de FAMEs de 94,3% poids/poids a été obtenu en utilisant la biomasse humide (83,8% d'humidité) en seulement 5 min de temps de réaction, ce qui représente le rendement le plus élevé. En comparaison avec le procédé de transestérification en deux étapes, le besoin en méthanol est plus élevé (rapport méthanol: lipide 360: 1), mais en contrepartie le temps de réaction diminue de 16 h à 5 min en utilisant la transestérification en présence des ultrasons assistes par N-LS. Cela indique que le tensioactif (N-LS) a efficacement perturbé la paroi cellulaire et libéré les lipides, augmentant ainsi l'accessibilité du méthanol aux lipides corporels internes et exposant ainsi davantage le lipide à un contact direct avec les réactifs en peu de temps. Le temps de réaction réduit peut compenser le coût élevé du volume de réactifs nécessaire à la production industrielle du biodiesel. Les résultats obtenus dans cette étude sont statistiquement significatifs pour $p < 0,05$.

De plus, le coût du prétraitement de la perturbation des parois cellulaires (humides) à l'aide de N-Lauroyl sarcosine devrait être faible (0,48 $ N-LS/kg de biomasse sèche)

comparativement au procédé conventionnel utilisé pour libérer les lipides intracellulaires sans séchage ainsi que l'application des solvants organiques (chloroforme, méthanol et isopropanol). Dans le cas du procédé conventionnel (centrifugation, séchage), une grande quantité de solvants organiques est utilisée pour l'extraction des lipides et la transestérification, ce qui ajoute jusqu'à >4 $/kg de biomasse sèche à l'ensemble du processus. Ces solvants organiques peuvent être récupérés en utilisant la distillation, mais il s'agit d'un procédé à forte consommation d'énergie.

Les propriétés du biodiesel pur et du biodiesel mélangé (B100, B20, B10 et B5) étaient presque similaires à celles du biodiesel standard de l'ASTM. De plus, le biodiesel obtenu à partir de *Yarrowia lipolytica* (transestérification in situ prétraitée par N-LS et assisté par des ultrasons) a des propriétés de carburant comparables à celles du biodiesel obtenu à partir des microalgues et des champignons. Par conséquent, le biodiesel obtenu par prétraitement N-LS suivi d'une transestérification in situ assistée par des ultrasons et ayant des propriétés similaires au carburant standard conviendra à la production commerciale et à l'application comme carburant de transport ainsi qu'à la production d'électricité.

Pour réduire la concentration du N-lauroyl sarcosine nécessaire à la perturbation des parois cellulaires, une étude plus approfondie a été menée en utilisant l'acide nitreux libre (FNA) pour la perturbation de celles-ci. Les corps lipidiques dans la cellule microbienne ont été traités avec une concentration minimale de N-LS ce qui peut baisser le coût des produits chimiques et par conséquent le coût total de production du biodiesel.

Ensuite, un traitement développé en aval a été utilisé pour la biomasse de levure oléagineuse produite en utilisant des boues d'eaux usées pour connaître l'effet de la concentration des réactifs sur la rupture de la paroi cellulaire et le profil des acides gras. Par conséquent, la concentration de N-LS a été optimisée davantage pour cette étude et pour la diminuer, un prétraitement léger sans acide nitreux a été effectué pour décomposer la paroi cellulaire.

## 5.4 Réaliser la transestérification ex-situ en utilisant l'acide nitreux libre et la N-lauroyl sarcosine. (Chapitre II, Partie 4)

La biomasse de levure oléagineuse (*Trichosporon oléagineux* cultivé dans des boues d'épuration secondaires enrichies avec une solution de glycérol brut) a été utilisée dans cette étude pour la

perturbation de la paroi cellulaire humide en utilisant la N-Lauroyl sarcosine ou l'acide nitreux libre avec du chloroforme et du méthanol conventionnels (2:1 v/v) ou du pétro diésel. On a observé qu'avec l'augmentation de la concentration en N-LS, l'efficacité d'extraction des lipides augmentait et que l'efficacité maximale d'extraction des lipides de 98,11 ± 1,6 et 95,8 ± 1,1% poids/poids était atteinte à 75 mg N-LS/g de biomasse sèche dans le cas de l'utilisation du chloroforme/méthanol (1: 1) et le diesel de pétrole respectivement comme solvants. Cependant, dans cette étude, le traitement avec N-LS a provoqué une perturbation de la paroi cellulaire sans rupture des solides de boue et les lipides extraits dans la solution N-LS ont été séparés en interphase solvant en usant du chloroforme et du méthanol ou du gasoil diesel. De plus, moins de temps de contact (<5 min) entre la solution contenant des lipides après rupture de la paroi cellulaire et le solvant (chloroforme et méthanol ou pétro diésel) utilisé pour séparer les lipides conduit à la non-dissolution des solides de boue avec les lipides. Par conséquent, l'extraction des lipides assistée par la N-lauroyl sarcosine peut être utilisée avec succès pour l'extraction des lipides de la biomasse des boues par rapport aux autres méthodes d'extraction.

La rupture de paroi cellulaire assistée par l'acide nitreux (FNA) est une autre méthode d'extraction des lipides avec une augmentation de concentration de FNA de 10 à 80 mg FNA/g de biomasse, une augmentation de l'efficacité d'extraction des lipides et une efficacité maximale de 94,3 ± 1,6. On a obtenu 90,7 ± 1,1% en poids/poids à 40 mg de FNA/g de biomasse en utilisant respectivement du chloroforme et du méthanol (1: 1) et du pétro diésel comme solvant. Les recherches sur les espèces réactives de l'azote ont montré que le FNA et ses dérivés tels que l'oxyde nitrique (NO) et l'anhydride nitreux ($N_2O_3$) sont responsables de la dégradation des protéines et des polysaccharides. Perturber la paroi cellulaire et augmenter la concentration de FNA, peut également avoir un impact négatif sur le profil des acides gras. Par conséquent, une faible concentration de FNA doit être utilisée pour éviter la réaction inutile de FNA avec des lipides microbiens. Dans cette perspective, d'autres expériences ont été réalisées en utilisant une faible concentration de FNA suivie d'un traitement par N-LS.

L'efficacité d'extraction des lipides de la biomasse des boues en utilisant une faible concentration d'acide nitreux libre (10 mg de FNA/g de biomasse) était de 58,2 et 54,7% en poids/poids avec l'hexane ou le pétro diésel. Alors que l'extraction lipidique séquentielle avec de l'acide nitreux libre (10 mg FNA / g de biomasse) et une concentration de N-lauroyl sarcosine variant de 5 à 40 mg / g de biomasse augmente l'efficacité d'extraction des lipides de 58,2 à 95,1 et de 54,7 à 92,3% en utilisant l'hexane ou le diesel pétrolier. L'efficacité maximale d'extraction des lipides a été obtenue à une concentration de N-LS de 20 mg / g de biomasse. Les résultats expérimentaux et les

observations microscopiques ont révélé que la raison de l'efficacité accrue de l'extraction des lipides par traitement séquentiel utilisant FNA suivi d'un traitement N-LS était la dégradation de la membrane cellulaire et de la paroi cellulaire par un traitement FNA sans libération de lipides associée. Une fois que l'acide nitreux libère la paroi cellulaire autour de la biomasse de levure, N-LS forme progressivement une micelle autour des corps lipidiques à l'intérieur de la cellule microbienne et détruit la membrane phospholipide entourant les corps lipidiques et les lipides neutres ont été libérés et restaurés en utilisant des solvants.

Ces résultats sont en outre validés en utilisant un traitement séquentiel avec une faible concentration de FNA (10 mg de FNA / g de biomasse) et une extraction de lipides assistée par N-LS et une transestérification supplémentaire de l'huile en FAME sera acheminée. En utilisant une faible concentration de FNA et avec une augmentation de la concentration de N-LS, il y avait une augmentation de C16: 1, C18: 1, C18: 2 et C18: 3 et une efficacité maximale d'extraction des lipides a également été atteinte. Il a été suggéré que pour augmenter la stabilité à l'oxydation des EMAG (biodiesel), le pourcentage élevé d'acide gras insaturé est nécessaire pour éviter les problèmes d'allumage du moteur et de stockage.

## 5.5 Extraire de l'huile microbienne à partir de différentes matrices (ex. écumes) et ce, en présence de gasoil de pétrole, suivi de la conversion de ces huiles en biodiésel. Chapitre II, Partie 5)

La teneur en huile microbienne se varie selon le type de boues (les boues primaires, les boues secondaires et l'écume) en raison du processus de traitement des eaux usées. Les matières solides des boues primaires sont principalement constituées de lipides macromoléculaires, des fibres et des roches. Cependant, la boue secondaire est composée de solides en suspension avec des cellules microbiennes produites pendant le traitement biologique aérobie de traitement des eaux usées. L'écume est un mélange combiné de matières grasses, l'huile et la graisse (FOG) ; des fibres de cellulose, des poils et d'autres matières solides légères.

L'efficacité de l'extraction de lipides obtenue en utilisant de l'hexane comme solvant a été considérée comme 100%. L'efficacité de l'extraction de pétrole en utilisant du diesel comme solvant pour les boues primaires, les boues secondaires et l'écume ont été de 97, 98,1 et 97,8% p / p., respectivement. La nature physique de lipides sur les matrices de boues est similaire dans le cas des boues primaires, secondaires, et de l'écume. Par conséquent, la poudre séchée de l'écume a été utilisée pour optimiser les paramètres physiques (température, temps, l'agitation et

le volume de solvant) pour la récupération du biodiesel et des paramètres optimisés ont été en outre utilisés pour l'extraction de lipides à partir de boues primaires et secondaires.

Il y a eu un effet significatif de paramètres physiques tels que l'agitation, la température, le temps, la concentration en matières solides sur l'efficacité de l'extraction du biodiesel. Le solvant (hexane ou le diesel) peut dissoudre l'huile microbienne à partir de matières solides de boue ou de l'écume. La réutilisation du même solvant (filtrat) pour dissoudre l'huile d'écume ou des boues est connu à plusieurs reprises comme recyclage du solvant. Cependant, les résidus humides et secs ont considérablement affecté le taux de récupération des solvants% v / v et de l'efficacité d'extraction d'huile% en poids / poids à chaque fois de recyclage. La récupération du solvant et l'efficacité d'extraction de lipides est diminué de 12 à 14% v / v et de 13 à 19% v / v, respectivement avec un nombre croissant de recyclage de 0, 1 et 2, en utilisant les écumes sèches, l'hexane ou le diesel comme solvant. Toutefois, en cas d'écume humide, le taux de récupération du solvant et l'efficacité de l'extraction de lipides est diminué de 3 à 12% en poids / poids et de 4-9% en poids / poids, respectivement, avec un nombre croissant de recyclage de 0, 1 et 2 en utilisant l'hexane ou le diesel comme solvant. Ces résultats représentent que le solvant n'a pas été absorbé par les solides humides d'écume et que la nature non polaire du solvant empêche l'absorption avec l'humidité ou l'eau, qui est polaire. Par conséquent, l'utilisation d'écume humide pour la récupération d'huile microbienne peut réduire la contamination par les solvants dans les écumes.

Les paramètres optimisés pour les matières solides d'écume ont été utilisés pour séparer les lipides des matières solides lyophilisées de boues primaires et secondaires. L'utilisation de l'hexane et de diesel sous des paramètres physiques optimisées (60 ° C de température, temps de réaction de 60 min, agitation 300 rpm et 75 g de matières solides / solvant L) a donné une efficacité d'extraction de lipides de 97,3 et 96,1% en poids / poids (avec la boue primaire), respectivement. Dans le cas des boues secondaires, le rendement d'extraction de l'huile microbienne obtenue est de 95,2 et 96,3% p / p à l'aide d'hexane ou du diesel, respectivement. Le taux d'extraction d'huile maximal obtenu en utilisant la boue primaire et secondaire était presque identique à celui obtenu en utilisant l'écume, dans des conditions optimisées. Cela signifie que les paramètres optimisés peuvent être utilisés pour l'extraction de lipides en utilisant un autre type de matières solides des boues.

Les paramètres optimisés ont été validés à l'aide d'un kilogramme d'écume avec une teneur en eau de 73,8%. Dans cette étude, les résultats ont été presque similaire à celui obtenus

en utilisant 1 g d'écume à l'aide du diesel ou de l'hexane comme solvant avec une efficacité d'extraction d'huile de 94,1 et 92,3% en poids / poids, respectivement. L'huile récupérée à partir de boues primaires et secondaires et d'écume a été directement transestérifié sans séchage supplémentaire (avec solvant). Le rendement de conversion d'huile d'écume en FAME utilisant l'hexane et le diesel en tant que co-solvant était de 93,3 ± 1,9 et 95,2 ± 1,1% p / p, respectivement. L'efficacité de conversion plus ou moins similaire a été obtenue en utilisant des boues primaires et des boues secondaires. L'huile microbienne extraite de la boue primaire et secondaire a été essentiellement convertie en C14 et C16, tandis que l'huile d'écume a été convertie en C18: 1 et C18: 2. Il explique que les lipides neutres sont élevés dans les solides d'écume par rapport aux boues primaires ou secondaires. Les FAME obtenus à partir de lipides neutres sont très combustibles pour l'allumage du moteur.

Les lipides extraits de l'écume ont été établis comme matières premières alternatives pour la production de biodiesel, Mais a cause de la faible teneur en huile (30% p / p) présent dans l'écume, la levure oléagineuse dont sa croissance rapide et sa teneur enlevée en lipides 50% p / p, pourrait être considérée comme une attrayante matière première pour la production de biodiesel. Cependant, pour des raisons concernant la forte consommation d'énergie, la centrifugation comme une méthode utilisée pour la récolte de la biomasse, est une étape préliminaire dans un processus en aval de la production de biodiesel à partir de la biomasse oléagineuse. Par conséquent, un autre procédé pour la séparation de la biomasse du bouillon fermenté a été étudié.

# PARTIE 6: CONCLUSIONS ET RECOMMANDATIONS

## 6.1 Conclusions

1. Cette étude décrit la décantation de la biomasse de levure à l'aide d'alun et de chlorure de calcium comme coagulants avec ou sans dosage EPS. La présence d'EPS a augmenté le taux de sédimentation de la biomasse et la biomasse s'est stabilisée en 5 min avec un SVI faible (0,72 mL / g de biomasse). La biomasse décantée a été davantage perturbée par la N-LS pour libérer le lipide microbien intracellulaire et le lipide libéré a été séparé des débris cellulaires en utilisant du pétrodiesel. L'efficacité maximale de récupération des lipides de 94,7 ± 1,2% poids / poids a été enregistrée. Après la transestérification, le rendement maximal en biodiesel de 97,4 ± 1,1% a été obtenu. Par conséquent, ce procédé est une meilleure approche pour la production de biodiesel nécessitant moins d'énergie, de temps et de coût, car le séchage des cellules porteuses de lipides n'est pas nécessaire et les solvants toxiques pour la récupération des lipides sont éliminés. Ce procédé évite également le besoin d'un procédé de centrifugation coûteux et énergivore.

2. La méthode de désintégration cellulaire efficace et peu coûteuse pour la libération de lipides du processus de levure oléagineuse *Y.lipolytica* a été testée avec succès. La rupture de la cellule N-lauroyl sarcosine (N-LS) et la libération des lipides des cellules, suivie d'une séparation des lipides avec un volume plus faible de chloroforme et de méthanol (1: 1 v / v), ont révélé une efficacité d'extraction des lipides de 95,43% (w / w). Le temps requis pour obtenir un rendement d'extraction élevé a été réduit de 12 h dans un procédé classique, à savoir une rupture de cellule et une récupération de lipide assistée par chloroforme et methanol, à 8,8 minutes dans un procédé d'extraction assistée par N-LS. La température d'incubation (30,20 C) et la concentration de N-LS est de 48 mg N-LS / g de biomasse sèche. Ils étaient les paramètres les plus importants qui affectaient la rupture des cellules et la libération des lipides.

3. Les résultats obtenus dans cette étude ont révélé qu'il était possible de réduire le temps de réaction en utilisant le traitement par N-LS de la biomasse humide suivie d'une transestérification in situ assistée par ultrasons. Le rendement maximal de FAMEs a été obtenu dans les 5 minutes de temps de réaction en utilisant la transestérification assistée par ultrasonication assistée par N-LS, ce qui représente une beaucoup moins de temps par rapport à 12 h de réaction utilisé dans le procédé de transestérification en deux étapes. La composition des EMAGs obtenus par transestérification in situ avec (ou) sans traitement par N-LS était similaire à celle obtenue lors de

la transestérification en deux étapes. La conversion de la biomasse humide portant des lipides (83,8% d'humidité) en FAMEs en utilisant N-LS suivie d'une transestérification in situ assistée par ultrasonication pourrait être une approche prometteuse car elle élimine l'étape utilisation les solvants toxiques pour l'extraction lipidique et évite la lyophilisation ou le séchage de la biomasse humide, ce qui contribue à réduire la consommation d'énergie pour une production industrielle de biodiesel moins couteuse.

4. L'extraction des lipides assistée par N-LS en utilisant du chloroforme et du méthanol (1: 1) ou du gasoil de pétrole a procuré une efficacité d'extraction des lipides presque similaire mais l'extraction des lipides assistée par FNA a permis d'obtenir un maximum de lipides. Cependant, une concentration élevée de FNA a directement affecté le profil des acides gras. Par conséquent, l'extraction séquentielle des lipides en utilisant une faible concentration de FNA suivie du N-LS a été employée. Elle a donné l'efficacité maximale d'extraction de lipide de 95% (w / w) sans affecter le profil d'acide gras. Par conséquent, cette approche peut être bénéfique pour l'extraction des lipides et la production de biodiesel supplémentaire en utilisant la biomasse des boues.

5. La boue primaire, la boue secondaire et l'écume ont été utilisées pour l'extraction de l'huile. Cette dernière a ensuite été transestérifiée en biodiesel. Les paramètres physiques ont significativement affecté l'efficacité de l'extraction de l'huile des différentes boues solides et de l'écume en utilisant de l'hexane et du pétrodiesel comme solvant. Les paramètres optimisés pour une efficacité maximale d'extraction de l'huile étaient d'une température de 60 C, d'une agitation de 300 tr / min, d'une durée de 60 min et d'une concentration en solides de 50 g / l. L'efficacité de l'extraction d'huile obtenue en utilisant de l'hexane et du gasoil de pétrole était presque similaire dans tous les cas. La teneur en humidité dans les solides a affecté le pourcentage de récupération du solvant, mais il n'y a aucun impact sur l'efficacité de l'extraction du pétrole. Dans la transestérification ex-situ sans séchage de l'huile, il a été obtenu > 90% w / w en conversion en FAMEs. La présence de métaux a nui à la qualité du biodiesel, mais d'autres recherches sur la purification du biodiesel pour éliminer les contaminants pourraient être utiles pour établir l'huile d'écume comme matière première importante pour la production de biodiesel.

## 6.2 Recommandations

1) La biomasse de levure oléagineuse (*Yarrowia lipolytica* et *Trichosporon*) est constituée de 50% en poids de lipides. Cela signifie qu' 1 g de poids sec des cellules se compose de 0,5 g de lipides et de ± 0,5 g de biomasse dégraissée. La biomasse dégraissée est constituée de polysaccharides (chitine, glucane) et de protéines. Ces sous-produits ont une valeur économique élevée. Par

conséquent, une recherche rigoureuse doit être menée pour séparer ces produits de la biomasse dégraissée

2) La production du biodiesel à partir de la biomasse lipidique produite à l'aide des boues municipales a une certaine valeur économique. Mais le principal problème est que la boue municipale se compose d'une forte concentration de métaux et après la culture des lipides microbiens et la conversion de l'huile microbienne en biodiesel, les métaux contaminés dans le biodiesel deviendront un problème pour maintenir la norme ASTM. Par conséquent, d'autres recherches sont nécessaires pour éliminer ou purifier le biodiesel en utilisant la méthode de lavage à sec.

3) Après la séparation des lipides en utilisant du pétrodiesel, il y avait environ 1 à 3% v / v de pétrodiesel résiduel dans la biomasse dégraissée (ou) les solides de boues dans l'eau. Par conséquent, d'autres recherches sont nécessaires pour éliminer le gasoil de pétrole résiduel dans les eaux usées par centrifugation à deux phases.

4) Pour étudier d'autres solvants OS green tels que le carbonate de diméthyle ou le carbonate de polypropylène doivent être étudiés pour connaître la faisabilité du solvant pour la récupération des lipides.

5) Le bilan massique complet et le bilan énergétique doivent être réalisés pour l'extraction de l'huile d'écume et la conversion au biodiesel et la décantation de la biomasse pétrolifère et la perturbation de la paroi cellulaire en utilisant N-LS ou FNA et la transestérification pour le biodiesel.

# BIBLOGRAPHIE


Adam F, Abert-Vian M, Peltier G & Chemat F (2012) “Solvent-free” ultrasound-assisted extraction of lipids from fresh microalgae cells: A green, clean and scalable process. *Bioresour. Technol.* 114(0):457-465.

Adebayo G & Ameen O (2017) Physico-chemical properties of biodiesel produced from Jatropha curcas oil and fossil diesel. *Journal of Microbiology and Biotechnology Research* 1(1):12-16.

Alameldin H, Izadi-Darbandi A, Smith SA, Balan V, Jones AD & Sticklen M (2017) Production of seed-like storage lipids and increase in oil bodies in corn (Maize; Zea mays L.) vegetative biomass. *Industrial Crops and Products* 108:526-534.

Ali M & Watson IA (2015) Microwave treatment of wet algal paste for enhanced solvent extraction of lipids for biodiesel production. *Renewable Energy* 76:470-477.

Alvarez H & Steinbüchel A (2002) Triacylglycerols in prokaryotic microorganisms. *Applied microbiology and biotechnology* 60(4):367-376.

Anitescu G & Bruno TJ (2012) Liquid Biofuels: Fluid Properties to Optimize Feedstock Selection, Processing, Refining/Blending, Storage/Transportation, and Combustion. *Energy & Fuels* 26(1):324-348.

Arabolaza A, Rodriguez E, Altabe S, Alvarez H & Gramajo H (2008) Multiple pathways for triacylglycerol biosynthesis in Streptomyces coelicolor. *Applied and environmental microbiology* 74(9):2573-2582.

Brennan L & Owende P (2010) Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and sustainable energy reviews* 14(2):557-577.

Calderón V & Cerbón J (1992) Interfacial pH modulation of membrane protein function in vivo. Effect of anionic phospholipids. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1106(2):251-256.

Caridi A (2006) Enological functions of parietal yeast mannoproteins. *Antonie Van Leeuwenhoek* 89(3-4):417-422.

Chakraborty R & Sahu H (2014) Intensification of biodiesel production from waste goat tallow using infrared radiation: Process evaluation through response surface methodology and artificial neural network. *Applied energy* 114:827-836.

Chatzifragkou A, Fakas S, Galiotou-Panayotou M, Komaitis M, Aggelis G & Papanikolaou S (2010) Commercial sugars as substrates for lipid accumulation in Cunninghamella echinulata and Mortierella isabellina fungi. *European Journal of Lipid Science and Technology* 112(9):1048-1057.

Cheirsilp B, Suwannarat W & Niyomdecha R (2011) Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for lipid production from industrial wastes and its use as biodiesel feedstock. *New Biotechnol.* 28(4):362-368.

Chen C-Y, Yeh K-L, Aisyah R, Lee D-J & Chang J-S (2011) Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource technology* 102(1):71-81.

D'Hondt E, Martin-Juarez J, Bolado S, Kasperoviciene J, Koreiviene J, Sulcius S, Elst K & Bastiaens L (2018) Cell disruption technologies. *Microalgae-Based Biofuels and Bioproducts,* Elsevier. p 133-154.

da Cunha ME, Krause LC, Moraes MSA, Faccini CS, Jacques RA, Almeida SR, Rodrigues MRA & Caramão EB (2009) Beef tallow biodiesel produced in a pilot scale. *Fuel Processing Technology* 90(4):570-575.

Dey P, Mall N, Chattopadhyay A, Chakraborty M & Maiti MK (2014) Enhancement of lipid productivity in oleaginous Colletotrichum fungus through genetic transformation using the yeast CtDGAT2b gene under model-optimized growth condition. *PloS one* 9(11):e111253.

Dhanani T, Shah S, Gajbhiye NA & Kumar S (2013) Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera. *Arabian Journal of Chemistry* http://dx.doi.org/10.1016/j.arabjc.2013.02.015(0).

Dong T, Knoshaug EP, Pienkos PT & Laurens LM (2016) Lipid recovery from wet oleaginous microbial biomass for biofuel production: a critical review. *Applied Energy* 177:879-895.

Duarte SH, Ghiselli G & Maugeri F (2013) Influence of culture conditions on lipid production by Candida sp. LEB-M3 using glycerol from biodiesel synthesis. *Biocatalysis and Agricultural Biotechnology* 2(4):339-343.

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. *J. Amer. Oil Chem. Soc.* 84(2):181−187.

Fadhil AB & Ali LH (2013) Alkaline-catalyzed transesterification of Silurus triostegus Heckel fish oil: Optimization of transesterification parameters. *Renewable energy* 60:481-488.

Ferraz TPL, Fiúza MC, dos Santos MLA, Pontes de Carvalho L & Soares NM (2004) Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation. *J Biochem Bioph Methods* 58(3):187-193.

Folch J, Lees M & G.H. SS (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226(1):497-509.

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. *Bioresource Technology* 111(0):398-403.

Gardeli C, Athenaki M, Xenopoulos E, Mallouchos A, Koutinas AA, Aggelis G & Papanikolaou S (2017) Lipid production and characterization by Mortierella (Umbelopsis) isabellina cultivated on lignocellulosic sugars. *Journal of applied microbiology* 123(6):1461-1477.

Giroud S, Frare C, Strijkstra A, Boerema A, Arnold W & Ruf T (2013) Membrane phospholipid fatty acid composition regulates cardiac SERCA activity in a hibernator, the Syrian hamster (Mesocricetus auratus). *PloS one* 8(5):e63111.

Gouda MK, Omar SH & Aouad LM (2008) Single cell oil production by Gordonia sp. DG using agro-industrial wastes. *World Journal of Microbiology and Biotechnology* 24(9):1703.

Guldhe A, Singh B, Rawat I, Ramluckan K & Bux F (2014) Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production. *Fuel* 128:46-52.

Harun R, Singh M, Forde GM & Danquah MK (2010) Bioprocess engineering of microalgae to produce a variety of consumer products. *Renewable and Sustainable Energy Reviews* 14(3):1037-1047.

Hincapié G, Mondragón F & López D (2011) Conventional and in situ transesterification of castor seed oil for biodiesel production. *Fuel* 90(4):1618-1623.

Hsu C-J & Hsiau S-S (2015) A study of filtration performance in a cross-flow moving granular bed filter: The influence of gas flow uniformity. *Powder Technology* 274(0):20-27.

Islam MA, Heimann K & Brown RJ (2017) Microalgae biodiesel: Current status and future needs for engine performance and emissions. *Renewable and Sustainable Energy Reviews* 79:1160-1170.

Japar AS, Azis NM, Takriff MS & Yasin NHM (2017) Application of Different Techniques to Harvest Microalgae.

Jin G, Yang F, Hu C, Shen H & Zhao ZK (2012) Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast Rhodosporidium toruloides. *Bioresource Technology* 111(0):378-382.

Karmakar A & Mukherjee S (2017) Process optimization of biodiesel production from Neem oil. *Indian Journal of Agricultural Research* 51(6).

Kaya C, Hamamci C, Baysal A, Akba O, Erdogan S & Saydut A (2009) Methyl ester of peanut (Arachis hypogea L.) seed oil as a potential feedstock for biodiesel production. *Renewable Energy* 34(5):1257-1260.

Kitcha S & Cheirsilp B (2013) Enhancing lipid production from crude glycerol by newly isolated oleaginous yeasts: strain selection, process optimization, and fed-batch strategy. *BioEnergy Research* 6(1):300-310.

Klimek-Ochab M, Brzezińska-Rodak M, Żymańczyk-Duda E, Lejczak B & Kafarski P (2011) Comparative study of fungal cell disruption—scope and limitations of the methods. *Folia Microbiol* 56(5):469-475.

Klis FM, Boorsma A & De Groot PWJ (2006) Cell wall construction in Saccharomyces cerevisiae. *Yeast* 23(3):185-202.

Klis FM, Mol P, Hellingwerf K & Brul S (2002) *Dynamics of cell wall structure in Saccharomyces cerevisiae.* 239-256 p. http://femsre.oxfordjournals.org/femsre/26/3/239.full.pdf

Knothe G & Razon LF (2017) Biodiesel fuels. *Progress in Energy and Combustion Science* 58:36-59.

Leiva-Candia D, Tsakona S, Kopsahelis N, Garcia I, Papanikolaou S, Dorado M & Koutinas A (2015) Biorefining of by-product streams from sunflower-based biodiesel production plants for integrated synthesis of microbial oil and value-added co-products. *Bioresource technology* 190:57-65.

Li Q, Du W & Liu D (2008a) Perspectives of microbial oils for biodiesel production. *Applied microbiology and biotechnology* 80(5):749-756.

Li Y, Horsman M, Wu N, Lan CQ & Dubois-Calero N (2008b) Biofuels from microalgae. *Biotechnology progress* 24(4):815-820.

Liang M-H & Jiang J-G (2013) Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. *Progress in lipid research* 52(4):395-408.

Mata TM, Cardoso N, Ornelas M, Neves S & Caetano NS (2011) Evaluation of two purification methods of biodiesel from beef tallow, pork lard, and chicken fat. *Energy & Fuels* 25(10):4756-4762.

Matsakas L, Novak K, Enman J, Christakopoulos P & Rova U (2017) Acetate-detoxification of wood hydrolysates with alkali tolerant Bacillus sp. as a strategy to enhance the lipid production from Rhodosporidium toruloides. *Bioresource Technology*.

Miller N (2012) *Process design and modeling for the production of triacylglycerols (TAGs) in Rhodococcus opacus PD630*. (Massachusetts Institute of Technology).

Mitra D, Rasmussen ML, Chand P, Chintareddy VR, Yao L, Grewell D, Verkade JG, Wang T & van Leeuwen J (2012) Value-added oil and animal feed production from corn-ethanol stillage using the oleaginous fungus Mucor circinelloides. *Bioresource Technology* 107(0):368-375.

More TT, Yadav JSS, Yan S, Tyagi RD & Surampalli RY (2014) Extracellular polymeric substances of bacteria and their potential environmental applications. *Journal of Environmental Management* 144:1-25.

Munch G, Sestric R, Sparling R, Levin DB & Cicek N (2015) Lipid production in the under-characterized oleaginous yeasts, Rhodosporidium babjevae and Rhodosporidium diobovatum, from biodiesel-derived waste glycerol. *Bioresource technology* 185:49-55.

Nelson RG & Schrock MD (2006) Energetic and economic feasibility associated with the production, processing, and conversion of beef tallow to a substitute diesel fuel. *Biomass and Bioenergy* 30(6):584-591.

Nikolić NČ, Cakić SM, Novaković SM, Cvetković MD & Stanković MZ (2009) Effect of extraction techniques on yield and composition of soybean oil *Macedonian Journal of Chemistry and Chemical Engineering* 28 (2):173-179.

Nouha K, Kumar RS, Balasubramanian S & Tyagi RD (2017) Critical review of EPS production, synthesis and composition for sludge flocculation. *Journal of Environmental Sciences*.

Ochsenreither K, Glück C, Stressler T, Fischer L & Syldatk C (2016) Production strategies and applications of microbial single cell oils. *Frontiers in microbiology* 7:1539.

Pahl G (2008) *Biodiesel: growing a new energy economy.* Chelsea Green Publishing,

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, Komaitis M & Aggelis G (2004) Single cell oil (SCO) production by Mortierella isabellina grown on high-sugar content media. *Bioresource Technology* 95(3):287-291.

Prabakaran P & Ravindran AD (2011) A comparative study on effective cell disruption methods for lipid extraction from microalgae. *Lett. Appl. Microbiol.* 53(2):150-154.

Rawat I, Kumar RR, Mutanda T & Bux F (2013) Biodiesel from microalgae: a critical evaluation from laboratory to large scale production. *Applied energy* 103:444-467.

Rawat I, Ranjith Kumar R, Mutanda T & Bux F (2011) Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. *Applied Energy* 88(10):3411-3424.

Reijnders L (2018) Biofuels from Microalgae: Biodiesel. *Energy from Microalgae,* Springer. p 171-180.

Saka S & Kusdiana D (2001) Biodiesel fuel from rapeseed oil as prepared in supercritical methanol. *Fuel* 80(2):225-231.

Salim S, Bosma R, Vermuë M & Wijffels R (2011) Harvesting of microalgae by bio-flocculation. *J Appl Phycol* 23(5):849-855.

Shafiq SA (2017) Biodiesel production by oleaginous fungi before and after exposing of UV light. *EC Microbiology* 12:283-289.

Soldi RA, Oliveira AR, Ramos LP & César-Oliveira MAF (2009) Soybean oil and beef tallow alcoholysis by acid heterogeneous catalysis. *Applied Catalysis A: General* 361(1-2):42-48.

Takase M, Zhao T, Zhang M, Chen Y, Liu H, Yang L & Wu X (2015) An expatiate review of neem, jatropha, rubber and karanja as multipurpose non-edible biodiesel resources and comparison of their fuel, engine and emission properties. *Renewable and Sustainable Energy Reviews* 43:495-520.

Tang S, Chen M, Yang J, Ni Q, He D & Chen T (2007) Research of producing oil by Mortierella isabellina. *China Oils Fats* 32(12):35-37.

Uduman N, Qi Y, Danquah MK, Forde GM & Hoadley A (2010) Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. *Journal of Renewable and Sustainable Energy* 2(1):012701.

Van Gerpen J, Shanks B, Pruszko R, Clements D & Knothe G (2004) Biodiesel analytical methods. *National Renewable Energy Laboratory, Colorado* :37-47.

Verma M, Loha C, Sinha AN & Chatterjee PK (2017) Drying of biomass for utilising in co-firing with coal and its impact on environment–A review. *Renewable and Sustainable Energy Reviews* 71:732-741.

Vicente G, Bautista LF, Rodríguez R, Gutiérrez FJ, Sádaba I, Ruiz-Vázquez RM, Torres-Martínez S & Garre V (2009) Biodiesel production from biomass of an oleaginous fungus. *Biochem. Eng. J.* 48(1):22-27.

Wynn JP & Ratledge C (2005) Oils from microorganisms. *Bailey's Industrial Oil and Fat Products*.

Xu J, Du W, Zhao X & Liu D (2016) Renewable microbial lipid production from Oleaginous Yeast: some surfactants greatly improved lipid production of Rhodosporidium toruloides. *World Journal of Microbiology and Biotechnology* 32(7):1-9.

Yahyaee R, Ghobadian B & Najafi G (2013) Waste fish oil biodiesel as a source of renewable fuel in Iran. *Renewable and Sustainable Energy Reviews* 17:312-319.

Yuan J, Kendall A & Zhang Y (2015) Mass balance and life cycle assessment of biodiesel from microalgae incorporated with nutrient recycling options and technology uncertainties. *Gcb Bioenergy* 7(6):1245-1259.

Zhang X, Agrawal A & San KY (2012) Improving fatty acid production in escherichia coli through the overexpression of malonyl coA-Acyl carrier protein transacylase. *Biotechnology progress* 28(1):60-65.

Zhang X, Yan S, Tyagi RD, Drogui P & Surampalli RY (2014a) Ultrasonication assisted lipid extraction from oleaginous microorganisms. *Bioresource technology* 158:253-261.

Zhang X, Yan S, Tyagi RD, Surampalli RY & Valéro JR (2014b) Ultrasonication aided in-situ transesterification of microbial lipids to biodiesel. *Bioresource technology* 169:175-180.

Zhang X, Yan S, Tyagi RD, Surampalli RY & Valéro JR (2016) Energy balance of biofuel production from biological conversion of crude glycerol. *Journal of environmental management* 170:169-176.

Zhao X, Kong X, Hua Y, Feng B & Zhao ZK (2008) Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast Lipomyces starkeyi. *European Journal of Lipid Science and Technology* 110(5):405-412.

Zhu L, Zong M & Wu H (2008) Efficient lipid production with Trichosporonfermentans and its use for biodiesel preparation. *Bioresource Technology* 99(16):7881-7885.

# CHAPITRE II

# PARTIE 1

# RECENT DEVELOPMENTS OF DOWNSTREAM PROCESSING FOR MICROBIAL LIPIDS AND CONVERSION TO BIODIESEL

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## RÈSUMÈ

L'augmentation de la population mondiale et l'épuisement des ressources naturelles créent une demande urgente d'innovation radicale pour satisfaire aux besoins de base de la vie. Dans cette optique, des ressources d'énergie renouvelable non conventionnelles comme le biodiesel, ont été élaborées au cours des dernières décennies. Le biocarburant (par exemple le biodiesel) pourrait présenter une solution durable à la crise 'aliment vs carburant'. Durant le bio-raffinage, l'extraction des lipides à partir de lipides microbiens oléagineux fait partie intégrante de la libération des acides gras. L'extraction directe des lipides à partir d'une biomasse cellulaire humide au lieu d'une biomasse sèche, pourrait économiser les couts de déshydratation de cette dernière. Dorénavant, des recherches doivent être mises en œuvre afin d'établir des approches robustes et applicables à une échelle industrielle, pour l'extraction des lipides à partir d'une biomasse humide. Cette revue a pour but de mettre le point d'une façon critique sur la perturbation cellulaire, la purification et la récupération des lipides, afin d'appuyer la possibilité de l'extraction des lipides à partir d'une biomasse cellulaire humide et par la suite une transestérification efficace.

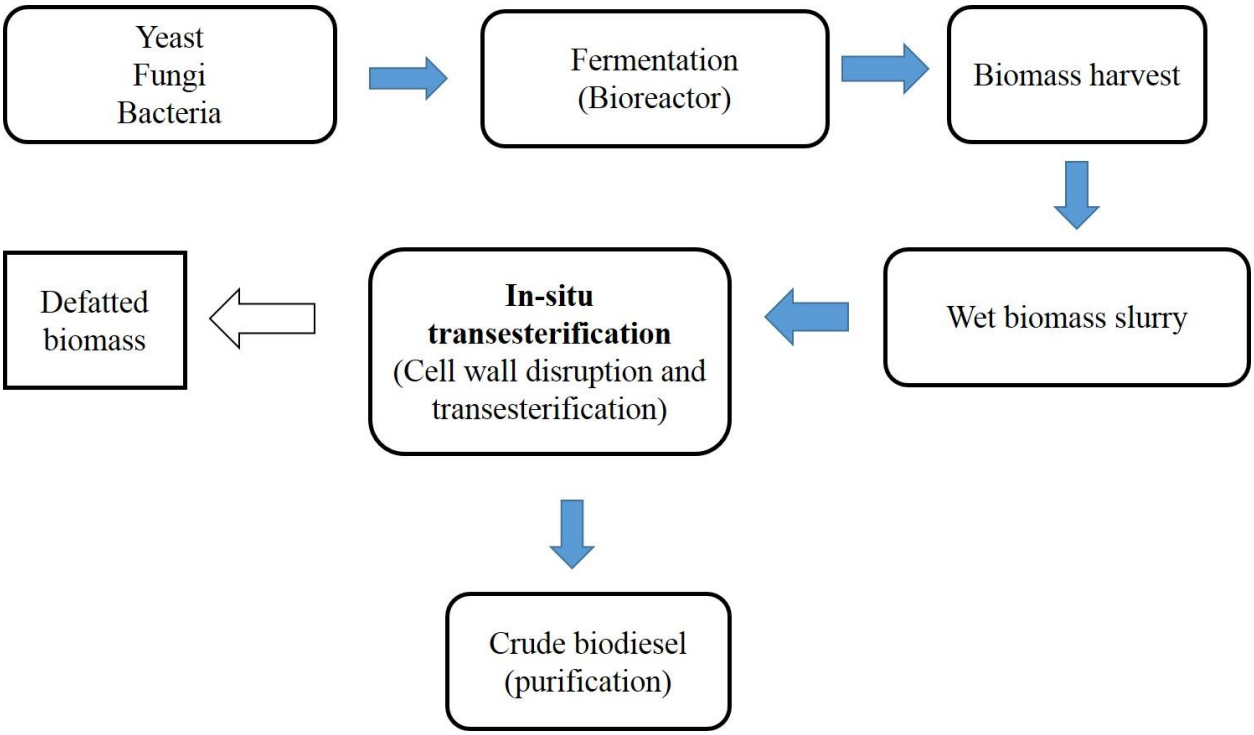
## ABSTRACT

With increasing global population and depleting resources, there is an apparent demand for radical unprecedented innovation to satisfy the basic needs of lives. Hence, non-conventional renewable energy resources like biodiesel have been worked out in past few decades. Biofuel (e.g. Biodiesel) serves to be the most sustainable answer to solve if non-edible oil is used. In biorefinery process, lipid extraction from oleaginous microbial lipids is an integral part as it facilitates the release of fatty acids. Direct lipid extraction from wet cell-biomass is favorable in comparison to dry-cell biomass because it eliminates the application of expensive dehydration. However, this process is not commercialized yet, instead, it requires intensive research and development in order to establish robust approaches for lipid extraction that can be practically applied on an industrial scale. This review aims for the critical presentation on cell disruption, lipid recovery and purification to support extraction from wet cell-biomass for an efficient transesterification.



**Keywords:** Biomass harvesting, wet biomass, cell wall disruption, lipid recovery, transesterification, biodiesel purification

## GRAPHICAL ABSTRACT



## 2.1 INTRODUCTION

Worldwide fossil fuel (non-renewable fuel) demand is increasing day by day and its depletion concerns over greenhouse gas emission (GHG). With this rapid consumption, the oil resources will be exhausted within 40 years (Shafiee & Topal, 2009). Hence, the development of renewable fuels is attracting researchers. In order to mitigate the heavy reliablity on the raw material (vegetable oil or animal fat) availability, it is necessary to find some alternative feedstock to produce biodiesel. It has been widely reported that oleaginous microorganisms could be used as raw materials for producing biodiesel (Munch et al., 2015; Patel et al., 2015; Sitepu et al., 2014). Various studies have been done using Oleaginous microorganisms (lipid-producing microorganisms) like yeast, fungi, microalgae, and bacteria they can accumulate lipids in the form of TG (triglycerides), FFA (free fatty acids), sterols, polar lipids, hydrocarbon, and pigments. During downstream processing (bio-refinery process), harvested biomass is fractionated into biofuel, value-added co-products, and energy in order to create cost-effective biomass-based industry (Grima et al., 2013).

The biggest obstacle to biodiesel production from an oleaginous microorganism is the high cost (Amanor-Boadu et al., 2014; Benemann et al., 2011; Santander et al., 2014). It requires around minimum 6-8 $ to produce per gallon of biodiesel from an oleaginous microorganism which was only 2 to 3 $ for per gallon biodiesel produced from vegetable oil and animal fat (Davis et al., 2011; Delrue et al., 2012; Ramos Tercero et al., 2014). Autotrophic microorganisms (microalgae) use sunlight as driven power to convert carbon dioxide to lipid which requires zero cost in carbon utilization. However, the cultivation requires large land occupation and the lipid accumulation is slow (Bellou et al., 2014; Meng et al., 2009). Heterotrophic microorganisms are promising to produce lipid due to their ability to accumulate high lipid content and rapid growth rate. The downstream processing such as, biomass harvesting, drying, cell wall disruption and transesterification are costly, which is the main cause of high biodiesel production cost (Koutinas et al., 2014). In order to lower the cost, multiple steps of downstream processing need to be reduced.

In literature, vigorous reviews have been published on downstream processing using microalgae as a feedstock. But there was a lack of knowledge using oleaginous yeast, fungi, and bacteria as a feedstock for biomass harvesting, wet cell wall disruption, and in-situ transesterification to obtain final product biodiesel.

This review mainly highlights important considerations involved since last five years (2013 – 2017) of literature on downstream processing of biodiesel obtained from oleaginous yeast, fungal and bacterial lipids. Advancement in biomass harvesting and mechanism of wet biomass cell wall disruption with various recent technologies has been introduced. This review presents a critical discussion about lipid recovery and its mechanism using organic and environmentally friendly solvents and its effects after lipid separation. This review contains in-depth analysis and discussion about lipid extraction and transesterification from wet biomass slurry and recent trends of biodiesel purification and challenges for researchers to make biodiesel an industrially feasible economical process.

## 2.2 OLEAGINOUS MICROORGANISMS FOR LIPIDS PRODUCTION

All microbes including prokaryotes and eukaryotes like fungi and yeast are known to produce lipids for regular cellular metabolism and structural purposes but recent research has identified many microbes mostly yeast and algae which are found to be accumulating a significant amount of intracellular lipids in the form of lipid vesicles which account for over 20% of dry biomass weight. These organisms are classified as oleaginous microorganisms (Liang & Jiang, 2013). From last one decade, several studies have been done on microbial lipid production using yeast, fungi, and bacteria under lab scale and it consists of 30 to 80% wt lipid content. Origin of single cell oil dates back to 1985 when first single cell oil was produced from *Mucor circinelloides.* Since then many new microbes have been discovered, such as, *Cryptococcus sp, Lipomyces sp, Rhodosporidium sp, Rhodotorula sp, Trichosporon sp, Yarrowia sp, Aspergillus sp, Mortierrela sp, Thamnidium sp, Candida sp, Zygosacchromyces sp, Zygorhynchus sp, Mucor sp, Torulopsis sp and Pichia sp* (Levering et al., 2015). Table 2.1.1, gives a non-exhaustive list of the oleaginous microorganism employed for the production of single cell oil so far.

**Table 2.1 1 Different types of lipids found in oleaginous microorganisms.**

| Name of Organism | Neutral lipids (N.L) % w/w | Polar lipids (P.L) % w/w | References |
|---|---|---|---|
| **Fungi and yeast** | | | |
| *Cryptococcus curvatus* ATCC20509 | 66.0 | 15.5 | (Liang et al., 2012) |
| | 35.9 | 7.6 | (Gong et al., 2014) |
| *Cryptococcus* sp. | 63.5<br>61.3 (fed batch) | 9.4,<br>10.8 (fed batch) | (Chang et al., 2013) |
| *Lipomyces starkeyi* DSM 70295 | 56.39 | 13.3 | (Angerbauer et al., 2008) |
| *Lipomyces starkeyi* | 47 | 17.2 | (Huang et al., 2014) |
| *Microsphaeropsis* sp | 32.5 | 8 | (Xiaowei & Hongzhang, 2012) |
| *Rhodosporidium toruloides* 21167 | 63.63 | 22 | (Wang et al., 2012) |
| *Rhodosporidium toruloides* AS2 1389 | 69.66 | 26.7 | (Xu et al., 2012) |
| *Yarrowia lipolytica* SKY 7 | 58.77 | 11.4 | (Yellapu et al., 2016) |

## 2.3 BIOMASS HARVESTING

The initial step in the downstream processing for biodiesel production is the biomass harvesting from the fermented broth. Due to a tiny cell size of yeast, fungi, and bacteria (less than 5 µm in diameter), separation of biomass from the medium is the key bottleneck for the biodiesel production. After lipid accumulation, harvesting is the preliminary step for processing of biomass to biofuel, where water removal from yeast, fungi, and bacteria by centrifugation accounts 20-30% of total production cost (Dickinson et al., 2017).

**Table 2.1 2 Different harvesting technologies used for microbial biomass separation**

| Harvesting method | | Advantages | Disadvantages | References |
|---|---|---|---|---|
| Centrifugation | • Microalgae, yeast, fungi, and bacteria | • Fast method.<br>• 95-100% efficiency. | • Expensive method.<br>• High energy requirements. | (Dassey & Theegala, 2013) |
| Chemical coagulation/Flocculation | • Microalgae. | • Low energy requirement<br>• 95% efficiency | • Chemical flocculants may be expensive<br>• Recycling of culture medium is limited | (Barros et al., 2015) |
| Flotation | • Microalgae | • Feasible for large-scale application.<br>• Low-cost method.<br>• 90-95% efficiency. | • Oversized bubbles break up the floc<br>• Unfeasible for marine microalgae harvesting. | (Kurniawati et al., 2014) |
| Filtration | • Microalgae and fungi | • Allows the separation of shear sensitive species.<br>• 70-89% efficiency | • High operational and maintenance cost.<br>• Membrane replacement and pumping represent the major associated costs. | (Zhao et al., 2017) |
| Magnetic based separation | • Microalgae | • High recovery efficiencies.<br>• Recovery and utilization of nanoparticles | • Nanoparticles are cost effective.<br>• It is under lab-scale study. | (Yang et al., 2018) |

### 2.3.1 Recent trends in biomass harvesting

Harvesting method has a great importance for economics and industrial biodiesel production using microbial lipid. The harvesting method depends upon characteristics of the microorganism. A vigorous research was done and critical reviews were published on biomass harvesting using microalgae, a brief overview was presented in Table 2.1.2 such as centrifugation, coagulation, filtration, and flotation. A huge knowledge gap has been observed in literature on biomass (yeast, fungi, and bacteria) separation without any intensive and cost-effective process.

According to literature, till date biomass (Yeast, fungi, and bacteria) harvesting was done by using batch centrifugation (Dassey & Theegala, 2013). Recently in our lab, oleaginous yeast biomass has been harvested by settling using extra polymeric substances (EPS) as a bioflocculant with a combination of calcium chloride and biomass settling was observed in less than 10 min (unpublished data).

## 2.4 EXISTING TECHNOLOGIES FOR CELL WALL DISRUPTION

Another critical challenge in biodiesel production from microbial lipid is cell wall disruption followed by lipid recovery. Recently researchers have a major focus on cell wall disruption using wet biomass. Depending upon the nature of the material, mechanism of cell wall disruption can change. Cell wall disruption using dry biomass is based upon physical mechanism that directly acts upon cell wall in presence of co-solvent as a medium.

Cell disruption is the most important step in lipid extraction from the microbial biomass because the efficiency of this step has a direct influence on subsequent downstream processing efficiency (Senanayake & Fichtali, 2006). Microbial cells synthesize both extracellular and intracellular products, where extracellular products can be easily separated by filtration or centrifugation; while recovery of intracellular products (lipid in the form of bilayer cell membrane and lipid droplets in the cytoplasm) requires cell disruption. Traditional lipid extraction methods developed by Folch (Folch et al., 1957) and Bligh & Dyer (Bligh & Dyer, 1959) requires a co-solvent system, a mixture of a non-polar solvent (chloroform) and a polar solvent (methanol), to extract the lipids from the dry biological material. The total dry lipid obtained from the microbial biomass was considered as 100% (w/w) and it was compared using different alternative and economic technologies to know the lipid extraction efficiency. In literature, cell wall disruption was much reviewed. Therefore, in this section, Table 2.1.3, an overview of mechanical cell wall disruption techniques and critical discussion of recent biomass disruption technologies will be explained.

### 2.4.1 Mechanical Cell Disruption Methods

Mechanical cell disruption results in non-specific cell wall breakdown due to high shear stress, abrasion. Mechanical cell disruption methods show great industrial potential due to their less dependency on species and applicability on an industrial scale (Klimek-Ochab et al., 2011). Bead milling, homogenization, and ultrasonication are commonly used mechanical methods. Therefore, these methods were briefly explained in following sections.

**Bead Milling:** Bead milling is an effective and suitable method for a wide range of microbes. Compaction and shearing action of glass, ceramic, or steel beads result in cell disruption. Disruption efficiency depends on the size and type of beads, agitation velocity, cell concentration, flow rate, bead loading, and microorganisms (Doucha & Lívanský, 2008). Bead milling has been proved to be an effective disruptive method for algal species, e.g., *Botryococcus* sp., *Chlorella* P12, *Chlorella vulgaris, Scenedesmus* sp. (Doucha & Lívanský, 2008; Lee et al., 2010) , yeast species, e.g., *Rhodotorula gracilis, Candida boidinii, S. cerevisiae, S. carlsbergensis* (Channi et al., 2016), e.g., *Bacillus cereus, Rhodococcus* sp*., E. coli* as well as for fungal species, e.g., *Penicillium citrinum,* (Klimek-Ochab et al., 2011).

**Table 2.1 3 Comparison of various Cell Disruption Methods bacterial species**

| **Microorganism** | **Type** | **Cell disruption technology** | **Moisture content %** | **Lipid recovery %(w/w)** | **Limitations** | **References** |
|---|---|---|---|---|---|---|
| *Scenedesmus sp.* | Microalgae | Enzymatic | 93.2 | 75 | Need specific enzyme cocktails for every microorganism. Very expensive | (Taher et al., 2014) |
| *Scenedesmus sp.* | Microalgae | Surfactant-MTAB*, 3_DAPS* | - | 98 | Requires subsequent process to remove the detergent | (Lai et al., 2016) |
| *Chlamydomonas reinhardtii* | Microalgae | Osmotic shock | 99 | 84 | High cost of additives | (Lee et al., 2010) |
| *Nannochloropsis occulata* | Microalgae | SDS* | 30 | 98 | Efficiency of the method depends on surfactant concentration | (Salam et al., 2016) |
| *Nannochloropsis sp* | Microalgae | Oligomeric surfactant | 30 | 78.8 | | (Wu et al., 2017) |
| *Yarrowia lipolytica* | Yeast | Detergent | 83.2 | 98.2 | | (Yellapu et al., 2016) |
| *Trichosporon oleaginous* | Yeast | Ultrasonication | - | 100 | Non-specific cell disruption. High heat generation. Generation of harmful free radicals | (Zhang et al., 2014) |
| *Rhodotorula glutinis* | Yeast | Pressurized $CO_2$ | - | 99 | High energy consumption | (Duarte et al., 2017) |
| *Rhodosporidium diobovatum* | Yeast | Ionic liquid | 80 | 97.1 | Not suitable for large scale | (Ward et al., 2017) |
| *Cryptococcus curvatus* | Yeast | Acid digestion | 95.2 | 98.9 | Not applicable for industrial process. Acid will corrode reactor | (Yu et al., 2015) |
| *Mortierella isabelina* | Fungi | Soxhlet | 97.5 | 100 | High energy and solvent consumption | (Yu et al., 2015) |

| | | | | | |
|---|---|---|---|---|---|
| *Mucor fragilis AFT7-4* | Fungi | Soxhlet | - | 95.4 | (Huang et al., 2015) |

**Ultrasonication:** Ultrasonication attributes to formation, growth and collapse of gas bubbles. Microscopic bubbles at various nucleation sites in fluid were formed during ultrasonication, which has two phases, namely, rarefaction and compression phase. The bubbles grow during the rarefaction and are compressed during compression phase, which cause the collapse of the bubbles. Ultrasonication has been widely applied in industry for protein extraction, chemical synthesis, disinfection, and cell disruption with reduced chemical addition. The study has been performed by utilization of ultrasonication on lipid extraction from *Nannochloropsis oculata* (Adam et al., 2012). The highest lipid yield was 0.21% w/w which is lower than solvent (chloroform and methanol) extraction yield (5.47% w/w). In another study by Zhang et al. (2014), ultrasonication (50 Hz, 2800 W) was applied for lipid extraction from *Trichosporon oleaginous* and SKF-5 (an oleaginous fungal strain). They compared the efficiency of water, methanol, hexane and 1:1 v/v chloroform/methanol under ultrasonication. In case of *Trichosporon oleaginous,* highest lipid recovery was 43.2% with hexane, 10.2% with water, 75.7% with methanol and 100% w/w with chloroform/methanol. Similarly, for SKF-5, 100% w/w lipid recovery was obtained with chloroform/methanol at ultrasonication frequency of 50 Hz with 2800 W power input for 15 min as compared to water (9.3% w/w), methanol (65.1% w/w) and hexane (33.2% w/w). So, more efforts are required to increase lipid recovery with ultrasonication.

### 2.4.2 Non-Mechanical Cell Disruption Methods

#### 2.4.2.1 Physical Methods

**Microwaves assisted lipid extraction:** Microwave-assisted lipid extraction is an efficient extraction process that results in an increased yield and quality within short time. The mechanism of microwave technology works on non-ionizing electromagnetic oscillating waves. In the range of 300 MHz to 300 GHz, they generate heat in the polar material by the electric field- induced polarization and the reorientation of the molecules that results in friction (Jeevan Kumar et al., 2017). These microwaves interact with free water molecules present inside the cell and give a hasty non-uniform rise in temperature resulting in increased intracellular pressure, thereby, causing spontaneous cell rupture. Lipid extraction can be potentially quick and inexpensive with microwave-assisted solvent extraction with the following benefits- a) requirement of the reduced amount of organic solvent (eg: chloroform and methanol) b) elimination of pre-drying of biomass, c) increased yield in comparison to simple thermal treatment (Rakesh et al., 2015).

The limitations of microwave use at industrial scale, involves damage of PUFA (Polyunsaturated fatty acids) due to development of heat and free radicals, hence affecting the product quality (Günerken et al., 2015).

**Electroporation:** Electroporation is used for lipid extraction from yeast, cyanobacteria, diatoms, and other microalgae (Coustets et al., 2013; Coustets et al., 2015; Sheng et al., 2011). In this system, two electrodes (anode and cathode) are connected with an electrical power supply in a PEF (pulsed electric field) treatment chamber and then the aqueous medium (culture medium) is passed between the electrodes by applying a voltage of 0.5 V to 50 kV. The electrical power is pulsed at a frequency range of 1 Hz to 50 kHz. Pulsation results in the fracture of the cell wall, thereby, releasing oil content. PEF does not lead to cell flocculation, and hence no cellular component come out of the cell. The fractured cells then undergo healing, thereby, remain viable. Thus, this same microbial batch can be reused for further high valued materials (HVM) extraction (Reep & Green, 2012)

Several recent studies have been conducted on PEF process for lipid extraction. Flisar et al. (2014) have investigated the effect of PEF on lipid extraction from *Chlorella vulgaris* in a continuous flow system. *Chlorella vulgaris* consists of 50-58% of lipid content based on dry biomass weight. In this study, PEF treatment chamber was fabricated with stainless steel as electrodes with a gap of 15mm between them. They obtained 50% lipid yield (wt%) when an electric field strength of 2.7 kV/cm was applied for 21 pulses in 100 µs. Eing et al. (2013) also used stainless steel as electrodes in PEF treatment chamber with a 4mm gap between them. Here, an electric field strength of 35 kV/cm was applied to the target sample (*Auxenochlorella protothecoides*) for 1 µs duration and obtained 22% lipid yield. In another study done by (Zbinden et al., 2013), lipid has been extracted from *Ankistrodesmus falcatus* using this approach. At an electric field strength of 45 kV/cm for 100 ms, PEF resulted in the electroporation of 90% of algal cells and thus led to 6.1 mg/L lipid yield. Liu et al. (2011) have reported electroporation of 87% of the cells of *Synechocystis* PCC 6803 by applying electric field strength of 35 $kWh/m^3$ that resulted in 25-75% lipid recovery. Thus, PEF has high potential to be used at high scale due to its low energy consumption which makes it economical.

2.4.2.2 Chemical Cell Disruption Methods

**Organic Solvent Extraction:** Biocompatible organic solvents have been used for lipid milking (removal of accumulated lipid without killing the oleaginous microbes so that these microbes can undergo repetitive milking) from microalgae. Here, the microbial biomass is exposed to a

biocompatible hydrophobic solvent that is absorbed by the cells. The solvent creates pores and openings in the cell membrane and results in the secretion of lipids (inside cytosol) outside the cells. The partition coefficient of the biocompatible solvent should be high (log P >5) in order to obtain highly efficient extraction and also to maintain a separation between the extracellular chemicals and the aqueous cytosolic content so that cell culture can be prevented from being contaminated (Dong et al., 2016). The high partition coefficient prevents irreversible membranous damages (such as uncontrolled cracks and holes), thereby, extends the cell life for milking. The concept of algal milking using this process has been demonstrated to extract β-carotene from *Dunaliella salina* culture using a biphasic reactor (consisting of two phases- an aqueous phase and a biocompatible organic solvent phase) (Jackson et al., 2017).

**Surfactant-assisted lipid extraction:** Surfactant-assisted lipid extraction is non-toxic and uses biodegradable chemicals and has a potential for the cell wall disruption without requiring any specific equipment (Zeng et al., 2007). Surfactants are differentiated by hydrophobic and hydrophilic moieties. Cell membranes possess negative charges because of functional groups. As a result, they can be disrupted easily using a hydrophobic domain (Jeevan Kumar et al., 2017). Surfactant application in enzyme isolation is well studied, where cationic, anionic and zwitter ions are employed. The state-of-art of using surfactant is for wet biomass disruption. Recently few papers have been published on the subject. (Lai et al., 2016) investigated Myristyltrimethylammonium bromide (MTAB)- and 3-(decyldimethylammonio)-propanesulfonate inner salt (3-DAPS)-surfactants for lipid recovery from wet biomass slurry of *Scenedesmus* sp and lipid extraction efficiency was almost 100% as compared with standard chloroform and methanol (2:1) method. A similar study was conducted by Yellapu et al. (2016) using N-lauroyl sarcosine (N-LS) as a biodegradable anionic surfactant for lipid extraction from oleaginous yeast wet biomass with 82.3% (w/w) moisture content. The maximum lipid extraction efficiency obtained was 98.2 % w/w in less than 10 min reaction time. There are similar studies using different surfactants, such as oligomeric surfactant and sodium dodecyl sulphate (SDS), lipid recovery in both cases was greater than 90% w/w (Salam et al., 2016; Wu et al., 2017) also showed that the technique has an important potential in the development of an industrial-viable approach for lipid extraction. However, studies on variables (concentration of surfactant, reaction temperature and pH) that limit the efficiency of surfactant action are scanty and systematic research should be carried out.

**Supercritical Fluid Lipid Extraction:** In recent years, supercritical fluid extraction (SFE) has grabbed considerable attention (Duarte et al., 2017). SFE achieves the lipid extraction by manipulating the chemicals, which behave as both a liquid and a gas in their critical temperature

and pressure. In critical stage, solvating power of the compound used in SFE is increased and then it plays as a solvent to extract the product from cells. Mostly, carbon dioxide is used due to its low viscosity (<100 μPa.s), high diffusivity (<0.1 $mm^2/s$), and suitable critical temperature (31.1 °C) and pressure (72.8 atm). In an extraction vessel, oil-bearing substances contact with supercritical carbon dioxide for certain time (several hours). During the process, oil will be solubilized in $CO_2$ and extracted. $CO_2$ which contains oil is then collected and depressurized to allow the escape of $CO_2$, and finally, oil is obtained. The application of supercritical $CO_2$ lipid extraction from microorganisms has been extensively reported.

## 2.5 Separation of microbial lipids for transesterification

The foremost requirement for industrialization of biodiesel production using microbial lipid is the efficient extraction of lipid from biomass. The downstream recovery of microbial lipid in conventional method requires a large amount of chemical solvent which contributes around 70-80% of the total biodiesel production cost (Dong et al., 2016). Moreover, the chemical solvents employed for lipid extraction (chloroform, methanol) have high toxicity and flammability which consequently raise a concern regarding its impact on the environment. The lipid in microbial cells is enclosed by the solid matrix. Therefore, the ideal solvent should be able to penetrate solid matrix and solvate the lipid. In last decades, a large number of studies were performed for developing the efficient and green process for lipid extraction; this section discussed the recent research efforts taken towards the optimization of extraction procedure, their challenges, and impact of physical properties of solvent on extraction and green environmentally friendly solvent extraction techniques for biodiesel production.

### 2.5.1 Lipid separation mechanism and their challenges

The extraction of lipid from microbial biomass is a two-step process. In the first step, physical, chemical and enzymatic disruptions of the cell wall are performed by various means (Refer to section 3.2). The second step involves the use of a chemical solvent for oil recovery, and it is associated with specific conditions such as temperature and processing time. In the conventional method, the definite proportion of chloroform and methanol were used for extraction of microbial oil (Bligh & Dyer, 1959). With the advancement in the field of science, various extraction techniques have been developed for lipid extraction, such as ultrasonication assisted, microwave assisted, supercritical fluid extraction, pressurized fluid extraction, Soxhlet extraction and many more. In the microbial cell, lipid exists in three forms which are neutral lipids, free fatty acids, and polar lipids. Principally, the neutral lipid exists as globules in the cytoplasm of the cell and they

form complex with the non-polar organic solvent through van der waal forces and diffuse out from the cell via concentration gradient separation. While polar lipids attached to protein in cell membrane via hydrogen bonding requires a polar solvent to disrupt the strong binding between the polar lipid and membrane proteins. However, some neutral lipid complexes with the polar lipid and hence not extracted via non-polar organic solvent, therefore in order to ensure efficient and complete recovery, co-solvent mechanism or mixture of polar and non-polar organic solvent were utilized. In the co-solvent system, polar solvent breaks the hydrogen bond between the neutral lipid and polar lipid followed by its van der waal interaction of non-polar solvent which surrounds the neutral lipid and comes out via diffusion. Once the lipid comes out from the cell membrane with solvent, the lipid portion is recovered by addition of non-polar organic solvent and water which perform biphasic separation of lipid molecules from the other contaminants such as carbohydrates and proteins. The choice of solvent for lipid extraction from microbial cells depends on factors like initial lipid content, solvent-cellular interaction, type of microorganism and reaction time (Ranjan et al., 2010).

The mixture of chloroform and methanol is the most commonly used solvent for extraction due to its characteristic feature of being fast and quantitative, and also it does not require the complete dewatering of biomass, rather the water present in the cell works as ternary substance and helps in complete extraction of polar and neutral lipid (Jose & Archanaa, 2017). However, high toxicity of the chloroform and methanol limits its application on an industrial scale. A study reported that hexane could be more suitable for oil recovery because it was found to be more selective for neutral lipids which in turn reduces the downstream purification step, however, hexane was unable to extract polar lipids which cause the loss of lipid that ultimately affects the economy of the process. This study clearly demonstrates that the efficiency of extraction depends on the polarity of the solvent. However, complete extraction of lipid via solvent was not reported until date.

The physical disruption technique is a prerequisite for the complete and efficient recovery of lipid. In a study of mechanistic assessment of lipid extraction, it was reported that neither soxhlet method (with hexane) nor Bligh and Dyer method was able to disrupt the cell completely (Ranjan et al., 2010). Boyd et al. (2012) investigated the use of Switchable Hydrophilicity Solvent (SHS) N, N-dimethylcyclohexylamine without any prior treatment of cell disruption such as sonication or microwave heating. The study reported 22 wt% recovery of oil from microalgal biomass whereas the lipid extraction efficiency by the conventional method was 52%. Zhang et al. (2014) evaluates the efficiency of lipid extraction of four solvent after ultra-sonication treatment

(Water, hexane, methanol, chloroform and conventional chloroform-methanol mix) and observed 100% lipid extraction efficiency of chloroform at low temperature and shorter time duration. These studies strongly support the necessity of physical disruption technique before solvent extraction.

The recovered lipid from wet microalgae was subjected to treatment using persulfate-based oxidation with ferric chloride as a coagulant in order to eliminate the dewatering step. In this study, microalgal cells were first harvested by adding 200 mg $L^{-1}$ of $FeCl_3$ (as a coagulant) and extraction was performed using persulfate based oxidation by addition of potassium persulfate, which eventually lead to the recovery of 95% of lipid (Seo et al., 2016).The persulfate based extraction does not require organic solvent for extraction process and can be directly applied to wet biomass.

### 2.5.2 Effect of physical properties of solvent upon lipid extraction

The physical properties of a solvent such as partition coefficient, density, and solubility of water are the critical parameters which determine the efficient extraction of lipid from the solvent.

The partition coefficient is the quantitative measurement of an organic compound distributed between the organic and aqueous phase. The partition coefficient of solvent defines the polarity of solvent which in turn determines the degree of interaction of solvent with the lipid molecule. However, a hydration shell enclosed the polar lipids because of electrostatic attraction of water. Therefore, it requires additional energy input for the extraction (Dong et al., 2016). The solubility of solvent in the water affects the recovery of the solvent after extraction. A polar solvent such as methanol, ethanol, isopropanol is miscible in water, which means these solvents require the additional step of distillation for the recovery. A large difference in density of solvent and water help in the formation of the biphasic system.

### 2.5.3 Green Recovery of lipid

Traditional extraction procedures require harsh organic solvent which has reported to have a high environment, health and safety risk score (Zbinden et al., 2013). These limitations of the organic solvents lead to the investigation of green recovery system with an eco-friendly and natural solvent for lipid extraction. Pulsed electric field, lipolytic enzyme degradation, simultaneous distillation and extraction process, solvent-free extraction via non-woven fabric are the research effort taken towards the development of clean and green extraction system (Liu et al., 2011; Shang et al., 2015; Tanzi et al., 2013; Zbinden et al., 2013).

A recent study on utilization of non-woven fabric demonstrates the solvent-free extraction of lipid from the yeast *Rhodotorula glutinis.* The fermented broth of *Rhodotorula glutinis* was concentrated and homogenized in order to rupture the cell wall. The non-woven fabric (using polypropylene) was then immersed in the fermented broth, which adsorbed lipid with other impurities. Then the lipid was recovered from the fabric by mechanical extrusion, and the recovery of 10.4g of oil per gram of fabric was reported (Shang et al., 2015). This technique seems to be beneficial in terms of oil separation, recyclability, and environment-friendly features.

Ionic liquids are non-aqueous organic salts which consist of asymmetric organic cation and an inorganic or organic anion. Their non-volatile nature and thermal stability make them a suitable option for green recovery of lipid. Until date only one study was conducted using ionic liquids for recovery of microalgae lipid, the study reported a meager lipid content of 19% wt; however, conventional Bligh and Dyer's method process resulted in only 11% wt lipid recovery in the same study (Cooney & Benjamin, 2016). Although the extraction efficiency was quite low compared with other methods, the technical and economic viability is important. Therefore, research efforts should be directed to explore the potential of the ionic liquid as a solvent. In our lab, we identified petroleum diesel can act as a co-solvent to recover microbial lipid after cell wall disruption and transesterification (unpublished data). And this process will also help to avoid further blending of petroleum diesel and fatty acid methyl esters (FAMES).

All the extraction procedure investigated so far, either employing a green extraction process or organic solvent demonstrate the dependency of extraction process on lipid composition, type of lipid fraction (neutral or polar lipid), and their interaction with a membrane protein. An ideal extraction process should not only efficiently recover oil but also reduce the contamination, increase mass transfer and simplify downstream processing. Therefore, further research has to be carried out regarding the scalability, extraction efficiency, and energy consumption and downstream process.

## 2.6 Microbial lipid to biodiesel conversion (Transesterification)

Biodiesel is produced by transesterification of triglycerides present in the microbial lipids, plant oils and animal fats in the presence of catalyst and alcohol to produce the fatty acid alkyl esters (FAAE) and glycerol as a byproduct. Transesterification of microbial lipids to biodiesel is being carried out by both homogeneous and heterogeneous catalysts. The homogeneous alkali catalysts such as sodium hydroxide (NaOH) or potassium hydroxide (KOH) have been mostly used for transesterification due to certain advantages such as faster reaction under mild reaction

conditions of low temperature and atmospheric pressure. However, due to the presence of high content of free fatty acids in the microbial lipids, homogeneous alkali catalysts are not suitable for transesterification process as they lead to formation of soap in the presence of free fatty acids, which causes the difficulty in biodiesel separation and further purification process (Hidalgo et al., 2013). To overcome this limitation, the acid catalysts such as sulfuric acid ($H_2SO_4$) or hydrochloric acid (HCl) have been considered as they can be used in the presence of free fatty acid content higher than 1%. However, they require higher temperature as well as higher reaction time as compared to alkali catalysts (Vonortas & Papayannakos, 2014). In various studies, both acid and alkali catalysts have been used. Primarily, the acid catalyst is being used to reduce the free fatty acid content to less than 1%, thereafter, alkali catalyst is being considered to conduct transesterification of triglycerides to FAAE. Enzyme catalytic process has gained the attention of researchers since last decade due to certain advantages such as accessibility for every feedstock, insensitivity to free fatty acid content as well as high purity of products (Channi et al., 2016). However, high production cost, the unstable behavior of enzymes as well as lower conversion yield as compared to homogeneous alkali and acid catalysts makes this process less considerable at the industrial scale biodiesel production process.

The use of heterogeneous catalysts such as alkali exchanged zeolite, potassium exchanged alumni, etc. for transesterification process has been considered as one of the emerging technology due to their advantage for removal of undesirable free fatty acid impurities, easy recovery, and production of cleaner biodiesel. Moreover, removal of washing and purification steps from the process steps due to the usage of heterogeneous catalysts makes them much preferable for transesterification process (Degirmenbasi et al., 2015). In spite of certain advantages, these catalysts require high temperature and pressure as well as longer reaction time due to the formation of three different phases of reactants. However, researchers are continuously working to overcome these limitations (Lee & Wilson, 2015).The catalysts play an efficient role in transesterification process. However, the transesterification process of microbial lipid to biodiesel is a challenging process due to the presence of high water content in the biomass. There is two type of transesterification methods for microbial lipids.

### 2.6.1 Conventional method

The conventional method of biodiesel production using microbial lipids include multiple steps such as biomass drying, microbial cell disruption by mechanical, chemical or biological methods, oil extraction, separation, and transesterification. These multiple steps involved in conventional

methods are considered as highly energy intensive as they require high temperature, a large number of solvents and longer reaction time, which adds up to high biodiesel production cost (Cheirsilp & Louhasakul, 2013). Moreover, use of toxic organic solvents in conventional transesterification method is deeming them unfeasible for industrial-scale biodiesel production. However, it has been reported that the drying step of biomass consumes a huge amount of energy and the researchers are trying to develop the method for production of biodiesel using wet biomass to avoid the drying step. Very few studies have been reported using wet biomass and further transesterification using conventional method. The first study was reported by Nagle and Lemke (1990), where wet concentrated microalgal biomass was used after harvesting and successful extraction of lipids by using 1-butanol, ethanol, hexane, and 2-propanol. The high recovery yield of 90% (w/w) was reported for lipids using 1-butanol as a solvent and high conversion yield of 93% was reported using conventional method. Most recently, Yellapu et al. (2016) reported the detergent assisted lipid extraction approach for wet biomass of yeast *Yarrowia lipolytica* and used "response surface methodology" for optimization of principal parameters to obtain maximum lipid extraction efficiency of 95.3% (w/w). Further, transesterification was performed using conventional chloroform and methanol method and lipid to FAME conversion efficiency of 94.3% (w/w) was achieved.

Even though researchers are shifting towards lipid extraction using wet biomass and further transesterification process, but intensive research and development are required to establish the robust and economic process to be used at industrial scale biodiesel production. Moreover, the necessity to reduce the multiple steps involved in the conventional method as well as to reduce the use of solvents has shifted the researchers towards direct transesterification, which is also referred as in-situ transesterification.

### 2.6.2 In-situ or direct transesterification

In this process, biomass is treated with the methanol and catalyst (acid or base catalyst) in the single reactor, which results in the reactive extraction of lipids as FAAE (Fatty acid acyl esters). The methanol serves two functions, one as extraction agent and another as esterification agent. In some of the studies, an additional solvent such as chloroform or hexane is being used for easy extraction of oil from the microbial cells and also to enhance the contact of microbial oil with the esterification agent (Cao et al., 2013). Direct transesterification process has several advantages such as the elimination of multiple steps, reduction in the use and the potential loss of solvents during the extraction process and consequently reducing the processing units and

costs. Several studies have been conducted for direct transesterification of dry microbial biomass. Thliveros et al. (2014) reported 97.7% FAME yield from yeast *Rhodosporidium toruloides* by using 4g/L of NaOH at 50$^{o}$C in 10 h reaction time in the presence of methanol. In another study by Carvalho et al. (2017), conventional and in-situ transesterification reaction were performed using dried and wet microbial biomass obtained from fungal strain *Mucor circinelloides* in the presence of a heterogeneous catalyst supported on alumina as well as ethanol. Both reactions achieved high FAME yield of 97% (w/w). However, the conventional method has not been recommended due to higher energy intensive process as well as the use of huge amounts of toxic organic solvents.

The direct transesterification of lipids present in wet microbial biomass has been investigated using homogeneous acid catalysts. Liu and Zhao (2007) reported the acid catalyzed in-situ transesterification using wet microbial biomass obtained from two yeasts *Lipomyces starkeyi*, *Mortierella isabellina,* and one fungus *Rhodosporidium toruloides* and high FAME yield of up to 90% (w/w) was obtained using 0.2mol/L of $H_2SO_4$ at 70$^{0}$C in 20h reaction time in the presence of the methanol. In another study conducted by (Vicente et al., 2009), direct and conventional transesterification reactions were compared for wet biomass obtained from fungal strain *Mucor circinelloides* in the presence of three different solvent systems, chloroform: methanol, chloroform: methanol: water. The direct transesterification reaction gave high purity FAME of >99% as compared to conventional transesterification (91.4- 98%) using an acid catalyst for 8h at 65$^{0}$C in the presence of methanol to oil molar ratio of 60:1. Im et al. (2015) also reported in-situ transesterification of the wet microbial biomass of microalgae *N. oceanica* and obtained high FAME conversion yield of 91.1% using 0.3g of $H_2SO_4$ catalyst for 90 min at 95$^{o}$C in the presence of chloroform and methanol.

For direct transesterification reaction, high amount of methanol as well as sulfuric acid is required, which is not feasible at industrial scale biodiesel production as the use of methanol could be costly and presence of sulfuric acid can corrode the reactor. Therefore, researchers are developing advanced strategies for in-situ transesterification in order to decrease the use of a solvent as well as sulfuric acid.

### 2.6.3 Factors affecting in-situ transesterification and advanced strategies used

Though direct transesterification offers shorter processing time, less use of solvents and lower production cost of biodiesel from microbial biomass as compared to conventional transesterification, there are many factors, which affect the conversion efficiency of in-situ

transesterification. Water content, cell wall disruption, selection of catalyst as well as solvent extraction are the important factors that need to be discussed (Yousuf et al., 2017). Therefore, further investigation is required to improve these factors and researchers are continuously working to make this process feasible from lab scale to industrial scale.

**Moisture content:** The moisture content present in the microbial biomass significantly affects the efficiency of direct transesterification process and hence biodiesel production costs. Three types of effects have been discussed by Sathish et al. (2014): a) reversible reaction, i.e., hydrolysis of biodiesel into methanol and free fatty acids, b) shield the oil, thereby interference in reaction, c) deactivation of the acid catalyst due to competition of ions present in the water with protons present in the reaction. Hence, with increased moisture content, the conversion efficiency of lipids to FAME decreases (Hidalgo et al., 2013). Ehimen et al. (2010) also reported the similar results with an increase in moisture content from 0 to 72 % (w/w). In another study reported by Im et al. (2015), effect of moisture content on the product yield was studied by fixing the microalgal cell weight and increasing the moisture content from 0 to 90 wt.% and drastic decrease in the product yield was observed with the increase in moisture content more than 50 wt. %.

In spite of the limitations of transesterification reaction due to the presence of moisture or water level, the energy associated with the drying process is very high and hence the production cost. Therefore, it is necessary to use the wet microbial biomass for in-situ transesterification process. Kim et al. (2015) used the wet microalgae biomass for in-situ transesterification in the presence of HCl catalyst and methanol. Here, a mixture of wet algal cells, HCl and methanol were heated at 95 $^{o}$C, resulting in <90% FAME yield. The high affinity of HCl with water resulted in low impact of moisture content on FAME yield and 15 wt.% higher FAME yield was obtained as compared to the $H_2SO_4$ catalyst. In order to improve the FAME yield using wet biomass, a number of other techniques have been implemented such as increasing methanol dosage, integrating mechanical processes and using supercritical methanol. The efficiency of in-situ transesterification process can also be improved by integrating microwave or ultra-sonication technologies in order to improve the mass transfer rate between immiscible phases and subsequently reducing the reaction time even by using wet biomass (Hidalgo et al., 2013).

**Cell wall disruption:** The disruption of microbial cell wall during direct transesterification is very important in order to release the lipids outside the microbial cells and further partitioned into solvents such as hexane and pentane (Halim et al., 2012). The knowledge of the structure of microbial cell wall is important for the choice of suitable cell disruption method. The oil-rich microalgae cell wall is comparatively thick and tough as compared to prokaryotic cells. The yeast

cell wall is also rigid due to the presence of polysaccharides and proteinaceous network, which provide integrity and shape to the cells and provide stability in the osmotic environment (Backhaus et al., 2013). Therefore, cell disruption method has to be integrated with direct transesterification process in order to obtain high FAME yield. Several methods of cell disruption such as ultrasonication, microwave, and supercritical processes have been developed to disrupt the cell wall and to bring out lipids from inner compartments of microbial cells to the solvents. (Zhang et al., 2016) reported the ultrasonication assisted biodiesel production using dried biomass-derived lipids and in-situ transesterification process was performed. For lipid recovery, ultrasonication process along with chloroform and methanol (1:1 v/v) mixture exhibited the best performance among all the solvents (hexane, methanol) and 95.3% (w/w) recovery was reported. Ultrasonication assisted in-situ transesterification gave maximum biodiesel yield of 95% (w/w) within 20 min reaction time as compared to 24h, without ultrasonication. Sara et al. (2016) also compared the microwave and ultrasonication assisted in-situ transesterification for dried biomass of *Trichosporon oleaginous* and maximum FAME conversion of 99% (w/w) was achieved with microwave assisted in-situ transesterification in the presence of 183 : 1 molar ratio of methanol to lipid and 2% (w/w) NaOH within 20 min at 100°C. In case of ultrasonication assisted in-situ transesterification, 95.1% (w/w) FAME yield was obtained by using 183 : 1 molar ratio of methanol to lipid and 3% (w/w) NaOH in 20 min at 25°C. Jazzar et al. (2015)used supercritical methanol for in-situ transesterification without catalyst and achieved 45.62 wt.% biodiesel yield.

The other techniques of cell destabilization for the wet biomass includes the use of surfactants, ionic liquids and use of nanoparticles, which are known to cause weakening of the cell wall (Park et al., 2015). The surfactants have been reported to enhance FAME yield along with catalyst for wet microbial biomass as they have high water tolerance ability and hence can cause the disruption of cell as well as phospholipid membrane layer. Yellapu et al. (2016) reported the N- Lauroyl sarcosine (N-LS) assisted ultrasonication aided in situ transesterification for biodiesel production using oleaginous yeast wet biomass. The maximum FAMEs yield of 96.1 ± 1.9 and 71 ± 1.4% (w/w) was obtained with or without N-LS treatment respectively in 24 h reaction time. The maximum FAMEs yield after N-LS treatment of biomass followed by with or without ultrasonication revealed 94.3 ± 1.9% and 82.9 ± 1.8% w/w respectively using methanol to lipid molar ratio 360:1 and catalyst concentration 360 mM (64 µL $H_2SO_4$/g lipid) within 5 and 25 min reaction time, respectively. (Yoo et al., 2014) studied cell disruption using wet biomass of microalgae using functional membrane coated with a cationic polymer [tertiary-amine cations deposited on poly-dimethylaminomethylstyrene (pDMAMS) film] and gained a cell disruption yield

of 26% in 6h reaction time. However, the cell disruption yield was comparatively low but this process can also be combined with in-situ transesterification process as it was proposed to be a simple and efficient process.

**Catalyst selection:** In the transesterification reaction, catalyst selection plays an important role. During in-situ transesterification, acid catalysts are recommended due to the presence of high moisture content in the biomass. However, heterogeneous catalysts (acid and base, a mix of solid acid and solid base) have gained more attention due to easy separation, regeneration, reusability as well as easy product purification (Dong et al., 2016). Solid acids (silica-based, carbon-based, zeolite based, polymer-based, zirconia-based and hydroxyapatite based) are also preferred due to problems of corrosion and the environmental problem associated with the disposal of liquid acid catalysts. Solid super acids have the ability to perform simultaneous esterification and transesterification of fatty acids and hence can be used easily for a high content of free fatty acids. However, the limitations of heterogeneous catalysts such as longer reaction time and lower reaction rate have been considered as challenging aspects of ongoing research. Ma et al. (2015) reported the in-situ heterogeneous transesterification of microalgae using combined microwave and ultrasound irradiation. By using KF/CaO catalyst prepared by wet impregnation method along with microwave and ultrasound technology gave 93.07 ± 2.39% FAME yield in the presence of 12 wt. % of catalyst and a methanol to biomass ratio of 8:1 at 60 °C for 45 min. It was reported that the combination of US and MW (US–MW) irradiation could overcome the limitations associated with the use of heterogeneous catalyst and has been successfully designed and well documented for product synthesis, decrease in reaction time and energy consumption, improved and enhanced yield, and selection of products (Zbancioc et al., 2014).

In other studies, researchers also performed the direct enzymatic (lipase) transesterification of wet microbial biomass. Tran et al. (2013)reported the direct enzymatic transesterification of *Chlorella vulgaris* lipids using immobilized *Burkholderia* lipase as a catalyst and obtained 95.7% of FAME conversion. The wet microalgae biomass with 86–91% water content was pre-treated by sonication to disrupt the cell wall and then directly mixed with methanol and solvent in the presence of immobilized *Burkholderia* lipase with 1.65 molar ratio of hexane/methanol at 45$^{o}$C and 500-600 rpm. Navarro López et al. (2016) also performed the optimization for the production of FAME using wet *Nannochloropsis gaditana* microalgal biomass by direct enzymatic transesterification. The wet microalgal biomass was homogenized at 140 MPa to enhance cell disruption and high FAME conversion of 99.5% was achieved using oil: mass ratio of 0.32 with methanol/oil and t-butanol/oil ratios of 4.6 and 7.1 $cm^3\ g^{-1}$, respectively,

at 40 °C for 56 h. However, FAME conversion decreased to 57% after catalyzing three reactions with the same lipase. In addition, the presence of moisture, methanol, and biodiesel also contributed to the degradation of lipase immobilization support in N435. The key point of direct enzymatic transesterification technology is that the microbial biomass should have a high lipid content to obtain an efficiency of 90% FAME conversion, using lower biocatalyst loading and better lipase recycle efficiency. However, this technology is not economical and feasible at industrial scale for microbial biomass with the low lipid content. In the latest study by Kim et al. (2017), in-situ transesterification was performed without any catalyst by combining hydrothermal liquefaction (iTHL) technology with in-situ transesterification. It was found that the chlorinated hydrocarbon solvents such as dichloromethane, chloroform, and dichloroethane (DCE), improve FAME production by providing hydrogen chloride in an ionized form that can act as an acid catalyst. The most effective solvent is DCE with the FAME selectivity of 91.85% at 185.08°C with 4.69 mL ethanol and 1.98 mL DCE/g of dry algal cells.

**Solvent extraction:** In most of the direct transesterification studies, extraction of lipids from wet microbial biomass was done by using solvents such as chloroform, hexane, 2-propanol, and ethanol (Park et al., 2015). The use of a solvent for direct transesterification reaction facilitates extraction and increases the contact between oil and esterification agents, thus ensuring superior ester formation. In the study reported by Li et al. (2011), the reaction mixture of wet microalgae biomass along with methanol and the sulfuric acid catalyst was stored at 120°C and was supplemented with 2, 4, 6, 8 and 10 ml of hexane over a reaction time of 120 min. It was observed that with the increase in hexane content in the reaction mixture (from 2 to 10 ml), FAME yield increased significantly from 16.6% to 94.5% as hexane enhanced the solubility of the oil.

However, the heating requirement for the solvent extraction step also requires high energy consumption and also suffers from challenges in the solvent extraction and scale-up process. The idea of direct transesterification of fatty acids in lipid without the use of solvent extraction step could substantially reduce both the time and solvent and biodiesel production cost (Halim et al., 2012). Cheirsilp and Louhasakul (2013) developed a method for direct transesterification without using nonpolar solvent. The FAME yield of 58% was obtained by using 125:1 molar ratio of methanol/biomass for 6h, while 65-69% FAME yield was obtained with an increase in methanol/biomass ratio to 209:1 in 1h. Liu and Zhao (2007) also achieved 60% FAME yield using direct transesterification of oleaginous yeast in the presence of methanol and sulfuric acid within the longer reaction time of 20h. Nevertheless, the use of excess methanol is a cost-effective

process as compared to traditional solvent extraction method because it is recoverable and reusable for the next batch.

## 2.7 Purification of biodiesel

After the trans-esterification reaction, the biodiesel-glycerol mixture contains many impurities like metal ions, water, acid, soap which needs to be separated in order to have better fuel performance and emission characteristics (Shirazi et al., 2013). Many downstream purification processes of biodiesel have been reported in the literature like dry-washing, wet-washing, and membrane separation technology. However, for actual purification process, it is inevitable to separate glycerol from the biodiesel as the pre-treatment step. It is usually done by gravitational settling, which involves lengthy separation of polar denser phase including glycerol from lighter non-polar phase (mono-alkyl esters of long-chain fatty acids) with both phases containing impurities.

If in-situ transesterification is performed using biomass, then the first step is to separate biomass from the trans-esterified mixture using filtration or centrifugation. The reported studies for biodiesel purification using in-situ trans-esterified mixture were mostly done by wet-washing technique. A study has been reported where ultrasonication assisted in-situ transesterification was performed using algal biomass and then the biomass was filtered followed by settling/ phase separation of the filtrate, wet-washing of filtrate using water and drying using anhydrous sodium sulphate (Suganya et al., 2014). Another process has been reported where biodiesel was produced using microwave mediated in-situ transesterification of algal biomass. Once crude biodiesel was obtained, alcohol was devolatilized using vacuum distillation. N-hexane was mixed with the remaining product of vacuum distillation and passed through centrifugation. Three layers were obtained after centrifugation- upper organic phase containing biodiesel, lower aqueous phase containing glycerol, alcohol and other impurities and algal biomass layer. The uppermost layer was treated with anhydrous sodium sulfate followed by filtration and the purified biodiesel was analyzed for purity and impurities (Patil et al., 2013). The schematic diagram for biodiesel purification is shown in Figure 2.1.1.

### 2.7.1 Biodiesel glycerol separation

**Salt assisted gravitational settling vs Centrifugation:** Although salt assisted gravitational settling has applications in batch process, centrifugation can be employed for the continuous process where the oil is continuously fed into the trans-esterification reaction and continuous

purification takes place. In such scenario, gravitational settling, which requires longer incubation time is not feasible.

The continuous centrifuge can be employed for glycerol-biodiesel separation as the exit streams contain two liquid phases with two different densities. Due to high centrifugal force, settling is faster than the gravitational method. Centrifugation is apt where biodiesel is produced using in-situ transesterification. In this situation, separation of biomass and aqueous impurities including metal ions take place simultaneously (Patil et al., 2013). Although, continuous centrifuge has many advantages over salt-assisted gravitational settling method in terms of process time and productivity salt assisted gravitational method can be effective in a batch process and smaller scale biodiesel production industries due to lower capital investments, lower operational and maintenance cost. Moreover, it is 4 times faster than conventional gravitational settling method.

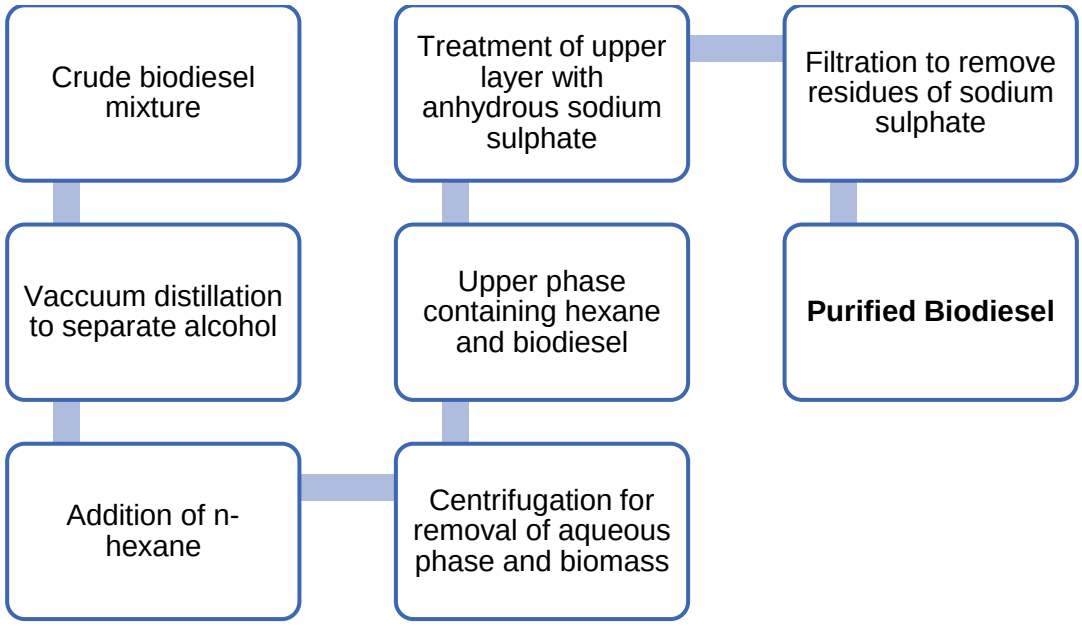



**Figure 2.1. 1 Scheme of biodiesel purification of in-situ trans-esterified biomass**

### 2.7.2 Biodiesel Purification techniques

Once biodiesel is separated from the glycerol, it still contains soap, metal ions, water, acid ions, catalyst, residual alcohol and bound glycerol in form of mono-, di- and triglycerides. There are various techniques for biodiesel purification including wet washing, dry washing and membrane technology. The biodiesel purified after several techniques should meet quality standards as specified by ASTM (American society of testing and materials) and EN (European Union) (Banga et al., 2014).

**Wet-washing:** The common methodology for wet-washing is highlighted in Figure 2.1.2. The glycerol free biodiesel is washed with water/ acid/ organic solvents followed by phase separation for 10-60 min. Two phases will be obtained after phase separation – (1) washed water/ solvent/ acid with impurities and (2) treated biodiesel. The process is repeated 2-3 times to obtain pure biodiesel.

(Mendow et al., 2012) reported biodiesel purification with two-different wet-washing techniques: two consecutive washing; (1) washing with aqueous solution of 5 wt.% HCl (aqueous phase: 30% v/v with respect to the biodiesel phase) followed by water saturated with $CO_2$ (30% v/v of water relative to the biodiesel phase); (2) washing with neutral water (10% v/v relative to biodiesel phase) followed by water saturated with $CO_2$ (30% v/v of water relative to the biodiesel phase). Later, the treated biodiesel obtained after phase separation was allowed for stripping with nitrogen at 80-100$^{o}$C for removing residual water. The results of the study indicated that second method of washing with neutral water followed by water saturated with $CO_2$ was more effective in removing acidity (0.32 mg KOH/g) as the first method with HCl imparted some $H^+$ ions in the mixture increases the acidity value (1.29 mg KOH/g). According to the international standards, the maximum value of acidity in purified biodiesel should be less than 0.5 mgKOH/g. However, both the methods were successful in reducing the soap and glycerine content to 0 from initial values of 11.28 g soap/kg biodiesel and 0.39% glycerine content.

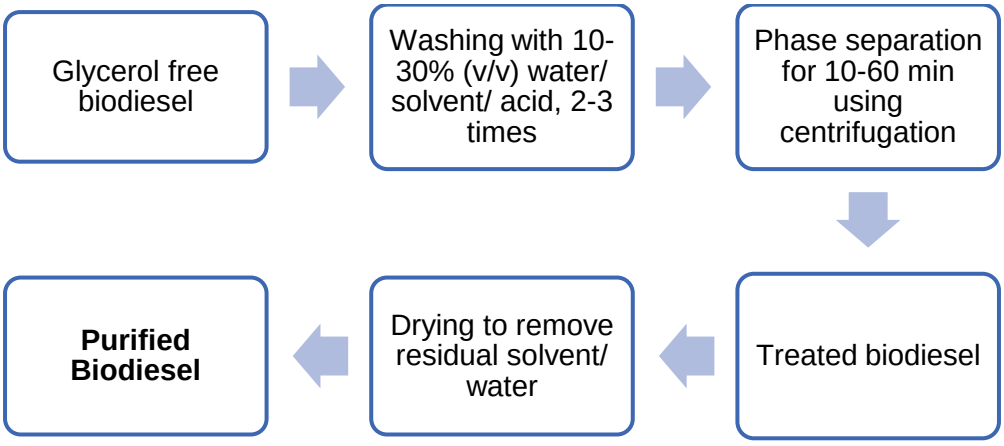



**Figure 2.1. 2 wet-washing technique for biodiesel purification**

Besides, water and acid, organic solvents like glycerol have also been used for biodiesel purification (Berrios et al., 2011). Biodiesel purification was performed in single or multiple steps with different concentrations (5, 10 and 15 wt%). The mixture was vigorously shaken followed by

settling for 10 min and centrifugation step for 10 min to obtain the final product. It was found that 15% wt glycerol was more effective than distilled and tap water in reducing the acid value and water content. Not only with regard to acid value and water content, glycerol was effective in purifying biodiesel as per international quality standards (European Union 14214). It can be concluded that by using water as a solvent in wet-washing technique imparts water content in the biodiesel while glycerol being hydrophilic in nature, it is soluble in water and hence it is able to remove water content as per quality standards.

However, wet-washing techniques have many disadvantages like huge amount of wastewater produced during the process, use of centrifugation for the phase separation making the process more expensive and more energy intensive, 2-3 times washing increasing the operation time, requirement of additional drying step and use of more holding tanks leading to the decreased productivity of the operation. Besides it, using water and acid in wet-washing methods imparts high water content and acidity values in the biodiesel respectively, not meeting the international quality standards of biodiesel.

**Dry washing - Ion-exchange resins:** Dry-washing method does not use solvents in biodiesel purification. Dry-washing can be attained using ion-exchange resins like AMBERLITE®, PUROLITE®, and LEWATIT®. These resins having negatively charged sulphonate groups ($SO_3^{2-}$) are able to bind positively charged impurities like water, glycerol, metal ions, acid ions and soap leading to purified biodiesel. (Banga et al., 2014) has reported a comparative study of purification of Jatropha Curcas based biodiesel using ion-exchange resins like Amberlite® BD10 DRY, Purolite® PD206, and Tulison® T-45BD and the results were compared with wet washing methods. Here, crude biodiesel treatment with Amberlite® with 3% concentration at 65$^{o}$C for 25 min was most effective in removing soap, potassium, and methanol but the treated biodiesel didn't meet ASTM standards for water and acid value. Similar values were obtained after treatment with Purolite® at 3% concentration. Also, the temperature of 65$^{o}$C was more effective than room temperature even at low concentration of resin. This is because the temperature had an impact on the adsorption capacity of the resin, since, at room temperature, the resin is surrounded by water layers leaving no site available for the binding of other impurities while at high temperature, the water is removed from the surface sites of the resins, which can bind with other impurities. Hence, Amberlite® was effective in removing free glycerol and bonded glycerol, potassium ions and residual methanol up to quality standards.

**Adsorbents:** Dry-washing is also accomplished by using adsorbents like silica, Magnesol® which are inorganic in nature and have the high surface area, which can adsorb all sorts of impurities irrespective of their nature. They have excellent mechanical properties, very good solvent stability, and good chemical resistance. Due to these reasons, their application in biodiesel purification is apt. In one of the studies, a single step biodiesel purification has been attained with silica as an adsorbent (Manuale et al., 2014). In the study, 100 $cm^3$ of biodiesel were treated with silica Trisyl 3000 (1g and 3 g) at different temperatures varying between 50°C-90°C for different contact times, 15-100 min. Vacuum condition of 0.2 bar was maintained for the treatment. After the treatment, treated biodiesel was filtered and liquid obtained was analyzed for impurities. It was found that reaction time of 90 min, a reaction temperature of 90°C, adsorbent concentration of 1.1% and a vacuum pressure of 0.2 bar gave maximum adsorption capacity towards methanol, soap, water and other impurities. At high temperatures residual methanol and water were evaporated, reducing their content in purified biodiesel. This technology is advantageous as it is a single step purification method with no pre-treatment to remove excess methanol and glycerol.

### 2.7.3 Recent advancements in biodiesel purification techniques

**Simultaneous production of FAMEs and purification using ion-exchange resins**: In one of the studies, simultaneous production of high-quality biodiesel and glycerine from Jatropha oil using ion-exchange resins as catalysts and adsorbents has been reported (Shibasaki-Kitakawa et al., 2013). The cation-exchange resin, Diaion PK208LH acted as the catalyst for transesterification reaction and the anion-exchange resin, Diaion PA306S were used to adsorb impurities. The temperature of each resin was maintained at 50°C by hot-water circulation through the jacket. The solution mixture of crude oil and methanol was fed to the bottom of the first column and elute coming from the final column was analyzed for reactants, FFA, triglyceride, products and FAME. The reaction was stopped when FAME concentration in the elute from the final column started decreasing due to loss of anion exchange resins catalytic activity. The operating conditions were first optimized with different flow-rates and reaction time. It was found that feed flow-rate of 0.233 $dm^3$/h with time between 4-16 h gave maximum FAME yield as after 16 h unreacted triglycerides started increasing.

The biodiesel purified using anion exchange resin Diaion PA306S met the EN 14214 quality standard values for the impurities and FAME content. The process has many advantages like no requirement of upstream processing for refining the crude biodiesel, simultaneous trans-esterification and purification reducing the process time and increasing the productivity while

glycerine adsorbed by anion-exchange resin can be easily recovered by supplying methanol. No decrease in catalytic activity of cation exchange resin while decrease in catalytic activity of anion exchange resin was observed which can be regenerated by sequential pass of i) methanol to recover glycerine, ii) acetic acid in methanol to displace fatty acid ion from resin, iii) NaOH aqueous solution to displace acetic acid ion, iv) deionized water to remove NaOH solution and v) methanol to restore the resin. The process could be helpful at continuous large scale operation as scale-up of column operation can be easily performed based on FAME productivity per hour per anion-exchange resin's weight ($dm^3$/h/kg-resin weight). However, a large number of solvents required to regenerate the anion-exchange resin is a slight disadvantage of the process.

In one of the studies, solid waste from ceramic industry (chamotte clay) was used as glycerol adsorbent for biodiesel purification by dry washing method (Santos et al., 2017). In the study, a face-centered composite design was used to analyze the combined effect of chamotte concentration (varied between 2-8% w/v) and temperature (varied between 30-50$^o$C) on glycerol removal. Based on graphical optimization models, optimum glycerol concentration (2.4 wt%) and temperature (45$^o$C) were determined. Glycerol removal reached 1282 mg/g of resin within 30 min adsorption time. Biodiesel obtained using biological (immobilized lipase) and chemical catalysts (Niobium oxide impregnated with sodium) was purified using chamotte clay and free glycerol was removed as per ASTM standard (< 0.02% wt). High adsorption capacity can be related to high silica (56 % w/w) and alumina content (36 % w/w) with porous structure and large surface area. But, chamotte clay was unable to be regenerated with organic solvents at 50$^o$C. However, it has benefits like chamotte-glycerol composite can be reused in a brick formulation. Chamotte clay is a low-cost material with good adsorption capacity, which can be a promising adsorbent in biodiesel purification. But non-regeneration of the adsorbent is the main concern for its use in industrial processes. However, studies need to be conducted for its regeneration at high temperatures.

In another study, raw sugarcane bagasse was used for biodiesel purification using dry washing technique (Alves et al., 2016). Raw sugarcane bagasse was first cleaned with distilled water and dried at 80$^o$C for 24 h. Crude biodiesel of 100 mL was treated with adsorbent loading concentration ranged from 0.1-3% (w/v) at 120 rpm and 30$^o$C for 120 min. It was observed that 0.5% w/v sugarcane ash resulted in 40% removal of crude glycerol to bring down the glycerol content in biodiesel to less than 0.02% wt. These results for biodiesel purification were comparable to that of Magnesol®. However, sugarcane bagasse ash (obtained by heating raw sugarcane bagasse at 700$^o$C for 4 h) performed poorly as compared to raw sugarcane bagasse

due to its low content of the cellulosic material. From the adsorption kinetics, it was concluded that with the addition of 3 wt% sugarcane bagasse, the necessary glycerol removal was achieved after only 10 min of the adsorption process. The process has many advantages like low-cost adsorbent with lower process time as compared to wet-washing technique. However, there are disadvantages of the process, such as, it was unable to remove water as per ASTM standard and regeneration studies have not been performed on the adsorbent.

**Use of membrane technology:** In one of the studies, solvent-resistant polymeric membranes were synthesized and used for biodiesel purification (Torres et al., 2017). The synthesized nanofiltration membranes were composed of poly (vinylidene fluoride) (PVDF) as a support and poly(dimethylsiloxane) as a coating material. The membranes were prepared by phase inversion process. MWCO (Molecular weight cut-off) of the membrane was evaluated by passing organic solutes (300-1000 g/gmol) with ethanol that was based on rejection coefficients. The membrane showed high stability during adverse conditions like pH 12 and 60$^{o}$C temperature. Rejection coefficient of impurities was calculated based on following formulae, % R = (1 – Cp/Cr)*100 where Cp = concentration of impurity in permeate and Cr = concentration of impurity in the retentate. The experiments conducted at 60°C, pH 12 & 15 bar pressure revealed that rejection coefficients of 70% glycerol, 69% glycerides were obtained with a permeate flux of 7.4 M/m$^2$.h. High membrane stability was displayed with flux recovery ratio of 0.94-0.95 even after 20 cycles of use. Moreover, the presence of alcohol in biodiesel had little effect on rejection coefficients. Membrane separation in biodiesel can be economical as they have lower capital and operating costs. However, high membrane purchase cost is a disadvantage of membrane technology at industrial scale and the presence of high amount of soap in the crude biodiesel can lead to concentration polarization and membrane fouling.

**Fiber-based bio sorbents:** Yang et al. (2017) reported biodiesel purification using fiber-based dry-washing technique; BD-Zorb, sawdust, and wood shavings. Biodiesel purification was conducted in 3 cylindrical separator funnels (125 mL) filled with 18 g of adsorbent while biodiesel was allowed to pass with a flow-rate of 100 mL/h. The results revealed that BD-Zorb exhibited the best performance for soap removal capacity from the crude camelina biodiesel. The soap removal capacity of BD-Zorb, sawdust and wood shavings were 51.1 mg/L, 24.4 mg/L, and 9.5 mg/L. However, acid content (for BD-Zorb) and water content of biodiesel purified from three bio sorbents did not meet biodiesel quality standards indicating that the additional steps are required to decrease the acid and water content of biodiesel purified by fiber-based dry washing. A lower purification capacity of sawdust and wood shavings implied more frequent replacements of

adsorbents, leading to increased labor costs. Table 4 represents various advantages and disadvantages of different biodiesel purification techniques.

## 2.8 Current challenges and future prospects

There are many technical challenges that must be resolved for profitable biodiesel production. A major challenge is to reduce the high feedstock cost by using low-cost feedstock, including waste cooking oil (WCO), algal oil, and animal fats, etc. However, these feedstocks also contained high amounts of free fatty acids (FFAs) and water that may lead to saponification, thereby, require further pretreatment and purification steps. These problems must be addressed in order to produce sustainable biodiesel.

### 2.8.1 Vegetable oil and Non-food crops as feedstock for biodiesel

Microalgae have been proved to solve most of the energy-crop associated problems. But main drawback using microalgae is that their growth rate is very slow. Therefore, use of heterotrophic microorganisms (Yeast and fungi) is alternative approach due to their fast growth and less rigid cell wall than microalgae. However, the extraction technologies require major advancements for the sustainable commercial production.

### 2.8.2 Biomass harvesting

Several harvesting technologies (Flocculation, Auto-flocculation, magnetic separation etc.,) are developed to separate microalgae. But there are not many studies in the literature on techniques for harvesting of fast growing heterotrophic microorganisms such as yeast, fungi, and bacteria.

### 2.8.3 Effects of moisture and FFA

Presence of FFA and moisture content (contaminants) in the feedstock can badly affect transesterification process. Due to these types of contaminants in the feedstock, acid catalysts are used for transesterification reaction. However, there are several problems using an acid catalyst such as a) it will increase water content in the biodiesel b) reaction temperature higher than 80$^{o}$C is required c) Lipids to biodiesel conversion efficiency will be low and d) it will corrode reactor tank. Therefore, further research is need to be conducted to remove water and free fatty acids from feedstock using an economical process.

### 2.8.4 Supercritical alcohol process

Supercritical alcohol process takes only 4 to 10 min of residence time to generate biodiesel due to efficient mixing (Deshpande et al., 2017). However, there are some limitations with this process owing to the requirement of high temperature and pressure. The major limitation is the scale-up of the process to the commercial level adding extra cost for high energy requirement and high alcohol: oil (42:1) molar ratio. Researchers are trying to employ co-solvents, including $CO_2$, CaO, and hexane, in order to control the operating conditions, thereby, increasing product yield (Duarte et al., 2017). This became possible due to increase in homogeneity of reactants with the help of co-solvent. Supercritical $CO_2$ is another eco-friendly co-solvent that can be obtained at affordable cost. It can be safely recovered from the reaction via depressurization. This supercritical process combined with co-solvents helps in an increase in product yield, reduction in process time and a significant decrease in overall production cost. However, detailed systematic research is required in this field.

### 2.8.5 Use of co-solvents

Co-solvents, e.g. MTBE (methyl tertbutyl ether), THF (tetrahydrofuran), increase the rate of reaction and also overcome the mass transfer limitations. They help in the production of high-quality FAMEs under moderate conditions, i.e., 30 °C for 10 min. But there is a need for large “leak proof” reaction vessels. Also, the co-solvents must be completely removed from the product.

**Table 2.1 4 Advantages and disadvantages of various biodiesel purification techniques**

| | Advantages | Disadvantages |
|---|---|---|
| **Wet-washing** | • Excellent in removing soap, methanol and free glycerol | • Not effective in reducing water content - Centrifugation step required for phase separation<br>• Large amount of wastewater discharge - Additional drying step to remove water present in the crude biodiesel.<br>• Not applicable in continuous operation |
| **Dry-washing using resins** | • Excellent in removing soap, methanol and free glycerol.<br>• Lower capital investments.<br>• Less energy intensive.<br>• No wastewater production.<br>• Applicable in continuous operation.<br>• No drying step required.<br>• Methanol used during regeneration can further be re-used during trans-esterification reaction | • Most of the resins are shipped with $H^+$ ions, impart acidity and water in the treated biodiesel - Chemical composition of the resin, sometimes is difficult to predict<br>• No reported studies on performance of resin after regeneration |
| **Dry-washing using commercial adsorbents** | • High FAME yield, adsorbs residual water.<br>• Less energy intensive.<br>• No wastewater production.<br>• No drying step required.<br>• Faster than wet-washing technique | • Adsorbents are usually non-recyclable, frequent replacements of adsorbent leads to increased labour costs |

| | | |
|---|---|---|
| **Dry-washing using industrial/ agricultural wastes** | • No wastewater discharge.<br>• Environmental benefits - Used waste can act as soil corrective/ brick formulation | • Regeneration studies not reported<br>• Wastes are unable to remove all the impurities (water and acidity) from the crude biodiesel; needs further treatment increasing the costs |
| **Membrane technology** | • High fuel quality and excellent performance.<br>• Lower capital and operating cost.<br>• Performance comparable to wet-washing technique | • High soap content can foul the membranes, leading to frequent replacements of membranes which are expensive |

### 2.8.6 Biodiesel/glycerol separation and FAME quality

Separation of FAMEs and glycerol is a necessary step due to their soluble nature. This is usually done by phase separation. However, an excess of unreacted methanol in the reaction increases the solubility of ester in glycerol and vice versa, thereby, increasing the post-production cost. Also, all the trace elements must be removed from triglycerides because the emulsion layer formed by these trace elements interfere with the separation of glycerol and makes the product expensive. In case of continuous biodiesel production process after transesterification, phase separation between biodiesel and glycerol will be time-consuming process. Therefore, further research needs to be conducted to separate crude glycerol from biodiesel with less time and low cost.

### 2.8.7 Biodiesel purification

Regeneration studies for the use of adsorbents and resins in dry washing biodiesel purification technique were missing in the literature. Regeneration studies should be conducted with different solvents and temperature to avoid frequent replacement of resins and adsorbents at large scale. Most of the studies reported for use of industrial/ agricultural wastes as adsorbents in biodiesel purification were conducted at lab-scale. Pilot scale studies should be conducted for their industrial feasibility along with techno-economic evaluation. Studies reporting membrane technology for biodiesel purification were conducted at lab-scale. Membranes which are resistant to soap and organic solvents should be looked upon to prevent fouling of membrane.

## 3. SUMMARY

Biomass harvesting is one of the major task for biodiesel production using oleaginous biomass. Micro-algal biomass harvesting using flocculation has been rigorously studied during last few years but still, there is no harvesting method available except centrifugation that can be applied to oleaginous yeast, fungi, and bacteria. Various physical and chemical technologies have been developed for lipid extraction. Each of the methods has its advantage and disadvantage. Physical method is clean but high energy consuming, while chemical extraction has the high possibility of contamination of the lipid due to the presence of the residual solvents when toxic organic compounds are used as solvents. However, lipid extraction from wet microbial biomass

faces several challenges such as very limited lipid accessibility, reduced mass transfer, and formation of stable emulsions. To eliminate these problems, surfactant assisted cell wall disruption is a novel approach to extract total lipid from the wet biomass under lab scale. The process needs to be scale up followed by techno-economic analysis to ascertain the overall cost of the extraction process and to guide on the improvements required at a large scale.

Numerous green ecofriendly extraction methods were investigated, which are highly efficient in lipid recovery. However, all the extraction procedure investigated so far either employing a green extraction process or utilized organic solvents; demonstrated the dependency of extraction process on lipid composition, types of lipid fraction (neutral or polar lipid), and their interaction with membrane protein. Further, an ideal extraction process should not only efficiently recover oil but also reduce the contamination, increase mass transfer and simplify the downstream processing. Therefore, further research has to be carried out regarding the scalability, extraction efficiency, and energy consumption and downstream processing. The techno-economic analysis of the whole extraction process is also required, which provides guidance for improvement and modification of the process.

Biodiesel purification has been achieved by wet-washing methods using distilled water, glycerol and acid in the reported studies. Both wet and dry washing are applicable where biodiesel is produced from oil while wet washing is more convenient when biodiesel is produced through in-situ transesterification using wet-biomass. Wet-washing using glycerol is more effective than using water and acid as they impart high water content and acid values, respectively in the treated biodiesel. Although they are excellent in removing soap, methanol and free glycerol, wet-washing method has several disadvantages: large amount of polluting wastewater produced during the operation, additional drying step for removal of water, requirement of centrifugation for phase separation after the process, requirement of holding tanks making their application in large scale continuous operation unfeasible. Membrane technology can be a good option for biodiesel purification as it produces zero-water discharge, is less energy intensive and more-ecofriendly. But there are concerns about membrane fouling due to high soap content in crude biodiesel, moreover, high cost of membrane is also a concern for their commercialization at large scale.

## 4. CONCLUSION

Currently, biodiesel industries are facing many challenges and competition among energy sources, advancement and acceptability of technologies. Lipid production using heterotrophic microorganisms is a substantial approach for microbial lipid production and conversion to biodiesel. But the process is under lab scale study due to high energy was required to harvest biomass, cell wall disruption and lipid recovery from wet biomass under biodiesel downstream process. Moreover, use of organic solvents for lipid separation can affect the cost and are industrially unsafe. Therefore, low cost industrial viable environment friendly solvents are necessary to be investigated for microbial lipid separation and conversion to biodiesel. Hence, a considerable effort of research is needed to obtain industrial acceptable biodiesel production process.

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# REFERENCES


Adam, F., Abert-Vian, M., Peltier, G., Chemat, F. 2012. “Solvent-free” ultrasound-assisted extraction of lipids from fresh microalgae cells: A green, clean and scalable process. *Bioresour. Technol.*, **114**(0), 457-465.

Alves, M.J., Cavalcanti, Í.V., de Resende, M.M., Cardoso, V.L., Reis, M.H. 2016. Biodiesel dry purification with sugarcane bagasse. *Industrial Crops and Products*, **89**, 119-127.

Amanor-Boadu, V., Pfromm, P.H., Nelson, R. 2014. Economic feasibility of algal biodiesel under alternative public policies. *Renewable Energy*, **67**(0), 136-142.

Angerbauer, C., Siebenhofer, M., Mittelbach, M., Guebitz, G. 2008. Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production. *Bioresource technology*, **99**(8), 3051-3056.

Backhaus, K., Rippert, D., Heilmann, C.J., Sorgo, A.G., De Koster, C.G., Klis, F.M., Rodicio, R., Heinisch, J.J. 2013. Mutations in SNF1 complex genes affect yeast cell wall strength. *European journal of cell biology*, **92**(12), 383-395.

Banga, S., Varshney, P., Kumar, N., Pal, M. 2014. Optimization of parameters for purification of jatropha curcas based biodiesel using organic adsorbents. *International Journal of Renewable Energy Research (IJRER)*, **4**(3), 598-603.

Barros, A.I., Gonçalves, A.L., Simões, M., Pires, J.C. 2015. Harvesting techniques applied to microalgae: a review. *Renewable and Sustainable Energy Reviews*, **41**, 1489-1500.

Bellou, S., Baeshen, M.N., Elazzazy, A.M., Aggeli, D., Sayegh, F., Aggelis, G. 2014. Microalgal lipids biochemistry and biotechnological perspectives. *Biotechnology Advances*, **32**(8), 1476-1493.

Benemann, J.R., Woertz, I.C., Lundquist, T.J. 2011. A techno-economic analysis of open pond microalgae biofiels production. in: *1st International Conference on Alage Biomass, Biofuels, and Bioproducts*. Wsetin St Louis, St Louis, USA.

Berrios, M., Martín, M., Chica, A., Martín, A. 2011. Purification of biodiesel from used cooking oils. *Applied energy*, **88**(11), 3625-3631.

Bligh, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*, **37**(8), 911-917.

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

Cao, H., Zhang, Z., Wu, X., Miao, X. 2013. Direct biodiesel production from wet microalgae biomass of Chlorella pyrenoidosa through in situ transesterification. *BioMed research international*, **2013**.

Carvalho, A.K.F., da Conceição, L.R.V., Silva, J.P.V., Perez, V.H., de Castro, H.F. 2017. Biodiesel production from Mucor circinelloides using ethanol and heteropolyacid in one and two-step transesterification. *Fuel*, **202**, 503-511.

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.

Channi, H.K., Gupta, S., Singh, H., Gill, S.S. 2016. Different biodiesel production technologies: A review. *International Journal of Research in Engineering and Applied Sciences*, **6**(9), 123-128.

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. *Bioresource technology*, **142**, 329-337.

Cooney, M.J., Benjamin, K. 2016. Ionic Liquids in Lipid Extraction and Recovery. in: *Ionic Liquids in Lipid Processing and Analysis*, Elsevier, pp. 279-316.

Coustets, M., Al-Karablieh, N., Thomsen, C., Teissié, J. 2013. Flow process for electroextraction of total proteins from microalgae. *The Journal of membrane biology*, **246**(10), 751-760.

Coustets, M., Joubert-Durigneux, V., Hérault, J., Schoefs, B., Blanckaert, V., Garnier, J.-P., Teissié, J. 2015. Optimization of protein electroextraction from microalgae by a flow process. *Bioelectrochemistry*, **103**, 74-81.

Dassey, A.J., Theegala, C.S. 2013. Harvesting economics and strategies using centrifugation for cost effective separation of microalgae cells for biodiesel applications. *Bioresour Technol*, **128**, 241-5.

Davis, R., Aden, A., Pienkos, P.T. 2011. Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy*, **88**(10), 3524-3531.

Degirmenbasi, N., Coskun, S., Boz, N., Kalyon, D.M. 2015. Biodiesel synthesis from canola oil via heterogeneous catalysis using functionalized CaO nanoparticles. *Fuel*, **153**, 620-627.

Delrue, F., Setier, P.A., Sahut, C., Cournac, L., Roubaud, A., Peltier, G., Froment, A.K. 2012. An economic, sustainability, and energetic model of biodiesel production from microalgae. *Bioresource Technology*, **111**(0), 191-200.

Deshpande, S.R., Sunol, A.K., Philippidis, G. 2017. Status and prospects of supercritical alcohol transesterification for biodiesel production. *Wiley Interdisciplinary Reviews: Energy and Environment*, **6**(5), e252-n/a.

Dickinson, S., Mientus, M., Frey, D., Amini-Hajibashi, A., Ozturk, S., Shaikh, F., Sengupta, D., El-Halwagi, M.M. 2017. A review of biodiesel production from microalgae. *Clean Technologies and Environmental Policy*, **19**(3), 637-668.

Dong, T., Knoshaug, E.P., Pienkos, P.T., Laurens, L.M. 2016. Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review. *Applied Energy*, **177**, 879-895.

Doucha, J., Lívanský, K. 2008. Influence of processing parameters on disintegration of Chlorella cells in various types of homogenizers. *Applied microbiology and biotechnology*, **81**(3), 431.

Duarte, S.H., Santos, P.d., Michelon, M., Oliveira, S.M.d.P., Martínez, J., Maugeri, F. 2017. Recovery of yeast lipids using different cell disruption techniques and supercritical CO2 extraction. *Biochemical Engineering Journal*, **125**, 230-237.

Ehimen, E.A., Sun, Z.F., Carrington, C.G. 2010. Variables affecting the in situ transesterification of microalgae lipids. *Fuel*, **89**(3), 677-684.

Eing, C., Goettel, M., Straessner, R., Gusbeth, C., Frey, W. 2013. Pulsed electric field treatment of microalgae—benefits for microalgae biomass processing. *IEEE Transactions on Plasma Science*, **41**(10), 2901-2907.

Flisar, K., Meglic, S.H., Morelj, J., Golob, J., Miklavcic, D. 2014. Testing a prototype pulse generator for a continuous flow system and its use for E. coli inactivation and microalgae lipid extraction. *Bioelectrochemistry*, **100**, 44-51.

Folch, J., Lees, M., Sloane-Stanley, G. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J biol Chem*, **226**(1), 497-509.

Gong, Z., Wang, Q., Shen, H., Wang, L., Xie, H., Zhao, Z.K. 2014. Conversion of biomass-derived oligosaccharides into lipids. *Biotechnology for biofuels*, **7**(1), 13.

Grima, E.M., González, M.J.I., Giménez, A.G. 2013. Solvent Extraction for Microalgae Lipids. in: *Algae for Biofuels and Energy*, (Eds.) M.A. Borowitzka, N.R. Moheimani, Springer Netherlands. Dordrecht, pp. 187-205.

Günerken, E., d'Hondt, E., Eppink, M., Garcia-Gonzalez, L., Elst, K., Wijffels, R. 2015. Cell disruption for microalgae biorefineries. *Biotechnology advances*, **33**(2), 243-260.

Halim, R., Danquah, M.K., Webley, P.A. 2012. Extraction of oil from microalgae for biodiesel production: a review. *Biotechnology advances*, **30**(3), 709-732.

Hidalgo, P., Toro, C., Navia, R. 2013. Advances in direct transesterification of microalgal biomass for biodiesel production. *Reviews in Environmental Science and Bio/Technology*, **12**(2), 179-199.

Huang, C., Chen, X.-F., Yang, X.-Y., Xiong, L., Lin, X.-Q., Yang, J., Wang, B., Chen, X.-D. 2014. Bioconversion of corncob acid hydrolysate into microbial oil by the oleaginous yeast Lipomyces starkeyi. *Applied biochemistry and biotechnology*, **172**(4), 2197-2204.

Huang, R., Cheng, J., Qiu, Y., Li, T., Zhou, J., Cen, K. 2015. Using renewable ethanol and isopropanol for lipid transesterification in wet microalgae cells to produce biodiesel with low crystallization temperature. *Energy Conversion and Management*, **105**, 791-797.

Im, H., Kim, B., Lee, J.W. 2015. Concurrent production of biodiesel and chemicals through wet in situ transesterification of microalgae. *Bioresource technology*, **193**, 386-392.

Jackson, B.A., Bahri, P.A., Moheimani, N.R. 2017. Repetitive non-destructive milking of hydrocarbons from Botryococcus braunii. *Renewable and Sustainable Energy Reviews*, **79**, 1229-1240.

Jazzar, S., Quesada-Medina, J., Olivares-Carrillo, P., Marzouki, M.N., Acién-Fernández, F.G., Fernández-Sevilla, J.M., Molina-Grima, E., Smaali, I. 2015. A whole biodiesel conversion process combining isolation, cultivation and in situ supercritical methanol transesterification of native microalgae. *Bioresource Technology*, **190**, 281-288.

Jeevan Kumar, S.P., Vijay Kumar, G., Dash, A., Scholz, P., Banerjee, R. 2017. Sustainable green solvents and techniques for lipid extraction from microalgae: A review. *Algal Research*, **21**, 138-147.

Jose, S., Archanaa, S. 2017. Environmental and Economic Sustainability of Algal Lipid Extractions: An Essential Approach for the Commercialization of Algal Biofuels. in: *Algal Biofuels*, Springer, pp. 281-313.

Kim, B., Im, H., Lee, J.W. 2015. In situ transesterification of highly wet microalgae using hydrochloric acid. *Bioresource technology*, **185**, 421-425.

Kim, B., Park, J., Son, J., Lee, J.W. 2017. Catalyst-free production of alkyl esters from microalgae via combined wet in situ transesterification and hydrothermal liquefaction (iTHL). *Bioresource Technology*, **244**(Part 1), 423-432.

Klimek-Ochab, M., Brzezińska-Rodak, M., Żymańczyk-Duda, E., Lejczak, B., Kafarski, P. 2011. Comparative study of fungal cell disruption—scope and limitations of the methods. *Folia microbiologica*, **56**(5), 469.

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**, 566-577.

Kurniawati, H.A., Ismadji, S., Liu, J.C. 2014. Microalgae harvesting by flotation using natural saponin and chitosan. *Bioresource Technology*, **166**, 429-434.

Lai, Y.S., De Francesco, F., Aguinaga, A., Parameswaran, P., Rittmann, B.E. 2016. Improving lipid recovery from Scenedesmus wet biomass by surfactant-assisted disruption. *Green Chemistry*, **18**(5), 1319-1326.

Lee, A.F., Wilson, K. 2015. Recent developments in heterogeneous catalysis for the sustainable production of biodiesel. *Catalysis Today*, **242**, 3-18.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., Oh, H.-M. 2010. Comparison of several methods for effective lipid extraction from microalgae. *Bioresource Technology*, **101**(1, Supplement), S75-S77.

Levering, J., Broddrick, J., Zengler, K. 2015. Engineering of oleaginous organisms for lipid production. *Current opinion in biotechnology*, **36**, 32-39.

Li, P., Miao, X., Li, R., Zhong, J. 2011. In situ biodiesel production from fast-growing and high oil content Chlorella pyrenoidosa in rice straw hydrolysate. *BioMed Research International*, **2011**.

Liang, M.-H., Jiang, J.-G. 2013. Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. *Progress in lipid research*, **52**(4), 395-408.

Liang, Y., Tang, T., Siddaramu, T., Choudhary, R., Umagiliyage, A.L. 2012. Lipid production from sweet sorghum bagasse through yeast fermentation. *Renewable Energy*, **40**(1), 130-136.

Liu, B., Zhao, Z. 2007. Biodiesel production by direct methanolysis of oleaginous microbial biomass. *Journal of Chemical Technology & Biotechnology*, **82**(8), 775-780.

Liu, X., Fallon, S., Sheng, J., Curtiss, R. 2011. CO2-limitation-inducible Green Recovery of fatty acids from cyanobacterial biomass. *Proceedings of the National Academy of Sciences*, **108**(17), 6905-6908.

Ma, G., Hu, W., Pei, H., Jiang, L., Song, M., Mu, R. 2015. In situ heterogeneous transesterification of microalgae using combined ultrasound and microwave irradiation. *Energy Conversion and Management*, **90**(Supplement C), 41-46.

Manuale, D.L., Greco, E., Clementz, A., Torres, G.C., Vera, C.R., Yori, J.C. 2014. Biodiesel purification in one single stage using silica as adsorbent. *Chemical Engineering Journal*, **256**, 372-379.

Mendow, G., Veizaga, N., Sánchez, B., Querini, C. 2012. Biodiesel production by two-stage transesterification with ethanol by washing with neutral water and water saturated with carbon dioxide. *Bioresource technology*, **118**, 598-602.

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

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. *Bioresource Technology*, **185**, 49-55.

Nagle, N., Lemke, P. 1990. Production of methyl ester fuel from microalgae. *Applied Biochemistry and Biotechnology*, **24**(1), 355-361.

Navarro López, E., Robles Medina, A., Esteban Cerdán, L., González Moreno, P.A., Macías Sánchez, M.D., Molina Grima, E. 2016. Fatty acid methyl ester production from wet microalgal biomass by lipase-catalyzed direct transesterification. *Biomass and Bioenergy*, **93**(Supplement C), 6-12.

Park, J.-Y., Park, M.S., Lee, Y.-C., Yang, J.-W. 2015. Advances in direct transesterification of algal oils from wet biomass. *Bioresource technology*, **184**, 267-275.

Patel, A., Sindhu, D.K., Arora, N., Singh, R.P., Pruthi, V., Pruthi, P.A. 2015. Biodiesel production from non-edible lignocellulosic biomass of Cassia fistula L. fruit pulp using oleaginous yeast Rhodosporidium kratochvilovae HIMPA1. *Bioresource Technology*, **197**, 91-98.

Patil, P.D., Reddy, H., Muppaneni, T., Schaub, T., Holguin, F.O., Cooke, P., Lammers, P., Nirmalakhandan, N., Li, Y., Lu, X. 2013. In situ ethyl ester production from wet algal biomass under microwave-mediated supercritical ethanol conditions. *Bioresource technology*, **139**, 308-315.

Rakesh, S., Dhar, D.W., Prasanna, R., Saxena, A.K., Saha, S., Shukla, M., Sharma, K. 2015. Cell disruption methods for improving lipid extraction efficiency in unicellular microalgae. *Engineering in Life Sciences*, **15**(4), 443-447.

Ramos Tercero, E.A., Domenicali, G., Bertucco, A. 2014. Autotrophic production of biodiesel from microalgae: An updated process and economic analysis. *Energy*.

Ranjan, A., Patil, C., Moholkar, V.S. 2010. Mechanistic assessment of microalgal lipid extraction. *Industrial & Engineering Chemistry Research*, **49**(6), 2979-2985.

Reep, P., Green, M.P. 2012. Procedure for extracting of lipids from algae without cell sacrifice, Google Patents.

Salam, K.A., Velasquez-Orta, S.B., Harvey, A.P. 2016. Surfactant-assisted direct biodiesel production from wet Nannochloropsis occulata by in situ transesterification/reactive extraction. *Biofuel Research Journal*, **3**(1), 366-371.

Santander, C., Robles, P.A., Cisternas, L.A., Rivas, M. 2014. Technical–economic feasibility study of the installation of biodiesel from microalgae crops in the Atacama Desert of Chile. *Fuel Processing Technology*, **125**(0), 267-276.

Santos, F.D., da Conceição, L.R.V., Ceron, A., de Castro, H.F. 2017. Chamotte clay as potential low cost adsorbent to be used in the palm kernel biodiesel purification. *Applied Clay Science*, **149**, 41-50.

Sara, M., Brar, S.K., Blais, J.F. 2016. Comparative study between microwave and ultrasonication aided in situ transesterification of microbial lipids. *RSC Advances*, **6**(61), 56009-56017.

Sathish, A., Smith, B.R., Sims, R.C. 2014. Effect of moisture on in situ transesterification of microalgae for biodiesel production. *Journal of Chemical Technology and Biotechnology*, **89**(1), 137-142.

Senanayake, S., Fichtali, J. 2006. Single-cell oils as sources of nutraceutical and specialty lipids: processing technologies and applications. *NUTRACEUTICAL SCIENCE AND TECHNOLOGY*, **5**, 251.

Seo, Y.H., Sung, M., Oh, Y.-K., Han, J.-I. 2016. Lipid extraction from microalgae cell using persulfate-based oxidation. *Bioresource technology*, **200**, 1073-1075.

Shafiee, S., Topal, E. 2009. When will fossil fuel reserves be diminished? *Energy policy*, **37**(1), 181-189.

Shang, S., Cai, D., Han, J., Qin, P., Tan, T. 2015. An organic solvents free bio-lipids extraction process using non-woven fabric from pretreated fermentation broth. *Chemical Engineering Journal*, **270**, 223-228.

Sheng, J., Vannela, R., Rittmann, B.E. 2011. Evaluation of cell-disruption effects of pulsed-electric-field treatment of Synechocystis PCC 6803. *Environmental science & technology*, **45**(8), 3795-3802.

Shibasaki-Kitakawa, N., Kanagawa, K., Nakashima, K., Yonemoto, T. 2013. Simultaneous production of high quality biodiesel and glycerin from Jatropha oil using ion-exchange resins as catalysts and adsorbent. *Bioresource technology*, **142**, 732-736.

Shirazi, M.M.A., Kargari, A., Tabatabaei, M., Mostafaeid, B., Akia, M., Barkhi, M., Shirazi, M.J.A. 2013. Acceleration of biodiesel–glycerol decantation through NaCl-assisted gravitational settling: a strategy to economize biodiesel production. *Bioresource technology*, **134**, 401-406.

Sitepu, I.R., Garay, L.A., Sestric, R., Levin, D., Block, D.E., German, J.B., Boundy-Mills, K.L. 2014. Oleaginous yeasts for biodiesel: Current and future trends in biology and production. *Biotechnology Advances*, **32**(7), 1336-1360.

Suganya, T., Kasirajan, R., Renganathan, S. 2014. Ultrasound-enhanced rapid in situ transesterification of marine macroalgae Enteromorpha compressa for biodiesel production. *Bioresource technology*, **156**, 283-290.

Taher, H., Al-Zuhair, S., Al-Marzouqi, A.H., Haik, Y., Farid, M. 2014. Effective extraction of microalgae lipids from wet biomass for biodiesel production. *biomass and bioenergy*, **66**, 159-167.

Tanzi, C.D., Vian, M.A., Chemat, F. 2013. New procedure for extraction of algal lipids from wet biomass: A green clean and scalable process. *Bioresource technology*, **134**, 271-275.

Thliveros, P., Kiran, E.U., Webb, C. 2014. Microbial biodiesel production by direct methanolysis of oleaginous biomass. *Bioresource technology*, **157**, 181-187.

Torres, J.J., Toledo Arana, J., Ochoa, N.A., Marchese, J., Pagliero, C. 2017. Biodiesel purification using polymeric nanofiltration composite membranes highly resistant to hard conditions. *Chemical Engineering & Technology*.

Tran, D.-T., Chen, C.-L., Chang, J.-S. 2013. Effect of solvents and oil content on direct transesterification of wet oil-bearing microalgal biomass of Chlorella vulgaris ESP-31 for biodiesel synthesis using immobilized lipase as the biocatalyst. *Bioresource Technology*, **135**(Supplement C), 213-221.

Vicente, G., Bautista, L.F., Rodríguez, R., Gutiérrez, F.J., Sádaba, I., Ruiz-Vázquez, R.M., Torres-Martínez, S., Garre, V. 2009. Biodiesel production from biomass of an oleaginous fungus. *Biochemical Engineering Journal*, **48**(1), 22-27.

Vonortas, A., Papayannakos, N. 2014. Comparative analysis of biodiesel versus green diesel. *Wiley Interdisciplinary Reviews: Energy and Environment*, **3**(1), 3-23.

Wang, Q., Guo, F.-J., Rong, Y.-J., Chi, Z.-M. 2012. Lipid production from hydrolysate of cassava starch by Rhodosporidium toruloides 21167 for biodiesel making. *Renewable Energy*, **46**, 164-168.

Ward, V., Munch, G., Cicek, N., Rehmann, L. 2017. Direct conversion of the oleaginous yeast Rhodosporidium diobovatum to biodiesel using the ionic liquid [C2mim][EtSO4]. *ACS Sustainable Chemistry & Engineering*.

Wu, X., Zhu, F., Qi, J., Zhao, L., Yan, F., Li, C. 2017. Challenge of biodiesel production from sewage sludge catalyzed by KOH, KOH/activated carbon, and KOH/CaO. *Frontiers of Environmental Science & Engineering*, **11**(2), 3.

Xiaowei, P., Hongzhang, C. 2012. Hemicellulose sugar recovery from steam-exploded wheat straw for microbial oil production. *Process biochemistry*, **47**(2), 209-215.

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.

Yang, J., He, Q.S., Niu, H., Corscadden, K., Caldwell, C. 2017. A Comparative Study on the Performance of Fiber-Based Biosorbents in the Purification of Biodiesel Derived from Camelina sativa. *Journal of Technology Innovations in Renewable Energy*, **5**(4), 123-132.

Yang, Y., Hou, J., Wang, P., Wang, C., Miao, L., Ao, Y., Xu, Y., Wang, X., Lv, B., You, G., Yang, Z. 2018. Interpretation of the disparity in harvesting efficiency of different types of Microcystis aeruginosa using polyethylenimine (PEI)-coated magnetic nanoparticles. *Algal Research*, **29**, 257-265.

Yellapu, S.K., Bezawada, J., Kaur, R., Kuttiraja, M., Tyagi, R.D. 2016. Detergent assisted lipid extraction from wet yeast biomass for biodiesel: A response surface methodology approach. *Bioresource technology*, **218**, 667-673.

Yoo, G., Yoo, Y., Kwon, J.-H., Darpito, C., Mishra, S.K., Pak, K., Park, M.S., Im, S.G., Yang, J.-W. 2014. An effective, cost-efficient extraction method of biomass from wet microalgae with a functional polymeric membrane. *Green Chemistry*, **16**(1), 312-319.

Yousuf, A., Khan, M.R., Islam, M.A., Ab Wahid, Z., Pirozzi, D. 2017. Technical difficulties and solutions of direct transesterification process of microbial oil for biodiesel synthesis. *Biotechnology letters*, **39**(1), 13-23.

Yu, X., Dong, T., Zheng, Y., Miao, C., Chen, S. 2015. Investigations on cell disruption of oleaginous microorganisms: Hydrochloric acid digestion is an effective method for lipid extraction. *European Journal of Lipid Science and Technology*, **117**(5), 730-737.

Zbancioc, G., Zbancioc, A.M., Mangalagiu, I.I. 2014. Ultrasound and microwave assisted synthesis of dihydroxyacetophenone derivatives with or without 1, 2-diazine skeleton. *Ultrasonics sonochemistry*, **21**(2), 802-811.

Zbinden, M.D.A., Sturm, B.S., Nord, R.D., Carey, W.J., Moore, D., Shinogle, H., Stagg-Williams, S.M. 2013. Pulsed electric field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae. *Biotechnology and bioengineering*, **110**(6), 1605-1615.

Zeng, G., Fu, H., Zhong, H., Yuan, X., Fu, M., Wang, W., Huang, G. 2007. Co-degradation with glucose of four surfactants, CTAB, Triton X-100, SDS and Rhamnolipid, in liquid culture media and compost matrix. *Biodegradation*, **18**(3), 303-310.

Zhang, X., Yan, S., Tyagi, R.D., Drogui, P., Surampalli, R.Y. 2016. Ultrasonication aided biodiesel production from one-step and two-step transesterification of sludge derived lipid. *Energy*, **94**(Supplement C), 401-408.

Zhang, X., Yan, S., Tyagi, R.D., Drogui, P., Surampalli, R.Y. 2014. Ultrasonication assisted lipid extraction from oleaginous microorganisms. *Bioresource technology*, **158**, 253-261.

Zhao, F., Chu, H., Zhang, Y., Jiang, S., Yu, Z., Zhou, X., Zhao, J. 2017. Increasing the vibration frequency to mitigate reversible and irreversible membrane fouling using an axial vibration membrane in microalgae harvesting. *Journal of Membrane Science*, **529**, 215-223.

# CHAPITRE II

# PARTIE 2

# OLEAGINOUS YEAST BIOMASS FLOCCULATION USING BIOFLOCCULANT PRODUCED IN WASTEWATER SLUDGE AND TRANSESTERIFICATION USING PETROLEUM DIESEL AS A CO-SOLVENT

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# RÈSUMÈ

Dans cette étude, un nouveau procédé de récolte de biomasse de levure oléagineuse (*Yarrowia lipolytica*) a été développé. Ce procédé consiste en une floculation, une perturbation de la paroi cellulaire et d'une extraction lipidique à l'aide du gasoil de pétrole comme solvant. Les floculants : chlorure d'aluminium, chlorure de calcium ainsi que la substance polymère extracellulaire (EPS), ont été évalués en termes de décantation de biomasse riche en lipides. La floculation de la biomasse était de 74,3% en présence de chlorure de calcium (36 mM) en combinaison avec EPS (5,85 mg EPS / g biomasse) et de 79% en présence d'alun (1,2 mM) en combinaison avec EPS (18 mg EPS / g biomasse). La vitesse de sédimentation de la biomasse était de 2,93 et 1,46 mm / s, respectivement. La biomasse déposée (166 g / L) a été traitée avec du N-lauroyl sarcosine (N-LS) pour perturber la structure cellulaire et libérer les lipides. Une efficacité maximale de récupération de lipides de 94,7 ± 1,2 (poids / poids) a été observée.

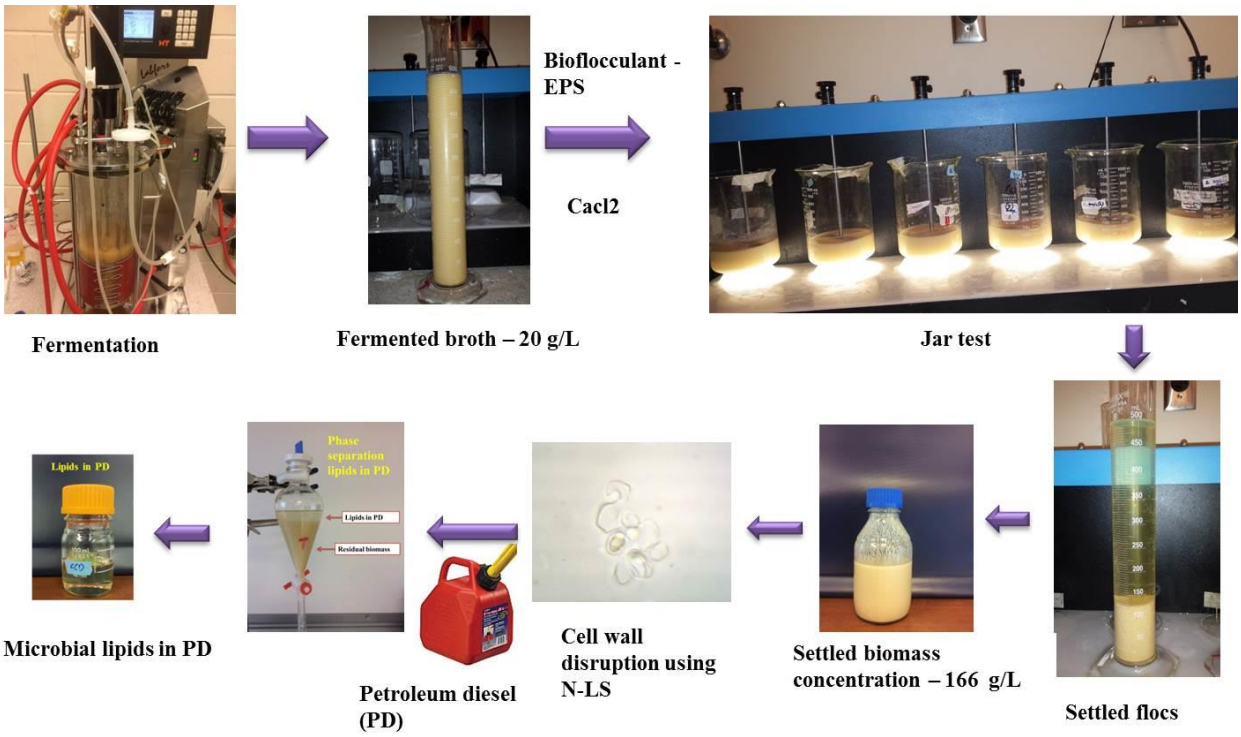
## ABSTRACT

In this research a new process of harvesting the oleaginous yeast biomass (*Yarrowia lipolytica*) by flocculation followed by cell wall disruption and lipid extraction with petroleum diesel as a solvent was developed. Alum and calcium chloride along with the extracellular polymeric substance (EPS) as a flocculant were evaluated for lipid bearing biomass settling. The maximum flocculation activity of biomass using calcium chloride (36 mM) in combination with EPS (5.85 mg EPS/g biomass) or Alum (1.2mM) with EPS (18 mg EPS/g biomass) was 74.3 and 79 % and the settling velocity was 2.93 and 1.46 mm/s, respectively. To have a similar efficiency of biomass settling, 3.07 times less dosage of EPS was required in combination with calcium chloride than required with Alum. Further, settled biomass (166g/L) was treated with N-lauroyl sarcosine (N-LS) to disrupt the cellular structure and release lipid. The released lipid was separated from cell debris and water using petroleum diesel (co-solvent) and maximum lipid recovery efficiency of 94.7 ± 1.2 (w/w) was observed.



**Keywords**: Oleaginous yeast, Flocculation, Extracellular polymeric substance, Ex-situ transesterification, Petroleum diesel.

## GRAPHICAL ABSTRACT



# 1. INTRODUCTION

In last four decades, the world energy consumption has increased by 5.6%. Due to this massive increase in energy consumption, the world is currently facing very critical challenges of environmental pollution and energy crisis (Milano *et al.*, 2016). The use of fossil fuels for energy generation and transportation fuel has led to high greenhouse gas emissions (Carbon dioxide, sulfur dioxide, nitrogen oxide and carbon monoxide), which have caused serious climatic changes (Demirbas, 2011).

Biofuels are one of the alternative sources of renewable energy as they provide cleaner energy generation and reduce the emissions of greenhouse gasses. There are various types of biofuels, out of these; biodiesel is produced from the different feedstocks such as plant oil, animal fats, and microbial oil from autotrophic microalgae, and heterotrophic oleaginous microorganisms (*Rhodococcus*, *Yarrowia lipolytica* and *Mortierella isableina* etc.). Oleaginous microorganisms are important feedstocks with lipid content up to 80% w/w (Alvarez *et al.*, 1997, Gao *et al.*, 2014, Li *et al.*, 2008).

The Biodiesel production from oleaginous microorganisms involves four steps: 1) Cultivation (or) fermentation, 2) harvesting, 3) cell wall disruption and lipid extraction and 4) microbial lipid transesterification. During last one decade, researchers are more concerned about the multiple steps involved in biodiesel production as they affect the cost of biodiesel process in a significant way. There are several limitations involved in each step such as carbon supplementation for microbial cultivation (Li *et al.*, 2008), harvesting of biomass using centrifugation, which is very expensive and energy intensive process (Uduman *et al.*, 2010). Lipid extraction and further lipid transesterification using toxic organic solvents, which are not acceptable due to environmental issues. Due to these many limitations, biodiesel production from oleaginous microorganisms is not able to generate a competition with fossil fuels (Milano *et al.*, 2016).

The downstream processing of microbial oil from oleaginous microalgae, yeast, and fungi is tedious due to very high water content of the biomass. Mostly, harvesting of biomass is conducted by using centrifugation, which is cost intensive and consumes very high energy of 1.2

kWh/h/$m^3$ (Uduman *et al.*, 2010). Therefore, to reduce the high centrifuge energy consumption, several studies have been performed of harvesting microalgae using flocculation (Salim *et al.*, 2012). The coagulation and flocculation process are being used in waste water industry from last few decades. Coagulation is the process of destabilization by charge neutralization. Once neutralized, particles no longer repel each other and can be brought together. Flocculation is the process, bringing together the destabilized or coagulated particles to form a large floc.

There are several coagulants such as Alum, ferrous sulfate, aluminum sulfate and calcium hydroxide, while flocculants include the polymers such as chitosan, polyacrylamide and biodegradable polymer like extracellular polymeric substances (EPS). EPS is produced by bacteria and is used as a flocculant for gravity settling of biomass for wastewater sludge and microalgae (Salim *et al.*, 2012), but there is no approach available for oleaginous heterotrophic yeast biomass harvesting using flocculation.

After biomass separation, cell wall disruption is mostly done by using mechanical (sonication, bead beater and microwave treatment), biological (enzymes) and chemical (organic solvents and acids) methods (Kim *et al.*, 2013). However, each and every method of lipid extraction has some disadvantages like energy consumption, heat generation and consumption of chemicals. Therefore, in recent literature, upgraded lipid extraction without drying process by using surfactants such as N-Laouryl sarcosine, SDS has achieved higher than 90% w/w extraction efficiency (Salam *et al.*, 2016, Yellapu *et al.*, 2016). For further separation of lipids, organic solvents such as chloroform and methanol, ethyl acetate, isoamyl alcohol, and hexane are mostly used (Kim *et al.*, 2013). However, the use of toxic organic solvents for lipid extraction and separation is not suitable for large-scale biodiesel production as they are expensive and hazardous to the environment (Dong *et al.*, 2016).

It is reported that biodiesel obtained from the plant oil or microbial oil has very low pour point and cloud point. Therefore, worldwide, biodiesel is blended with 5 - 20% of petroleum diesel, so as to increase the pour point and cloud point of biodiesel (Medeiros *et al.*, 2015). The petroleum diesel is a hydrophobic solvent, which is miscible with hydrophobic compounds such as microbial lipids. So, in this study, the hypothesis is made that due to hydrophobic nature of microbial lipids, petroleum diesel can be used as a solvent to separate the microbial lipids from disrupted cells.

There is no such literature present related to oleaginous yeast biomass harvesting using flocculation, followed by cell wall disruption using N-LS and further separation of microbial lipids using petroleum diesel. In this study, biomass harvesting using two different inorganic coagulants such as calcium chloride and alum was investigated in combination with extracellular polymeric substances (EPS) as a bioflocculant. Optimization of different parameters (pH, coagulants concentration, and bioflocculant concentration) for biomass settling was conducted. After settling, final biomass concentration and settling velocity were analyzed. The settled biomass was disrupted using N-LS, and microbial lipid was separated using petroleum diesel for further transesterification reaction.

# 2. MATERIALS AND METHODS

## 2.1 Strain, cultivation, and production

*Yarrowia lipolytica* SKY-7, an oleaginous yeast (isolated in our lab INRS-ETE Quebec, Canada) (Kuttiraja *et al.*, 2015) was grown in a medium containing crude glycerol with 78 % (w/w) glycerol (a by-product of biodiesel production) in a 15L fermenter with working volume 10L (Biogene, Quebec). The crude glycerol solution was obtained from a biodiesel producing industry in Quebec, Canada. The medium was supplemented with 10 g/L yeast extract, 2.7 g/L $KH_2PO_4$, 0.5 g/L MgSO4.7$H_2$0 and 1 g/L $Na_2HPO_4$ and initial C/N ratio was adjusted to 75. The fermenter was operated at constant pH 6.8 and temperature 28 $^oC$, and dissolved oxygen concentration was maintained above 30% of saturation. After 72 h of fermentation, the broth was heated in the fermenter at 80 ± 2$^oC$ for 10 min to kill the cells and to preserve the accumulated lipids inside the cells (Yellapu *et al.*, 2016, Zhang *et al.*, 2015). A sample of 25mL of heat treated fermented broth was collected and centrifuged at 8000 rpm for 10 min. The supernatant was discarded, and the pellet was re-suspended in hot tap water to remove residual glycerol and soap and then again centrifuged. The supernatant was discarded, and the pellet was kept in an aluminum cup for drying in hot air oven at 105$^oC$ until constant weight. Finally, oleaginous yeast dried biomass concentration was calculated by using Eq. (1).

$$\text{Biomass concentration (g/L)} = B_f - B_i / \text{Volume of the solution} \times 1000 \ldots.(1)$$

Where $B_f$ is final weight of the aluminum cup, and $B_i$ is the pre-weight of the aluminum cup. Biomass concentration was determined after each biomass settling experiment by drying the total settled biomass and using Eq. (1). The total lipid content in biomass was 44.3 ± 0.7% w/w. The cells were dried and total lipid content was determined by using (2:1) chloroform and methanol lipid extraction method (Bligh and dyer 1959).

## 2.2 Effect of alum and calcium chloride on zeta potential of fermented broth

Unless stated, heat treated fermented broth was used throughout the experiments. In the preliminary study, 1000 mL of fermented broth (initial biomass concentration 20g/L and pH 6.3) was transferred to a measuring cylinder of 1000 mL. Biomass settling was observed after 24 h without the addition of a coagulant, and then zeta potential was determined by using Zetaphoremeter IV (Zetacompact Z8000, CAD Instrumentation, France).

Alum and calcium chloride were tested separately for coagulation study to determine the best coagulant for biomass settling. The effect of coagulants concentration (Alum and calcium chloride) on the biomass settling was investigated by using jar test (Kurane *et al.*, 2014, Nouha, 2016). The solid salt (alum or calcium chloride) was added slowly to the fermented broth (biomass concentration 20 g/L, pH 6.3) with constant stirring at 100rpm until it dissolved. The salt addition was continued until floc formation was observed by naked eye.

## 2.3 Effect of pH on zeta potential of fermented broth

The effect of pH on zeta potential was measured by addition of coagulants (Alum -1.2 mM or Calcium chloride – 36mM) and mixed for 5 min using jar test and final pH was adjusted to 3, 5, 7, 9 and 11 with either 2N NaOH or 2N $H_2SO_4$ followed by measurement of zeta potential.

## 2.4 Effect of coagulant concentration on zeta potential of fermented broth

To find the required minimum concentration for floc formation of the heat treated fermented broth (20g/L biomass and pH 6.3), the coagulants concentration was varied from 0.6 - 1.3 mM for alum and 9 - 54mM calcium chloride. The coagulants were added slowly into the fermented broth (cell suspension 20 g/L, 1000 mL, pH 6.3) with continuous agitation at 100 rpm for 10 min. In each case, the pH after addition of individual coagulant was adjusted to 5 for alum and 9 for calcium chloride. After 10 min of mixing, the sample was drawn to measure zeta potential.

## 2.5 Settling velocity with coagulant

The solution having coagulant concentration (Alum – 1.2 mM or Calcium chloride – 36 mM) with maximum zeta potential (determined in the last section 2.4) was used to estimate the settling velocity. The sample was transferred to a 1000 mL measuring cylinder and allowed to settle. The solid-liquid interface height was recorded at 5, 10, 15, 20, 25 and 30 min. After 30 min, the sediment volume index (SVI) was determined, which is an estimate of the amount of water content present in the settled phase (sediments). The sediment volume index was calculated based upon the settled volume after 30 min. It is defined as volume (in milliliters) occupied per g of heat treated lipid bearing yeast biomass after settling. SVI was calculated by using Eq. (2).

$$SVI\ (mL\ g^{-1}) = Settled\ biomass\ volume\ (mL\ L^{-1}) / Biomass\ dry\ weight\ (mg\ L^{-1}) \times 1000\ (mg\ g^{-1}) \quad \text{..... (2)}$$

The solid-liquid interface height was recorded at 5, 10, 15, 20, 25 and 30 min to calculate the settling velocity (Eq. 3). The settling velocity is defined as the velocity in mm/s, to achieve complete biomass settling without further observed increase of settled floc volume.

Settling velocity = (Height of settled biomass at a certain time – Original height) / Time required to reach current height……. (3)

## 2.6 Combined effect of coagulant and bio flocculant on biomass settling

Biomass settling velocity with coagulant alone and with combined coagulant and bio flocculant was measured. The optimized concentration of coagulants ($Ca^{2+}$ or $Al^{3+}$) with a combination of slime extracellular polysaccharides (S-EPS or Bioflocculant) was used to study the biomass settling velocity. The S-EPS (crude) employed in this study was produced by *Cloacibacterium normanense* using wastewater sludge as a raw material (Nouha, 2016). The jar test was conducted using 1000 mL of heat treated fermented broth with biomass concentration of 20g/L and pH 6.3. Alum or calcium chloride was added slowly to make the concentration 1.2 mM and 36 mM, respectively. After that, pH was adjusted to 5 in the case of alum and 9 in case of calcium chloride. The S-EPS solution was diluted to obtain concentration of 6.5 g/L and added in different volumes (1-10 mL, to obtain a different concentration of EPS in the lipid bearing biomass suspension, mg EPS/g biomass) to the cell and coagulant suspension. The mixture was rapidly mixed at 120 rpm for an initial 5 min (to disperse the EPS throughout the mixture) and then slowly mixed at 80 rpm for an additional 15 min (to allow floc formation). Subsequently, samples were transferred to a measuring cylinder (1000mL) and allowed to settle.

After 30 min of sedimentation, the SVI of the settled slurry was determined as discussed in section 2.5.

The supernatant of the settled biomass (after 30 min settling) was collected to measure turbidity to calculate the flocculation activity (Eq.4).

Flocculation activity (%) = (B-A)/B × 100……(4)

Where ‘A’ is turbidity of the test sample supernatant with different EPS concentration in heat treated oleaginous fermented broth, and ‘B’ is the turbidity of the supernatant after heat treated oleaginous fermented broth settling after 30 min (Control - without EPS addition).

## 2.7 Effect of coagulants and bioflocculant on settling velocity with higher biomass concentration

The concentration of oleaginous microorganisms (biomass) in fermented broth varies from 10 to 60 g/L, depending upon the type of microorganism, type of fermentation (batch or fed-batch),

medium composition, fermentation time as well as substrate (Meng *et al.*, 2009). Due to this reason, the optimum parameters (pH, coagulant, and bioflocculant concentration) determined for biomass settling with initial biomass concentration of 20 g/L were used to study the biomass settling at higher initial biomass concentration of 40 and 60 g/L. The original biomass concentration in the cell suspension (fermented broth) was 20 g/L. Therefore, to achieve higher biomass concentration, the fermentation broth was centrifuged at 8000 rpm 15 min and biomass solids were re-suspended in the supernatant to obtain desired biomass concentration. Calcium chloride or alum was added slowly to 1000 mL of cell suspension (40 or 60 g/L biomass concentration) to reach zeta potential similar to that obtained for 20 g/L of biomass concentration. After that pH was adjusted to 5 (in the case of alum) and 9 (in the case of calcium chloride) with continuous agitation at 100 rpm for 10 min. A sample was withdrawn to measure zeta potential. Thereafter, 1 to 20 mL of S-EPS solution (S-EPS concentration 6.5 g/L) was added in the suspension and rapidly mixed at 120 rpm for an initial 5 min (to disperse the EPS throughout the mixture) and then slowly mixed at 80 rpm for additional 10 min**.** After that, the mixture was transferred to a 1000 mL measuring cylinder and allowed to settle. The solid-liquid interface height was recorded for 30 minutes to calculate settling velocity. The settled biomass slurry was readjusted to pH 7 and was stored at 4$^{o}$C for further study.

## 2.8 Cell wall disruption of the settled biomass using N-Lauroyl Sarcosine

Sample of 10 mL was taken from the settled biomass slurry (166 g/L, after settling of 20 g/L - initial biomass concentration) and was treated with N-lauroyl sarcosine (N-LS) concentration of 0.04 g/g dry biomass and vortexed to homogenize. The mixture of N-LS and biomass was incubated at 30 $^{o}$C for 15 min with agitation at 200 rpm (Yellapu *et al.*, 2016). After that petroleum diesel (50mL/g lipid) was added to N-LS treated mixture and kept for incubation at 70$^{o}$C for 10 minutes (Optimized conditions in our laboratory) to enhance emulsification of microbial lipids (present in N-LS treated solution) to petroleum diesel. The screw capped glass tubes containing the reaction mixture were tightly closed. After incubation, the mixture in tubes was kept for phase separation until clear top phase (approximately in 30 min) was obtained. Top phase consists of

microbial lipid in petroleum diesel and the bottom phase with residual defatted biomass and water and other intracellular products. The microbial lipid present in petroleum diesel ($L_{pd}$) was directly used for transesterification without drying. The transesterified microbial lipid to FAMEs (Fatty acid methyl esters) was analyzed using FT-IR and obtained FAMEs were used to calculate the weight of the microbial lipid separated using petroleum diesel.

The total lipid obtained by using petroleum diesel was calculated according to Equation (5):

$$\text{Lipid recovery efficiency of petroleum diesel (\% w/w)} = L_{pd} / L_c \times 100\ \% \text{ -------} \quad (5)$$

Whereas $L_{pd}$ is the weight of lipid (g) present in (10 mL of settled biomass, which is equal to 2.1 g dry biomass) 15 mL petroleum diesel. $L_c$ is the dry weight of lipid (Lc = 0.83g) obtained using dry cells and standard chloroform and methanol method (Bligh and dyer 1958) using 2.1 g dry biomass (or 10 mL of settled biomass 166 g/L)

A similar control experiment was conducted using (2:1) chloroform and methanol mixture (50 mL/g total lipids) employing wet cells instead of petroleum diesel to extract total lipids (from N-LS treated cell suspension) into chloroform phase (top phase) and bottom phase consists of residual defatted biomass, methanol, and water. The microbial lipid in chloroform phase was dried at 60$^{o}$C until constant weight ($L_{cm}$) was obtained.

The lipid recovery efficiency using N-LS treated wet cells and chloroform and methanol standard method (control) was calculated by eq (6)

$$\text{Recovery of lipid from cell suspension \% (w/w)} = L_{cm} / 0.83 \times 100\% \text{ ---------- (6)}$$

Where $L_{cm}$ is the weight of microbial lipid in chloroform phase extracted from N-LS treated samples.

## 2.9 Effect of petroleum diesel concentration on lipid separation

The effect of petroleum diesel concentration on lipid separation from N-LS disrupted lipid bearing cells was studied. The settled biomass (10 mL, biomass concentration 166 g/L) was treated with N-LS (as described above) and released lipids was separated using different concentration of

petroleum diesel (PD) 5, 10, 15, 20, 30, 40 and 50 (mL/g lipid) and the mixture was kept in a hot water bath 70$^{o}$C with agitation at 200 rpm for 15 min to enhance miscibility of microbial lipid in petroleum diesel. After incubation, the total mixture was allowed to phase separate and the separated microbial lipid in petroleum diesel phase. Transesterification of the recovered lipids was performed, FAMEs genertaed analyzed with FT-IR and lipid recovery efficiency was calculated (as discussed in section *2.10*).

## 2.10 Ex-situ transesterification of recovered lipids

The transesterification was conducted for different samples: 1) Dried microbial lipid recovered using chloroform and methanol (recovered by either standard method using dry cells or N-LS disrupted cells) and further dissolved using hexane (20 mg lipid/mL hexane); 2) Microbial lipid (obtained from N-LS disrupted cells) present in petroleum diesel (Ex-situ transesterification).

The transesterification process was conducted by adding sodium hydroxide as a catalyst dissolved in methanol (6:1 methanol to lipid molar ratio or 0.4 mL methanol per gram lipid) solution, and the final concentration of catalyst to lipid was 1% w/w (1g NaOH/100g lipid). The mixture was then heated to 55$^{o}$C for 2h. After the reaction, the mixture was cooled to room temperature, and 10 mL water (mL/g lipid) was added. After mixing, the mixture was kept for phase separation for 30 min. The top phase contained biodiesel in hexane (control), or biodiesel (BD) in petroleum diesel (PD) and the bottom phase contained residual catalyst, residual methanol, and glycerol. The biodiesel in hexane (control) was further purified as discussed in our previous study (Yellapu *et al.*, 2016). Moreover, the biodiesel present in petroleum diesel was washed with 2% w/w sodium bicarbonate (to remove excess water) and allowed to stand for phase separation until a clear solution was obtained in the top phase. Top phase contained biodiesel in petroleum diesel, and bottom phase contained residual water with sodium bicarbonate. The biodiesel present in petroleum diesel was directly quantified by using Fourier transform infrared spectrometer (FT-IR) to determine the percentage of biodiesel present in the petroleum diesel. In the case of control or N-LS disrupted cells (lipid recovered by chloroform and methanol as presented in section 2.8), the biodiesel concentration was determined by using GC-FID. The lipid conversion efficiency to biodiesel (% w/w) was calculated based upon the

concentration of biodiesel measured by GC-FID divided by the total lipid concentration × 100%. All experiments were conducted in triplicate, and the standard deviation was less than 5%.

The lipid conversion to biodiesel present in petroleum diesel was calculated using eq (7)

$$\text{Lipid conversion efficiency \% (w/w)} = B_{pd} \text{ (or) } B_c / 0.83 \text{ g} \times 100 \text{ \%} \quad \text{---------- (7)}$$

Where, $B_{pd}$ is grams of biodiesel in petroleum diesel. $B_c$ is biodiesel concentration obtained from control experiment.

## 2.11 Quantification of biodiesel using FT-IR

The biodiesel present in petroleum diesel was quantified using (FT-IR) Fourier transform infrared spectrometer (Nicolet™ is 50FT-IR, Thermoscientific Inc) equipped with standard KBx beam splitter with DTGS detector. The FT-IR scanning was conducted as per ASTM D7371 for sample analysis. 50 µL of a sample containing biodiesel in petroleum diesel obtained from transesterification was placed in a smart iTx-Diamond. Spectra were collected in 40 seconds (16 scans and 4 $cm^{-1}$ resolution). The data was collected using OMNIC™ spectroscopy software, and the chemometrics were performed using TQ Analyst™. The standard was prepared by using different concentration (B-1 to 100 % v/v) of pure biodiesel (obtained from trans-esterified sunflower oil). The Quantification of biodiesel was performed in triplicate, and the standard deviation was less than 5%. The biodiesel percentage was calculated based on the height and area of the peak obtained in standard calibration graph.

## 2.12 Effect of coagulants on biodiesel profile

The settled biomass obtained from each experiment (with or without coagulants or EPS) was lyophilized and then analyzed to study the effects of coagulants on biodiesel profile. The control lyophilized lipid bearing biomass (2 g) was used for lipid extraction using chloroform and methanol using standard method (Bligh *et al.*, 1959). After that, lipid transesterification was conducted using hexane as co-solvent, and biodiesel concentration was determined by using gas chromatography method as reported in Yellapu *et al.*, (2016).

# 3. RESULTS AND DISCUSSION

Heat treated oleaginous yeast fermentation broth with initial biomass concentration of 20 g/L was used for all biomass settling experiments. In the preliminary study, the fermented broth was transferred to 1000 mL measuring cylinder without the addition of a coagulant and no biomass settling was observed for 24 h at room temperature. It may be due to very high negative charge on the surface of yeast cell with zeta potential -54.3 mV. Zeta potential of yeast cells was comparatively higher as compared to microalgae cells (zeta potential – 20mV) (Ummalyma *et al.*, 2016). Therefore, a higher concentration of coagulant could be required for sedimentation of yeast cells. The alum and calcium chloride concentration required to be added to the fermented broth at which floc formation observed by naked eye was 1.2 mM (Alum) and 36mM (calcium chloride). The pH of the broth after addition of coagulants was 9 for calcium chloride and 5 in case of alum. The solution obtained was used to measure zeta potential.

## 3.1 Effect of pH on zeta potential of fermented broth

The inorganic coagulants, alum and calcium chloride, were used for coagulation of fermented broth. As shown in Figure 2.2.1, for calcium chloride - $Ca^{+2}$ (36 mM) as a coagulant, with increase in pH from 3 to 9, zeta potential increased from -53.2 to -22.5 mV and after that it became constant up to pH 11. However, in the case of alum (1.2 mM) as a coagulant, an increase in pH from 3 to 5, resulted in an increase in zeta potential from -42.1 to -29.3mV. As pH was further increased from 5 to 11, zeta potential decreased from -29.3 to -48.6 mV (Figure 2.2.1). It was reported that surface charge of particles is pH dependent **(**Ummalyma *et al.,* 2016**)**. Therefore, optimum pH obtained for floc formation in the case of alum was 5. Similar results were observed, when alum was used as a coagulant with waste water sludge where the optimum pH obtained was 5.5 (Amirtharajah *et al.*, 1982). The increase in zeta potential with increase in pH from 3 to 9 (36 mM concentration of $Ca^{2+}$,Figure 2.2.1) , could be due to neutralization of cell surface charge in this pH range (Cosa *et al.*, 2013). Thus, the optimum pH for calcium chloride and alum as a coagulant could be considered as 9 and 5, respectively.

## 3.2 Effect of coagulant concentration on zeta potential of fermented broth

With increase in calcium chloride concentration from 9 to 54 mM at pH 9, zeta potential increased from -42.3 to -22.5 until 36mM concentration and thereafter it became constant. Therefore, the optimum concentration for calcium chloride was determined to be 36mM (Figure 2.2.2 a). Further, an increase in alum concentration from 0.6 to 1.3 mM at pH 5, the zeta potential increased from -38.2 to -20.2 mV and the optimum concentration for coagulation process was found to be 1.2 mM (Figure 2.2.2.b). These results clearly show the effect of alum and calcium ions on the zeta potential of heat treated lipid bearing biomass. The charge reversal on biomass is attributed to the adsorption of alum or calcium ions at the particle-liquid interface. Also, increase in cations concentration can increase ionogenic group dissociation, and it can decrease re-adsorption of some ions in solution on the solid particle surface (DoymuŞ, 2007).

## 3.3 Biomass settling velocity with coagulant alone and combined coagulant and bioflocculant (S-EPS)

The S-EPS (bio flocculent) produced by *Cloacibacterium normanense* was used in combination with the coagulants (Calcium chloride or Alum) to enhance the lipid bearing biomass settling rate. The biomass settling was quantified by measuring interface height of the biomass. In control experiment, using coagulants without the addition of EPS, the interface height decreased slowly compared to the combination of individual coagulant with EPS (Figure 2.2.3). It was observed that with 36mM Calcium chloride and with EPS dosage of 7.15 and 6.5 mg/g biomass, the settling interface of biomass was almost similar. In the case of 5.85mg EPS /g biomass, settling of biomass was comparatively highest (Figure 2.2.3a) and was considered to be optimum dosage because biomass settling occurred within five min and lowest interface height (120 mm) was attained as compared to the other dosages of EPS (Figure 2.2.3a). In the case of alum, the interface height started decreasing with increase in EPS dosage from 7.8 to 18 mg EPS/g biomass. A minimum interface height (120 mm) was observed at 18 and 20.2 mg EPS/g biomass. Therefore, in case of alum 18 mg EPS/g biomass was considered optimum dosage of EPS for

biomass settling within 5 to 10 min (Figure 2.2.3b). From these results it was concluded that the combination of calcium chloride (coagulant) and EPS bio flocculant resulted in an interface height of 120 mm at EPS dosage of 5.85mg EPS/g biomass in approximately 5 min.

The biomass settling velocity was calculated based on the decrease in interface height during biomass settling (Figure 2.2.3) The biomass settling velocity after addition of alum (pH 5) or calcium chloride (pH 9) is presented in Table 2.2.1. Settling velocities for a combination of calcium chloride and EPS ranged between 0.75 -2.93 (mm/s), whereas in the case of alum and S-EPS combination the settling velocity ranged between 0.56 – 1.46 (mm/s). The settling velocity was maximum (2.93 mm/s) for combination of calcium chloride and EPS (5.85 mg/g biomass). Thereafter, the settling velocity decreased with increasing S-EPS dosage. In case of alum and S-EPS combination, the settling velocity increased with EPS concentration with maximum settling velocity of 1.46 mm/s at S-EPS dosage of 18mg EPS/g biomass. Thus, a maximum settling velocity was observed with calcium chloride (36mM) and S-EPS (5.85mg EPS/g biomass) combination and final biomass concentration obtained in the settled slurry was 166.7 g/L obtained within 5 min. In case of alum and S-EPS combination, a similar biomass concentration of 166.7 g/L was obtained in 10 min settling time at high S-EPS dosage (18mg/g biomass).

## 3.4 Flocculation activity

The flocculating activity was calculated for different dosage of S-EPS varying from 5.85 to 7.15 mg EPS/g biomass and 7.8 to 20.2 mg EPS/g biomass in the case of calcium chloride and alum as a coagulant, respectively (Figure 2.2.4a). The flocculating activity of 74.07% was achieved at 5.85mg S-EPS/g biomass using 36 mM of calcium chloride. However, there was slight decrease in the flocculating activity to 73.9 %, with increase in S-EPS dosage from 5.85 to 7.15 mg /g biomass (Figure 2.2.4a).The slight decrease in flocculating activity at higher dose of S-EPS might be due to over addition of the negatively charged S-EPS, generating strong repulsive forces between the biomass particles and the bioflocculant. These processes destabilized the suspended particles, increased the viscosity of the suspension, block the adsorption sites and noticeably reduce flocculation activity (Ho *et al.*, 2009). According to Nouha *et al.* (2016), the FTIR of S-EPS detected the presence of a relatively high quantity of hydroxyl (-OH) and carboxyl (-

COO-) groups. The presence of these groups is favorable for flocculation process to provide the surface charges, which helps in further binding with suspended particles and causes floc formation. However, the presence of these groups in excess may increase the negative charge and hence causes the repulsion between particles (Kavita *et al.*, 2014).

It was observed that in case of alum as a coagulant, the flocculation activity was 19.56 % at S-EPS dosage of 7.8 mg/g biomass. Further increase in S-EPS dosage (7.8 to 18 mg EPS/g biomass) resulted into decrease in turbidity and increase in flocculating activity (Figure 2.2.4b). A maximum flocculation activity of 79.3% was achieved at bioflocculant dosage of 18 mg EPS/g biomass. Further increase in S-EPS dosage to 20.2 mg EPS/g biomass, the flocculation activity was unchanged (Figure 2.2.4b). It can be explained that at a lower S-EPS dosage, less concentration of negatively charged groups were available to destabilize the negatively charged biomass particles, thereby decreasing the interaction of S-EPS and biomass particles, which led to increase in turbidity of the suspension and low flocculation activity.

Thus, from the foregoing observations it can be concluded that maximum flocculation activity (79%) was obtained by using 1.2 mM of alum and 18 mg S-EPS/g biomass whereas, 74% flocculation activity was observed using 36mM of calcium chloride and 5.85 mg S-EPS /g biomass. It was remarked that in case of calcium chloride, more than 3 times higher dosage of S-EPS was required to achieve almost similar flocculation activity as attained by using alum as a coagulant. These findings were in consistence with previously reported studies (Zheng *et al.*, 2008), where the optimum flocculation dosage of EPS was in the range of 1.0 to 20 mg/L and maximum flocculation activity were obtained by using 20 mg EPS/L bio-flocculant (EPS) dosage. It has been extensively documented that a lower concentration of bio-flocculant with a high flocculating efficiency will contribute in the cost reduction of biomass settling process.

Settling volume index (SVI) was calculated to determine the water content present in the settled biomass. In control experiment (with calcium chloride 36 mM and without EPS) the SVI was found to be 5.12 mL/g of biomass. The lowest SVI of 0.72 mL/g was observed with 5.85 mg S-EPS/g biomass using calcium chloride as cations, which was almost 7.1 times lower than the SVI obtained without addition of S-EPS. With increase in S-EPS concentration more than 5.85 mg/g of biomass, SVI started increasing and reached up to 3.38 mL/g of biomass (Figure 2.2.4a).

The maximum biomass concentration of 166.7 g/L in the settled slurry was obtained at S-EPS dosage of 5.85 mg S-EPS/g biomass with 0.72 (mL/g) SVI.

Settling velocity index for alum (1.2 mM) and varying dosage of EPS was presented in Figure 2.2.4b. In the control experiment (Alum 1.2 mM without the addition of EPS), the SVI was found to be 5.99 mL/g of biomass. As S-EPS dosage was increased from 7.8 to 20.2 mg EPS/g of biomass, the SVI started decreasing from 1.2 mL/g of biomass and reached to 0.71 mL/g of biomass at EPS concentration of 18 mg/g of biomass. The lowest SVI was obtained by using alum and S-EPS dosage of 18mg/g biomass. The lowest SVI obtained by using alum and S-EPS dosage of 18mg/g biomass was almost similar to that obtained by using calcium chloride and with S-EPS dosage of 5.85 mg/g biomass. It means that calcium chloride was found to be equally effective as alum.

## 3.5 Effect of coagulants and bioflocculant on biomass settling with higher biomass concentration

Effect of coagulant and bioflocculant concentration on biomass settling was further studied by using heat treated high biomass concentration (40 and 60 g/L). The zeta potential decreased with increase of biomass concentration in the suspension and decrease of zeta potential was much higher for alum (1.2mM) than calcium chloride (36mM) (Figure 2.2.5a). It explains that to maintain zeta potential of the suspension, the coagulant concentration need to be increased for charge neutralization. However, higher concentration of alum poses solubility problem.

Therefore, calcium chloride (concentration of 52 and 70 mM) was used for further study with 40 and 60 g/L biomass concentration. The S-EPS dosage of 5.85, 39.9 and 119mg EPS/g biomass was used for 20, 40 and 60g/L of biomass concentration, respectively, which was determined by adding S-EPS slowly in to the cell suspension. The S-EPS dosage was added until the point where floc formation was visible along with increase in floc size. The S-EPS dosage was calculated based upon the volume of S-EPS added. After settling, final biomass concentration obtained was 177 and 210 g/L for initial biomass concentration of 40 and 60 g/L, in 10 and 20 min settling time, respectively. A slow sedimentation begins at a higher biomass concentration of 60

g/L. In this context, the increase in polymer concentration could increase the viscosity of the fluid, which means that larger biomass particles or flocs could be formed by the viscous forces or bridging mechanisms (Chaiwong *et al.*, 2008). Figure 2.2.6 shows the change in solid liquid interface height for 40 and 60 g/L of biomass concentration. With increase in biomass concentration, interface height decreased slowly with respect to time. For 20g/L biomass concentration, the total biomass was settled within 5 min but in case of 40 and 60 g/L biomass concentration, the biomass settled in 10 and 20 min and the settling velocity was 1.25 and 0.64 mm/s, respectively.

## 3.6 Cell wall disruption of settled biomass using N-lauroyl sarcosine

The total lipid obtained after drying (lyophilization) and further extraction employing standard chloroform and methanol method was assumed as 100% lipid in the biomass. The total lipid obtained was 0.83 ± 0.07 g using 10 mL (2 ± 0.3 g dry biomass) of settled biomass. The lipid extraction efficiency 98.1±1.1 % w/w (0.81 ± 0.06 g) was obtained by using wet biomass slurry treated with N-LS and further microbial lipid recovery using chloroform and methanol (Table 2.2.2). It means the microbial lipid obtained by using wet or dry biomass is almost similar and loss of microbial lipid will be counted as statistical error and it can be negligible.

Whereas, the lipid recovery efficiency obtained with petroleum diesel as a solvent was 94.7 ± 1.2 % w/w. The previous researchers reported the use of non-polar (organic) solvents such as hexane, ethyl acetate and chloroform to separate the lipid after cell wall disruption (Table 2.2.2). But, use of organic solvents increases loss of energy due to solvent drying and it can directly affect biodiesel economics.

## 3.7 Effect of petroleum diesel concentration on lipid separation

The different volume of petroleum diesel (solvent) 5, 10, 15, 20, 30, 40 and 50 (mL/ g lipids) was used to separate the lipids after N-LS treatment (Table 2.2.3). Increase in petroleum diesel concentration from 5 to 50 mL/g lipids, the lipid separation efficiency was increased from 71.6 ± 0.5 to 95.1 ± 1.1 % w/w. The maximum lipid separation efficiency was achieved by using 20mL of petroleum diesel per gram of lipid. It was observed that when petroleum diesel concentration

of 5mL/g lipid was used, phase separation was observed in 60 min. whereas, in case of petroleum diesel concentration higher than 20 mL/g lipid, the phase separation was observed in less than 15 min. It explains that as the ratio between the water and solvent increased from 1:0.5 to 1:1.5, liquid-liquid separation increased, thereby decreasing the phase separation time.

## 3.8 Ex-situ transesterification of recovered lipids

The lipid separated from N-LS disrupted cells using petroleum diesel (co-solvent) was trans-esterified directly without any further drying (ex-situ). The maximum lipid conversion efficiency of 97.2% was observed at concentration of 20mL co-solvent /g of total lipid as determined optimum in this study (Table 2.2.3). As per the Canada biofuel annual report (2015), 2 to 5% (which is known as B-2 and B-5) biodiesel blended with petroleum diesel is considered a better-quality standard biodiesel. While increasing the petroleum diesel concentration, the final biodiesel % v/v can be less than the blending percentage of biodiesel. Therefore, in our future studies, we are going to recycle petroleum diesel to increase concentration of microbial lipid in petroleum diesel.

## 3.9 Quantification of biodiesel in petroleum diesel using FT-IR

The entire FTIR spectra of 8 standards containing mixture of biodiesel with oil were processed by using TQ analyst software (Thermo Fisher scientific, Canada). Each spectrum was applied to the software along with the known concentrations of the biodiesel for that spectrum and the selected regions were considered as characteristics of the biodiesel. The calibration of peaks was evaluated as reported by Mahamuni *et al.* (2009). The results of FTIR analysis of biodiesel obtained through transesterification in petroleum diesel and that of pure petroleum diesel (B-0%) was presented in Figure 2.2.7. For FT-IR spectrum of biodiesel, mid-range infrared (4000-400 $cm^{-1}$) was selected.

Figure 2.2.7 is divided into 3 sections: section 1 (3000 – 2500 $cm^{-1}$) corresponding to the CH stretching mode of olefins, which was present in both biodiesel and petroleum diesel. Section II (1800 – 1720 $cm^{-1}$) contains strong ester peaks at 1750 (the C=O vibration) and was present only in biodiesel. While section III (1376 – 1000 $cm^{-1}$) represents the methyl ester peaks and was

present only in biodiesel spectra, not in the petroleum diesel (Figure 2.2.7). The obtained peaks of methyl esters were almost similar with the peaks obtained by Mahamuni *et al.* (2009). The spectrum was selected based upon the peak height and area for quantification of biodiesel in petroleum diesel. Increase of area and peak size represents increase of biodiesel composition in PD.

## 3.10 Effect of coagulants on biodiesel profile

The biodiesel profile obtained from all types of biomass settling (with and without aid of EPS, chemical coagulants and control) was analyzed, and it mainly contained C-16 and C-18 fatty acids (Table 2.2.4). These results explained that there was no significant impact on biodiesel profile by using flocculants for biomass settling. Due to the presence of high C-18, the oxidation stability of biodiesel increases compared to plant oil, so the saturation point tends to increase boiling point and viscosity. It was thus concluded that coagulants did not cause any effect upon biodiesel and it could be substantial approach for industrial biodiesel production.

# 4. CONCLUSION

This study describes the yeast biomass settling using alum and calcium chloride as coagulants with or without EPS dosage. The presence of EPS increased the biomass settling rate and biomass settled within 5 min with low SVI (0.72 mL/g biomass). The settled biomass was further disrupted by N-LS to release intracellular microbial lipid and the released lipid was separated from the cell debris using petroleum diesel. The maximum lipid recovery efficiency of 94.7 ± 1.2 % w/w was recorded. After transesterification, maximum biodiesel yield of 97.4 ± 1.1% was obtained. Therefore, this process is a better approach for biodiesel production using less energy, time and cost because drying of lipid bearing cells is not required and toxic solvents for lipid recovery are eliminated. This process also obviates the need of expensive and high energy consuming centrifugation process.

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# 5. REFERENCES


Alvarez HM, Kalscheuer R & Steinbüchel A (1997) Accumulation of storage lipids in species of Rhodococcus and Nocardia and effect of inhibitors and polyethylene glycol. *Lipid / Fett* 99(7):239-246.

Amirtharajah A & Mills KM (1982) Rapid-mix design for mechanisms of alum coagulation. *Journal (American Water Works Association)*:210-216.

Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* 37(8):911-917.

Chaiwong N & Nuntiya A (2008) Influence of pH, electrolytes and polymers on flocculation of kaolin particle.

Cosa S, Ugbenyen AM, Mabinya LV, Rumbold K & Okoh AI (2013) Characterization and flocculation efficiency of a bioflocculant produced by a marine Halobacillus. *Environ Technol* 34(17-20):2671-2679.

Demirbas MF (2011) Biofuels from algae for sustainable development. *Applied Energy* 88(10):3473-3480.

Dong T, Knoshaug EP, Pienkos PT & Laurens LML (2016) Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review. *Applied Energy* 177:879-895.

DoymuŞ K (2007) The effect of ionic electrolytes and pH on the zeta potential of fine coal particles. *Turkish journal of chemistry* 31(6):589-597.

Gao D, Zeng J, Yu X, Dong T & Chen S (2014) Improved lipid accumulation by morphology engineering of oleaginous fungus Mortierella isabellina. *Biotechnology and Bioengineering* 111(9):1758-1766.

Halim R, Gladman B, Danquah MK & Webley PA (2011) Oil extraction from microalgae for biodiesel production. *Bioresour Technol* 102(1):178-185.

Ho Y, Norli I, Alkarkhi AF & Morad N (2009) Analysis and optimization of flocculation activity and turbidity reduction in kaolin suspension using pectin as a biopolymer flocculant. *Water Science and Technology* 60(3):771-781.

K N & Nv H (2016) Fourier Transform Infrared Spectroscopy and Liquid Chromatography–Mass Spectrometry Study of Extracellular Polymer Substances Produced on Secondary Sludge Fortified with Crude Glycerol. *Journal of Material Science & Engineering* 05(03).

Kavita K, Singh VK, Mishra A & Jha B (2014) Characterisation and anti-biofilm activity of extracellular polymeric substances from Oceanobacillus iheyensis. *Carbohydrate Polymers* 101:29-35.

Kim J, Yoo G, Lee H, Lim J, Kim K, Kim CW, Park MS & Yang JW (2013) Methods of downstream processing for the production of biodiesel from microalgae. *Biotechnol Adv* 31(6):862-876.

Kurane R, Takeda K & Suzuki T (2014) Screening for and Characteristics of Microbial Flocculants. *Agricultural and Biological Chemistry* 50(9):2301-2307.

Kuttiraja M, Krishna S, Dhouha A & Tyagi RD (2015) A Substrate-Based Approach for the Selection of Oil-Bearing Heterotrophs from Nitrogen-Deficient Soil for Lipid Production. *Applied Biochemistry and Biotechnology* 175(4):1926-1937.

Li Q, Du W & Liu D (2008) Perspectives of microbial oils for biodiesel production. *Applied Microbiology and Biotechnology* 80(5):749-756.

Mahamuni NN & Adewuyi YG (2009) Fourier Transform Infrared Spectroscopy (FTIR) Method To Monitor Soy Biodiesel and Soybean Oil in Transesterification Reactions, Petrodiesel−Biodiesel Blends, and Blend Adulteration with Soy Oil. *Energy & Fuels* 23(7):3773-3782.

Medeiros DL, Sales EA & Kiperstok A (2015) Energy production from microalgae biomass: carbon footprint and energy balance. *Journal of Cleaner Production* 96:493-500.

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

Milano J, Ong HC, Masjuki H, Chong W, Lam MK, Loh PK & Vellayan V (2016) Microalgae biofuels as an alternative to fossil fuel for power generation. *Renewable and Sustainable Energy Reviews* 58:180-197.

Nouha K (2016) Characterization of Extracellular Polymeric Substances (Eps) Produced by Cloacibacterium normanense Isolated from Wastewater Sludge for Sludge Settling and Dewatering. *Journal of Civil & Environmental Engineering* 05(06).

Salam KA, Velasquez-Orta SB & Harvey AP (2016) Surfactant-assisted direct biodiesel production from wet Nannochloropsis occulata by in situ transesterification/reactive extraction. *Biofuel Research Journal* 3(1):366-371.

Salim S, Vermuë M & Wijffels R (2012) Ratio between autoflocculating and target microalgae affects the energy-efficient harvesting by bio-flocculation. *Bioresource technology* 118:49-55.

Uduman N, Qi Y, Danquah MK, Forde GM & Hoadley A (2010) Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. *Journal of Renewable and Sustainable Energy* 2(1):012701.

Ummalyma SB, Mathew AK, Pandey A & Sukumaran RK (2016) Harvesting of microalgal biomass: Efficient method for flocculation through pH modulation. *Bioresour Technol* 213:216-221.

Yellapu SK, Bezawada J, Kaur R, Kuttiraja M & Tyagi RD (2016) Detergent assisted lipid extraction from wet yeast biomass for biodiesel: A response surface methodology approach. *Bioresour Technol* 218:667-673.

Zhang X, Yan S & Tyagi RD (2015) Strategies of preserving lipids in microorganism after fermentation. *Bioresource Technology* 192:718-725.

Zheng Y, Ye Z-L, Fang X-L, Li Y-H & Cai W-M (2008) Production and characteristics of a bioflocculant produced by Bacillus sp. F19. *Bioresource Technology* 99(16):7686-7691.

**Table 2.2. 1 Effect of coagulant and EPS dosage on settling velocity**

| Coagulant/Bio flocculant | Coagulant dosage (mM) | EPS dosage (mg EPS/g biomass) | Settling velocity (mm $s^{-1}$) |
|---|---|---|---|
| Calcium chloride | 36 | - | 0.75 |
| | | 5.85 | 2.93 |
| | | 6.5 | 1.2 |
| | | 7.15 | 0.61 |
| Alum | 1.2 | - | 0.56 |
| | | 7.8 | 0.62 |
| | | 13 | 0.71 |
| | | 15.6 | 0.75 |
| | | 18 | 1.46 |
| | | 20.2 | 1.46 |

**Table 2.2. 2 Comparison of lipid recovery efficiency obtained with different solvents**

| Oleaginous substance | Treatment conditions | Solvent | Lipid recovery efficiency % (w/w) | Reference |
|---|---|---|---|---|
| *Yarrowia lipolytica* | N-LS treatment | Chloroform/methanol (2:1) | 98.1± 1.1 | This study |
| *Yarrowia lipolytica* | N-LS treatment | Petroleum diesel | 94.7 ± 1.2 | This study |
| *Trichosporon oleaginous*[*] | Ultrasonication; 15 min, 25$^{o}$C | Chloroform/methanol (1:1) | 99.71 | Zhang *et al.,* 2014 |
| *Trichosporon oleaginous*[*] | Ultrasonication; 15 min, 25$^{o}$C | Hexane | 40.2 | Zhang *et al.,* 2014 |
| *Rhodosporidium toruloides* | Microwave pretreatment followed by enzyme treatment | Ethyl acetate | 86.9 | Jin *et al.,* 2012 |
| *Mucor circinelloides* | Ultrasonication; 30 min | Methanol | 35.72 | Mitra *et al.,* 2012 |
| *Nannaochloropsis* sp | Ultrasonication; 10 min | Water | 9.73 | Liang *et al.,* 2012 |

*****Dry biomass

**Table 2.2. 3 Optimization of petroleum diesel concentration to obtain maximum lipid separation efficiency % w/w (standard deviation less than 5%)**

| Solvents | Solvent volume (mL/g lipid) | Lipid separation efficiency % (w/w) | Lipid to biodiesel conversion efficiency % (w/w) |
|---|---|---|---|
| Chloroform/Methanol (2:1 v/v) | 50 | 100 | 96.1 ± 0.4 |
| Petroleum diesel | 5 | 71.6 ± 0.5 | 93.2 ± 1.1 |
| | 10 | 83.1 ± 0.8 | 95.3 ± 0.9 |
| | 15 | 88.7 ± 0.8 | 95.1 ± 1.2 |
| | 20 | 94.7 ± 1.2 | 97.2 ± 1.3 |
| | 30 | 94.5 ± 1.3 | 97.1 ± 1.3 |
| | 40 | 94.2 ± 0.9 | 96.9 ± 0.8 |
| | 50 | 95.1 ± 1.1 | 97.4 ± 1.1 |

**Table 2.2. 4 Comparison and effect of coagulants and bio flocculant on fatty acid profile (standard deviation less than 5%)**

| Fatty acids | Relative amount of fatty acids (% w/w) | | | | |
|---|---|---|---|---|---|
| | Control (without flocculation) | $CaCl_2$ (36mM) | $CaCl_2$ (36 mM) with EPS* | Alum (1.2mM) | Alum (1.2 mM) with EPS* |
| C 16:0 | 34.2 | 35.1 | 35.3 | 35.5 | 34.9 |
| C 16:1 | 2.9 | 3.1 | 3.0 | 2.7 | 3.1 |
| C18:1 | 9.2 | 10.2 | 9.9 | 10.2 | 10.4 |
| C18:2 | 47.8 | 47.2 | 48.0 | 47.9 | 47.8 |
| C18:3 | 10.1 | 9.9 | 10.3 | 10.0 | 9.8 |
| C20:1 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 |

*EPS – Extra polymeric substance

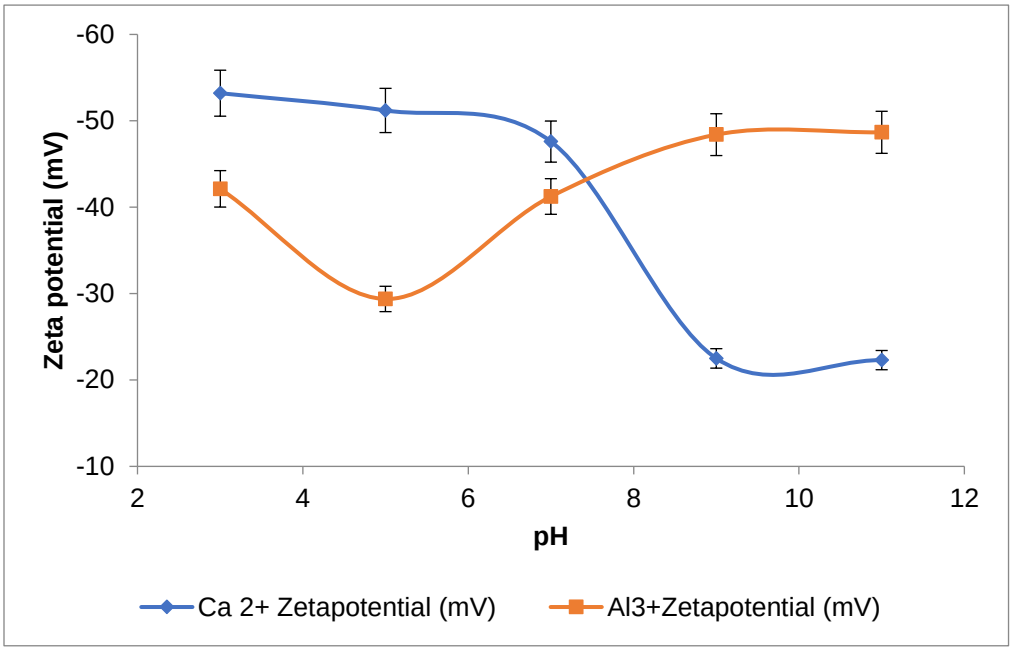



**Figure 2.2. 1 Effect of varying pH on zeta potential using Calcium chloride – $Ca^{2+}$ (36mM) and Alum – $Al^{3+}$ (1.2mM). (standard deviation less than 5%)**

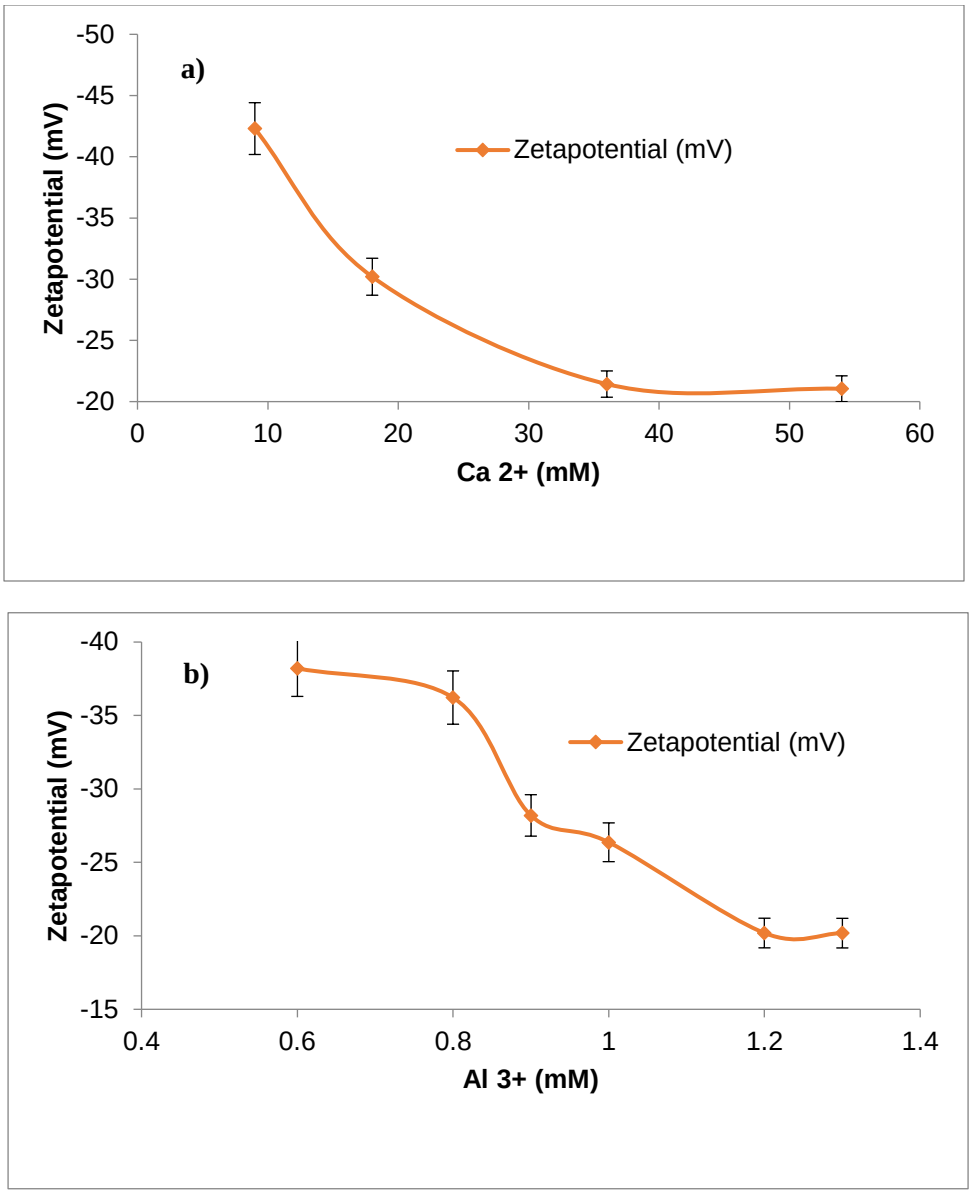
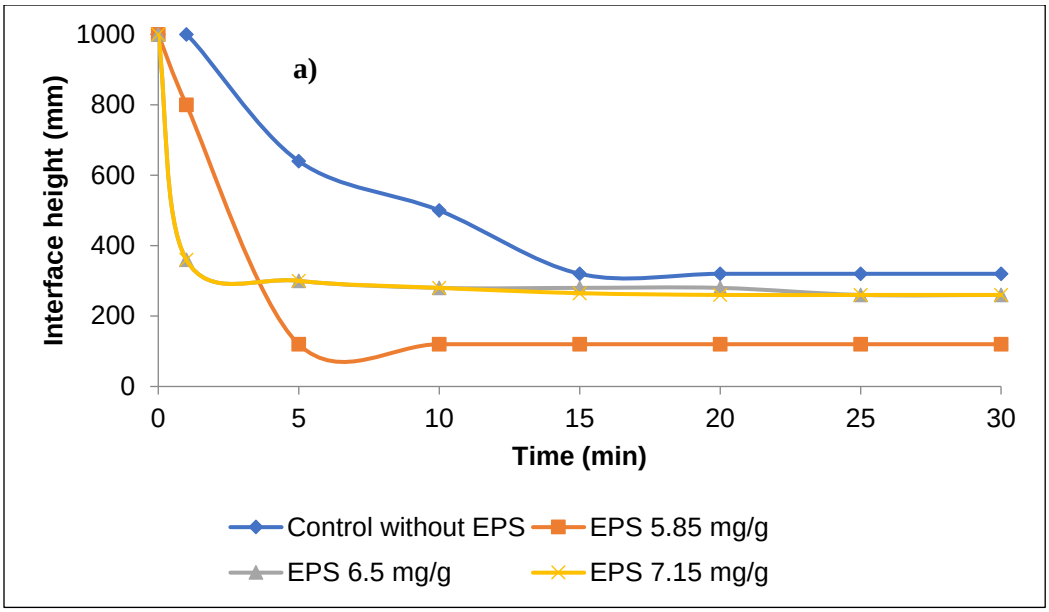



**Figure 2.2. 2 Variation of Zeta potential using different coagulant concentrations of a) Calcium chloride – $Ca^{2+}$ (9-54 mM) and b) Alum ($Al^{3+}$) (0.6 – 1.3 mM) (standard deviation less than 5%)**



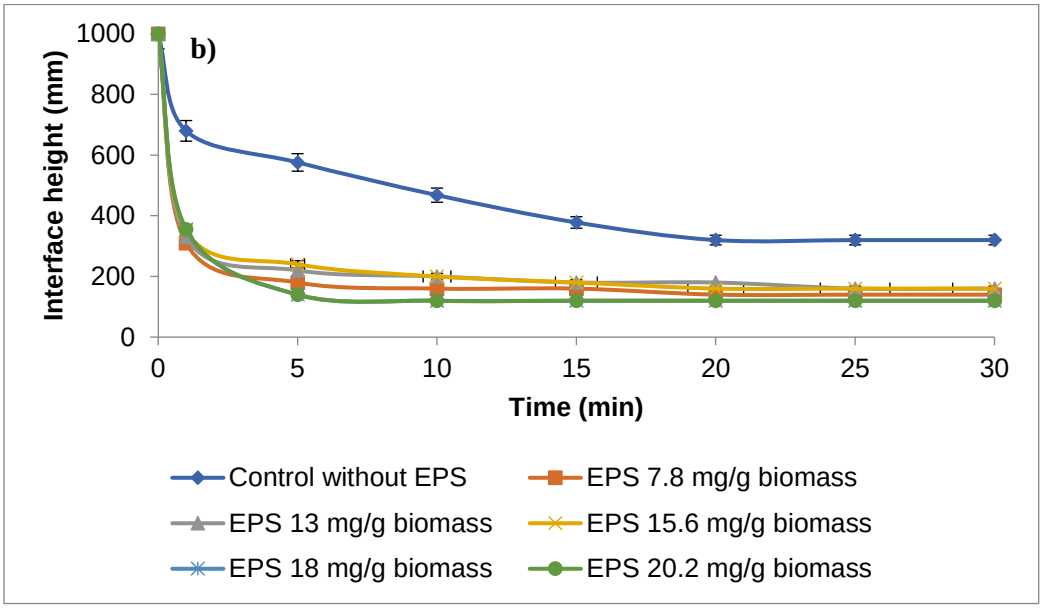
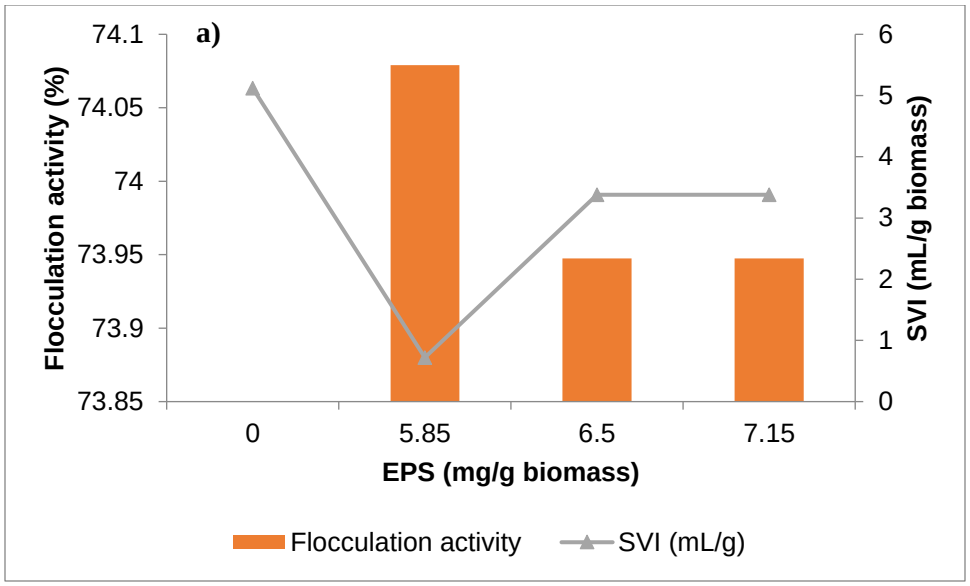




**Figure 2.2. 3 Solid liquid interface height of biomass settling with time: a) Calcium chloride - $Ca^{2+}$ (36mM) combination with different concentration of EPS mg/g biomass; b) Alum - $Al^{3+}$ (1.2mM) combination with different concentration of EPS mg/g biomass. (Standard d deviation less than 5%)**



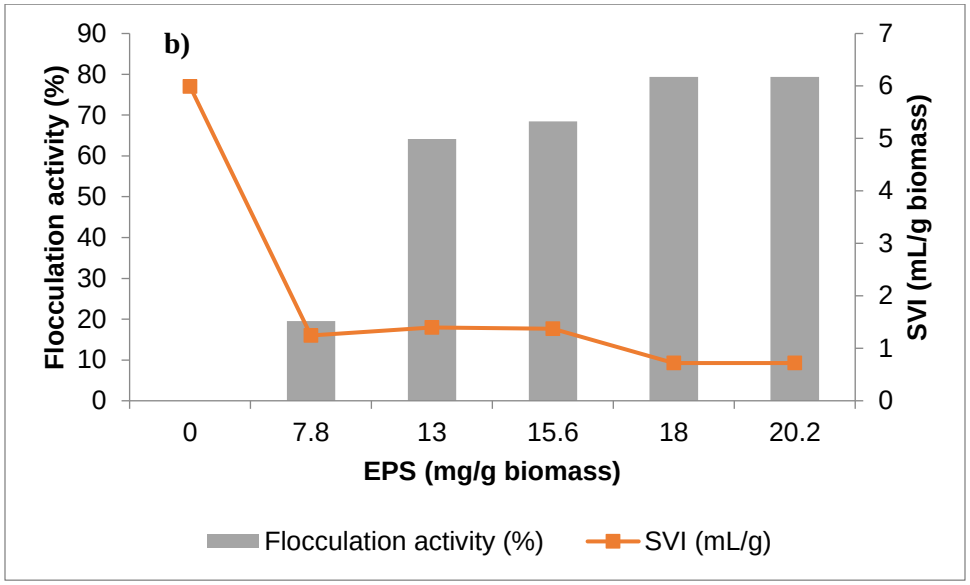




**Figure 2.2. 4 Flocculation activity and sediment volume index (SVI): a) $Ca^{2+}$ (36mM) with combination of different EPS concentrations b) $Al^{3+}$ (1.2mM) with combination of different EPS concentrations**

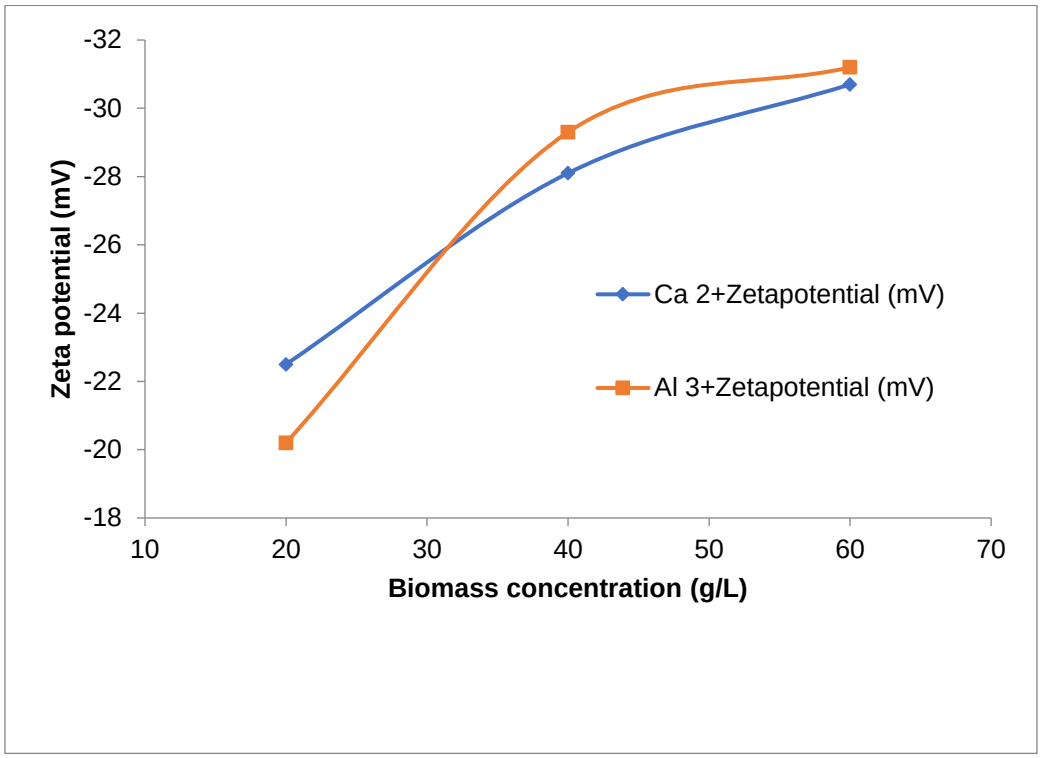



**Figure 2.2. 5 Effect of Calcium chloride - $Ca^{2+}$ (36mM) and Alum- $Al^{3+}$ (1.2mM) on zeta potential by varying different biomass concentrations (20, 40 and 60 g/L) (standard deviation less than 5%)**

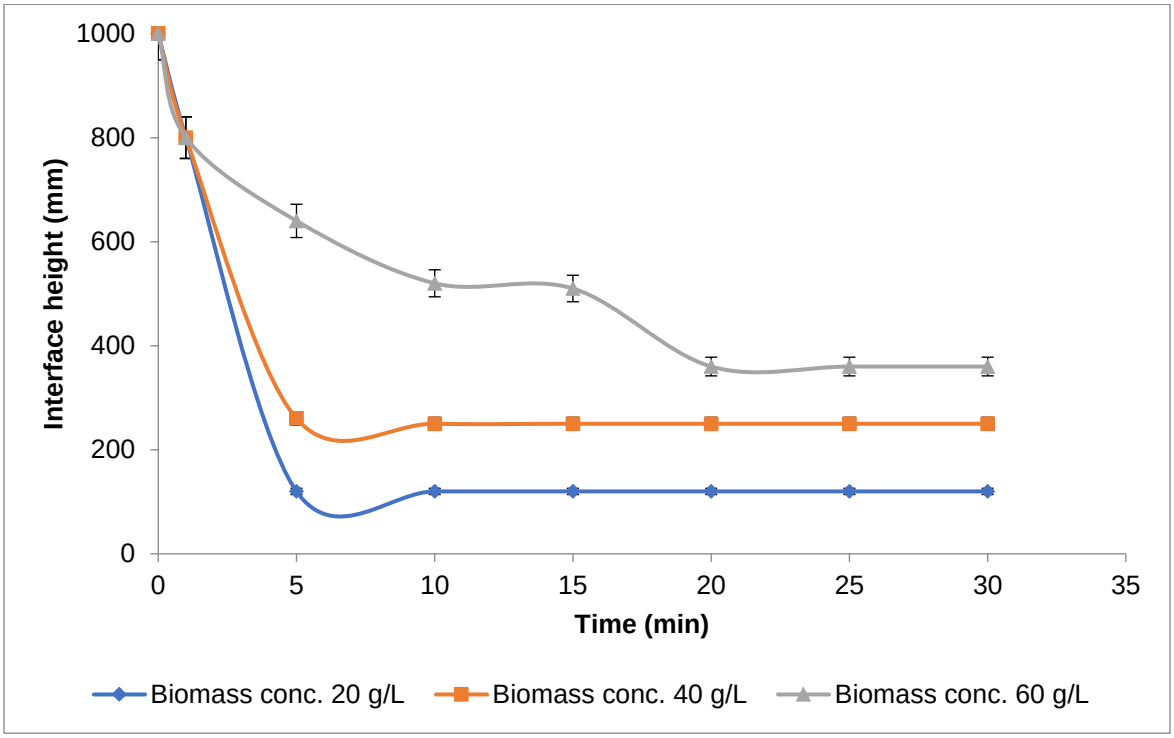



**Figure 2.2. 6 Variation of solid liquid interface height during biomass settling at different biomass concentration 20, 40 and 60 g/L using combination of Ca2+ (36, 52 and 70 mM) and different EPS concentration (5.85, 39.9 and 119 mg/g biomass). (Standard deviation less than 5%)**

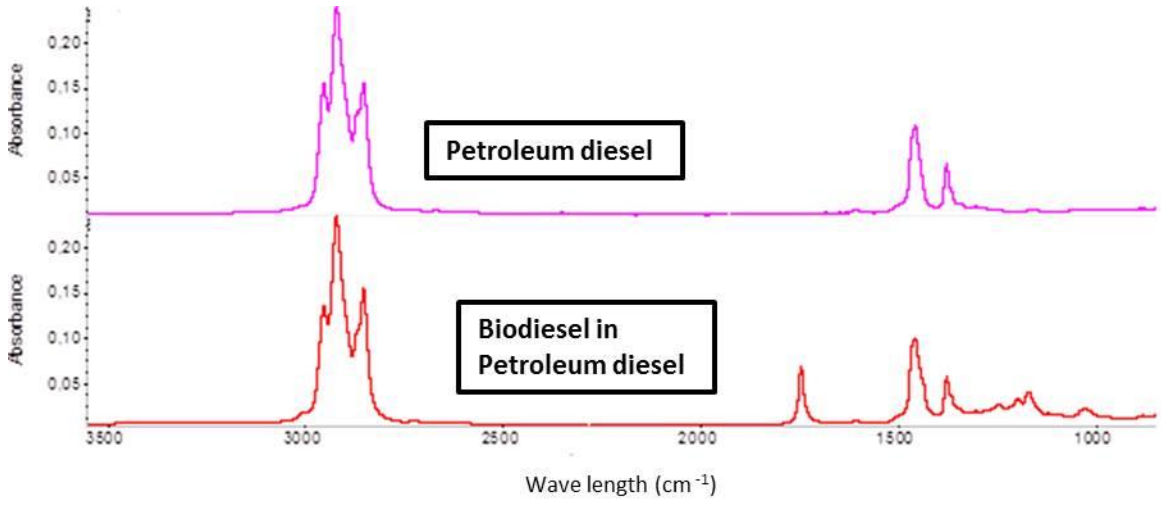



**Figure 2.2. 7 Variation of petroleum diesel and biodiesel spectra peaks using Fourier transform-Infrared spectroscopy (FT-IR)**

# CHAPTER II

# PARTIE 3

# DETERGENT ASSISTED LIPID EXTRACTION FROM WET YEAST BIOMASS FOR BIODIESEL: A RESPONSE SURFACE METHODOLOGY APPROACH

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# RÈSUMÈ

L'extraction des lipides à partir de la biomasse microbienne est un processus fastidieux et coûteux. Dans la présente étude, l'extraction de lipides de la culture de levure *Yarrowia lipolytica* SKY-7 a été réalisée à l'aide d'un détergent. La Méthodologie de surface de réponse (RSM) a été utilisé pour étudier l'effet de trois paramètres principaux (concentration de N-LS, temps et température) sur l'efficacité d'extraction des lipides microbiens % (p/p). La récupération maximale des lipides de 95,3 ± 0,3% p/p a été obtenue au niveau optimal des paramètres étudiés [concentration de N-LS 24,42 mg (égale à 48 mg N-LS/g de biomasse sèche), temps de traitement 8,8 min et température de réaction 30,2 ° C]. Tandis que l'extraction conventionnelle en utilisant le chloroforme et le méthanol a nécessité 12 heures à 60°C pour atteindre une récupération complète de lipides. L'étude a confirmé que le traitement de la biomasse de levures oléagineuses avec la N-lauroyl sarcosine, pourrait être une approche prometteuse pour la récupération des lipides microbiens à l'échelle industrielle.

# ABSTRACT

The lipid extraction from the microbial biomass is a tedious and high cost dependent process. In the present study, detergent assisted lipids extraction from the culture of the yeast *Yarrowia lipolytica* SKY-7 was carried out. Response surface methodology (RSM) was used to investigate the effect of three principle parameters (N-LS concentration, time and temperature) on microbial lipid extraction efficiency % (w/w). The results obtained by statistical analysis showed that the quadratic model fits in all cases. Maximum lipid recovery of 95.3 ± 0.3 % w/w was obtained at the optimum level of process variables [N-LS concentration 24.42 mg (equal to 48 mg N-LS/g dry biomass), treatment time 8.8 min and reaction temperature 30.2 $^{o}$C]. Whereas the conventional chloroform and methanol extraction to achieve total lipid recovery required 12 h at 60 $^{o}$C. The study confirmed that oleaginous yeast biomass treatment with N-lauroyl sarcosine would be a promising approach for industrial scale microbial lipid recovery.



**Keywords:** Lipid extraction, N-lauroyl sarcosine, Box-Behnken design, Biodiesel

# 1. INTRODUCTION

Biomass based biodiesel is a recent promoting approach to study alternative fossil based fuel due to concerns of decreasing oil reservoirs and less emission of greenhouse gasses (GHG) (Martínez *et al.*, 2015; Moser, 2011). The current research is focused on biotransforming industrial waste like crude glycerol, lignocellulosic waste, and municipal secondary sludge to renewable fuel (biodiesel) using heterotrophic oleaginous microorganisms (Capus *et al.*, 2016; Johnson *et al.*, 2007; Kumar *et al.*, 2009; Seo *et al.*, 2013). The microbial production of lipid will occupy less arable land and will not affect the food supply chain (Martínez *et al.*, 2015).

Biodiesel production utilizing oleaginous yeast consists of three major steps, microorganism cultivation (lipid accumulation), cell wall disruption and lipid extraction from the biomass, and transesterification. Lipid is energy storage (lipid droplets) and structural components of the cell membrane. The lipid droplets are enveloped by phospholipid membrane and outer cell membrane has to be disrupted to free the microbial lipid. There are two widely known methods, i.e. organic solvent extraction and mechanical pressing that have been used to extract lipid from lipid bearing substances. The main disadvantages of these methods are the low lipid yield and long process time required for extraction (Cheng *et al.*, 2011). Therefore, method with high lipid yield and less process time is required.

Traditionally chloroform and methanol-based lipid extraction is effective, but it is time consuming (8-12h), needs temperature up to 60 $^{o}$C and solvents are toxic having safety concern. Therefore, decreasing solvent volume and time of extraction are main factors for cost effective lipid extraction and safety reasons. Consequently, lipid extraction from dried biomass employing mechanical cell disruption processes such as bead milling, homogenization, microwave, ultra-sonication is an energy intensive process (Garoma *et al.*, 2016; Jin *et al.*, 2012; Lee *et al.*, 2010; Zhang *et al.*, 2014b). The non mechanical methods such as lytic enzyme treatment, alkali and acid (Jin *et al.*, 2012; Miranda *et al.*, 2012) are cost prohibitive for large-scale microbial lipid extraction.

The moisture content of the cell biomass (which is more than 80% on weight basis) needs to be removed by oven drying or lyophilization (dewatering) process before lipid extraction. Various researchers have investigated different methods of lipid extraction and in-situ transesterification from wet biomass including supercritical methanol (Patil *et al.*, 2011), Enzyme assisted extraction (Jin *et al.*, 2012), Ethanol (Yang *et al.*, 2014), Simultaneous distillation and extraction process (SDEP) (Tanzi *et al.*, 2013), osmotic shock (Yoo *et al.*, 2012), acid and base hydrolysis (Sathish *et al.*, 2012) and 3_DAPS (Lai *et al.*, 2016) Unfortunately, the most cases still require high energy input, time. Therefore, lipid extraction technologies are up to now within the laboratory scale. Therefore, there is lack of suitable industrial scale lipid extraction has not been developed.

N-lauroyl sarcosine (N-LS), an amino acid derived detergent (an anionic detergent, made up of amino acid sarcosine and fatty acid), is non-toxic, and biodegradable (Kippert, 1995). It can disrupt the cell wall by the formation of micelle at certain specific N-LS concentration, incubation time and temperature (Abraham *et al.*, 2008; Yadav *et al.*, 2014). It can be safely used for permeabilization of yeast cells to release intracellular enzyme activities (Yadav *et al.*, 2014). There are several chemical surfactants like Triton -100, sodium dodecyl sulfate (SDS),toluene and cetyltrimethyl ammonium bromide (CTAB) that have been used for yeast cells permeabilization (Abraham *et al.*, 2008; Kippert, 1995). But most of them are toxic and environmentally unsafe.

The objective of the present study is to investigate the wet biomass cell disruption for lipid extraction using N-lauroyl sarcosine. Response surface methodology was used to optimize the process parameters to obtain maximum lipid extraction efficiency. Three important parameters (N-LS concentration, incubation time and incubation temperature) were considered to study the impact on lipid extraction.

# 2. MATERIALS AND METHODS

## 2.1 Strain, production and harvesting conditions

*Yarrowia lipolytica* SKY-7, oleaginous yeast (isolated in our lab INRS-ETE Quebec, Canada) was used in this study (Kuttiraja *et al.*, 2015). The yeast strain was grown in a medium containing 500

mL of crude glycerol solution with 11% (w/v) glycerol (by-product of biodiesel production, obtained from a biodiesel producing industry in Quebec, Canada) and 8.5 L starch industry wastewater (SIW) in a 15L fermenter with working volume 10L, (Biogene, Quebec). SIW was obtained from a starch producing industry in Québec. The fermenter was operated at constant pH 6.8-7.0 and temperature 28 $^{o}$C and dissolved oxygen was maintained above 30% of saturation. After 72 h of fermentation, the broth was heat treated in the fermenter (to kill cells and preserve the accumulated lipid inside the cells) at 80 ± 2 $^{o}$C for 10 min (Zhang *et al.*, 2015). Thereafter, biomass was harvested by centrifugation at 8000 rpm for 10 min. The biomass was washed with warm water to remove residual glycerol and soap. To perform lipid extraction and to estimate biomass dry weight, 3.1±0.2g wet biomass (83.8 % moisture content) harvested from 25 mL fermented broth was used.

## 2.2 Conventional (chloroform-methanol assisted) lipid extraction

The standard chloroform and methanol extraction was used to determine the lipid content in the biomass (Bligh *et al.*, 1959; Folch *et al.*, 1957; Vicente *et al.*, 2009). The washed wet biomass pellet (3.1±0.2g) was mixed with 15 mL solvent mixture of chloroform and methanol (2:1 v/v), and then incubated for 4h in an agitator water bath at 60 $^{o}$C and 100rpm. The mixture was then centrifuged at 4000 rpm for 10 min. The mixture was separated in three different layers. The residual biomass was in the bottom layer, middle phase was lipid in chloroform and top layer methanol and water. The middle layer of chloroform containing lipid was pipetted out and transferred into a pre-weighed glass tube ($L_1$). The rest of the solution (containing cell debris, methanol) was again fortified with 15mL solvent mixture of chloroform and methanol (2:1 v/v) and again incubated for 4h at 60 $^{o}$C in the agitated water bath. After 4 h incubation, the solution was filtered using vacuum filtration. The filtrate was mixed with previously extracted solution (chloroform solution containing lipid) and the mixed solution was allowed to stand for phase separation. The bottom phase containing lipid in chloroform (the other phase was water and methanol) was collected and subjected to nitrogen sparging until total chloroform evaporated. The samples were further dried in an oven at 60 $^{o}$C until constant weight ($L_2$). The lipid recovery from the biomass calculated as.

$$CL\ \% = \frac{L2-L1}{DBW} \times 100\% \text{ --------- (1)}$$

The obtained lipid was stored for further transesterification study. Equation (1) CL represents weight obtained from conventional lipid extraction, $L_1$ expresses the pre-weighed glass tube and $L_2$ denotes the oven dried microbial lipid in a pre-weighed glass tube and DBW denotes dry biomass weight.

## 2.3 N-lauroyl sarcosine assisted lipid extraction

The lipid bearing wet biomass (3.1±0.2g wet biomass) harvested by centrifugation after fermentation was used in each experiment to extract lipid using N-lauroyl sarcosine (N-LS). The lipid extraction process was optimized by using response surface methodology (RSM) to minimize the number of experiments and to obtain a statistical validation of optimal experimental parameters. The following three parameters were optimized with constant volume two mL (3.1 g wet biomass): N-LS concentration (6.25 – 40 mg) (which is equivalent to 12.5 to 80 mg N-LS/g dry biomass), Temperature (25 – 35 $^{o}$C) and Time (5 – 20 min). After the N-LS reaction, 15 mL of 1:1 (v/v) chloroform and methanol solution was added to each sample (N-LS treated biomass solution) and the solution was filtered. A control study was also conducted each time where an equal volume of water without N-LS was added to wet biomass followed by addition of 15 mL of 1:1 (v/v) chloroform and methanol solution and the solution was filtered without incubation. The filtrate of each sample and the control was allowed to stand for 15 minutes for phase separation. The lipids were recovered in chloroform phase (the bottom phase), whereas water and methanol separated in the top phase. For each sample, the recovered chloroform phase was evaporate using nitrogen gas and simultaneously applying heat (65 $^{o}$C) to evaporate chloroform followed by drying at 60 $^{o}$C, as stated above. The lipids thus obtained were converted to FAMEs (Fatty acid methyl esters) through transesterification as described below in **section 2.6**.

## 2.4 Experimental design and statistical analysis

Response surface methodology (RSM) was applied to optimize the process variables to improve the lipid extraction efficiency using N-LS. Seventeen experiments were conducted using Box-

Behnken design (BBD) to study the impact of variables on lipid extraction (Table 2.3.1). A second order quadratic equation was used to fit the experimental data. Based on the response obtained, the statistical analysis such as analysis of variance (ANOVA), regression coefficient ($R^2$) was executed. To evaluate the importance of extraction parameters, 3D plots were prepared using the Design expert version 9.1. (Stat-Ease Inc., Minneapolis, USA). Finally, a validation experiment was conducted in triplicate and the correlation between the predicted and the experimental values were analyzed by correlation coefficient using Microsoft excel. The final response extraction efficiency % w/w was calculated (Table 2.3.1).

## 2.5 Large scale N-LS assisted lipid extraction

To further verify the optimum conditions determined by RSM, a higher scale experiment for lipid, the wet biomass 124, 248 and 372 ±2.5 g (equivalent to 20, 40 and 60 g dry biomass) obtained from 1, 2 and 3 L fermentation broth by centrifugation was mixed with water to make up to 80 mL containing 0.925 g N-LS (which is equivalent to 46.25, 23.12, 15.41 mg N-LS/g dry biomass) and kept at 170 rpm and 30 $^{o}$C for 12.5 minutes. The optimum conditions determined in the previous section was 124 g wet biomass suspended in water containing 0.925 g N-LS (final volume 80 mL). However, higher concentration of wet biomass (248 and 372 g in 80 mL solution) while treating with same concentration of N-LS was also tested to find out the change in the efficiency of lipid extraction. After incubation, 10 mL of each solution, in triplicate, was taken in different test tubes and 50 mL chloroform/methanol (1:1) solution per gram of lipid was added to each sample. The resulting samples were filtered without incubation and the filtrates were allowed to stand for 15 minutes for phase separation. Lower layer consists of chloroform with lipid and top layer with water and methanol. The chloroform phase was treated with nitrogen gas and simultaneously by heating at 65$^{o}$C to evaporate chloroform. Samples were further allowed to heat drying at 60$^{o}$C until constant weight. To determine the lipid weight, a parallel conventional lipid extraction or control experiment (i.e. without N-LS treatment as described in **section 2.2**) was conducted to calculate the recovery efficiency of lipids in the N-LS process.

## 2.6. Lipid transesterification

Lipids obtained by N-LS aided extraction was dissolved in hexane (25 mL hexane per gram lipid), then mixed with methanol (methanol to lipid ratio 6:1 or 0.4 mL methanol per gram lipid) containing sodium hydroxide as a catalyst with concentration of 1% w/w (1 g NaOH/ 100g oil). The mixture was then heated to 55 $^{o}$C for 2 h. After the reaction, NaCl solution was added (50 mL of 5% w/v NaCl solution per gram lipid) and the solution was allowed to stand for 15 min. FAMEs were extracted in hexane (top) phase. The bottom phase was again treated with hexane (25 mL per gram lipid, to remove non-recovered FAMEs) and FAMEs were separated and mixed with the fraction separated earlier. The FAMEs in hexane was washed with sodium bicarbonate solution (10 mL of 2% w/v solution per gram lipid), and the top hexane layer was then dried at 60 $^{o}$C in an oven (Halim *et al.*, 2011).

The FAMEs were re-dissolved in hexane (0.01 g lipid/10 mL hexane) and analyzed using a Gas Chromatograph coupled with FID (GC-FID) (Perkin Elmer, Clarus 500). The dimensions of the column used are 30 m x 0.25 mm, with a phase thickness of 0.2 µm. Helium was the carrier gas at a flow rate of 1.18 ml/min with the oven temperature 230 $^{o}$C. Transesterified sample of 1µl was injected with an automated sample injector and the sample analysis was performed with Agilent chem Station module software from Agilent technologies. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1,3-Dichlorobenzene was used as internal standard with concentration of 50 ppm.

## 3. RESULTS AND DISCUSSION

In this study, the lipid extracted from the wet biomass (containing 83.8% moisture content) employing conventional chloroform and methanol (2:1 v/v) lipid extraction method **(Section 2.2)** was assumed as 100% lipid in the biomass. The lipid obtained from the conventional lipid extraction process was 0.210 g per 3.1±0.2 g wet biomass of *Yarrowia lipolytica*. The ratio between wet and dry biomass was about 8.3:1 (i.e. 8.38 g wet weight was equal to 1g dry weight). Lipid extraction efficiency was the ratio of the total lipid extracted by N-LS treatment of the biomass and the lipid extracted by conventional method taking the same biomass weight.

## 3.1 Optimization of lipid extraction process

In order to obtain a complete cell disruption and higher lipid extraction efficiency, a response surface method (BBD) was employed. As shown in Table 2.3.2, significance of each term was determined by *p*-value (Prob > *F*). The terms A, B and C, AC and BC were significant, with very small *p*- values ($p < 0.05$) and the other term coefficient AB was not significant ($p > 0.05$).

The lack of fit test compares the residual error to the pure error from replicated design points. The lack of fit *F*-value of 6.28 is not significant as the *p*- value is > 0.05. The non-significant lack-of-fit showed that the model was valid for the present work. The optimum concentration of N-LS required for cell disruption, temperature and time could be expressed using the following equation:

$$\text{Extraction efficiency \%} = 99.62 + 2.41 \times A + 1.41 \times B + 1.90 \times C + 0.12 \times AB - 1.20 \times AC - 1.00 \times BC - 19.50 \times A^2 - 16.35 \times B^2 - 12.92 \times C^2$$

Where A (N-LS concentration), B (Temperature) and C (Time) are reaction conditions as shown in Table 2.3.3.

The coefficient of determination ($R^2$) of the model was 0.9995, which indicated a good fit between predicted values and the experimental data points. The model has high $R^2$ value; significant *F* -value, an insignificant lack of fit *p*-value, low standard deviation (0.55) and good coefficient of variance (0.71%). These results indicate a high precision in predicting the lipid extraction efficiency using N-LS. Therefore, the model was used for further analysis.

Predicted $R^2$ is a measure of how good the model predicts a response value. To be in reasonable agreement, the adjusted $R^2$ (0.9988) and predicted $R^2$ (0.9928) values should be with in ~ 0.20 difference of each other. The predicted and adjusted $R^2$ are close to 0.9988 with the difference of 0.0060, which is smaller than 0.2. It indicates that 99% of the variability in the response (extraction efficiency) could be explained by the model and provides accurate description of the experimental data indicating successful correlation among process parameters and extraction efficiency.

### 3.1.1 Effect of reaction variables on extraction efficiency percentage

The impact of reaction variables including N-LS concentration, temperature and time on extraction efficiency are in Table 2.3.3 and Figure 2.3.1. The lipid extraction efficiency increased up to N-LS concentration 23.12 mg (equal to 46.2 mg N-LS/g dry biomass), temperature 30 $^{o}$C and time > 8 min and there after started decreasing, whereas in Table 2.3.3 explains that by increasing the N-LS concentration from 6.25 to 40 mg (equal to 12.5 to 80 mg N-LS/g dry biomass) (actual factors: temperature at 30 $^{o}$C and time 12.5 min), lipid extraction efficiency increased (99.2 %) up to the mid-point of N-LS concentration and thereafter it decreased up to 82 %. A similar scenario of results was observed with combination of N-LS concentration and temperature factors (AB) (Figure 2.3.1a); lipid extraction efficiency increased up to 99.2% with N-LS concentration 23.12 mg and temperature 30 $^{o}$C and thereafter efficiency decreased.

At lower N-LS concentration, a decrease in lipid extraction efficiency % (w/w) might be due to incomplete cell wall disruption as surfactant only intercalates the membrane. (Kaur *et al.*, 2010). The Increase in the concentration of surfactant disrupts the membrane and form mixed micelles containing the surfactant, proteins and lipids. Concentration of the surfactant should be above critical micelle concentration (CMC) to maintain micelles. But above a particular concentration, micelles completely displaces the lipids and leaving only proteins within it (Maire *et al.*, 2000). The mechanism of permeabilization also depends on the interaction of the detergents with proteins and the lipids of the cell membrane, which results in pore formation in the membrane or perturbation of the membrane structure (Vasileva-Tonkova *et al.*, 2001).

In Table 2.3.1 there is variation in extraction efficiency with increase in temperature from 25 to 35 $^{o}$C (actual factors- N-LS concentration 23.12 and time 12.5 min). The maximum extraction efficiency obtained at 30 $^{o}$C while increasing the temperature, lipid extraction efficiency proportionally decreased from 99.2 to 84 %. It might be due to the fact that at this particular temperature (30 $^{o}$C), N-LS caused enough membrane distortion to release the intracellular lipid (Abraham *et al.*, 2008). For ionic detergents, hydrophobic and head group interactions change with temperature as micellization is greatly affected by varying temperature (Mohajeri *et al.*, 2012).The combination of temperature and time (BC) (Figure 2.3.1b). also gave maximum lipid

extraction efficiency around 30 $^{o}$C in only 12.5 min. incubation time (*p* value 0.0082) Table 2.3.3 indicates the maximum lipid extraction efficiency at 12.5 min incubation time and also in combination of (AC) N-LS concentration and time (Figure 2.3.1c), maximum extraction efficiency (99.7 %) observed at N-LS concentration 23.12 mg with significant *p* value 0.0032.

### 3.1.2 Process validation

Based on the above experimental design, a statistical prediction of optimal process parameters was performed. The predicted experimental variables vary as follows: N-LS concentration 24.42 mg (which is equivalent to 48 mg N-LS/g dry biomass), temperature 30.2 $^{o}$C and time 8.8 min. The desirability of the predicted values is 0.9. To verify the predicted model terms of optimized value of lipid extraction, one independent experiment in triplicate was conducted and extraction efficiency was found to be 95.43 %, which is approximately equal to the predicted extraction efficiency 95.8 % with ± 0.4 % error. This result proves that the predicted model terms are optimal for lipid extraction from wet biomass.

Thus, employing N-LS, the cells are disrupted and total lipids are released in 8.8 minutes. After cell disruption, the ratio of chloroform and methanol required for a complete separation from other components was 1:1 v/v, whereas the conventional method uses a higher ratio of chloroform and methanol (2:1 v/v) and 8-12h is required to achieve the similar results. Moreover, a complete lipid release from the cells was accomplished at 30 $^{o}$C with N-LS instead of 60 $^{o}$C for conventional chloroform-methanol extraction procedure. Different cell disruption methods such as microwave irradiation, bead beating, ultrasonication have been tested for lipid extraction from microalgae, yeast and fungi with a mixture of chloroform and methanol (Lee *et al.*, 2010; Zhang *et al.*, 2014) with lyophilized biomass. However, dewatering and drying the biomass is an expensive process for large scale operation of microbial lipid recovery process. Bonturi (2015) and co-workers used acid pretreatment by the addition of HCL followed by the treatment using commercial enzyme from *Trichoderma harzianium* (0.1g enzyme/g dry cell mass) for cell wall disruption. The lipid from the disrupted cells was extracted using hexane or combination of chloroform and methanol. Finally 42% and 48% lipids were recovered from *R.toruloides* and *L.starkeyi* in 4 h extraction time. Bogdan (2014) and co-workers used supercritical carbon dioxide for extracting lipids from fungi

mycelial and total fatty acids recovered was 45 wt%. The cost of biodiesel production is higher if lipid extraction consists of multiple steps. In our case, process is accomplished in a single step, the cell disruption time decreased to 8.8 minutes. The maximum extraction efficiency of 95.43 ±0.3 %w/w is comparable to other reports (Table 2.3.4).

## 3.2 Large scale N-LS assisted lipid extraction

On a large-scale process, the wet biomass 124, 248 and 372 ±2.5 g (equivalent to 20, 40 and 60 g dry biomass) obtained from 1, 2 and 3 L fermentation broth treated with 80 mL of solution containing 0.976 g N-LS (which is equivalent to 46.25, 23.15, 15.41 mg N-LS/g dry biomass) for 12.5 minutes gave 96.9± 1.1, 73.8 ± 0.6 and 42.9 ± 0.3 % w/w efficiency of lipid recovery. This suggests that detergent concentration should proportionally increase with biomass concentration and optimum concentration should be used [wet biomass 124 g, N-LS solution 80 mL with 0.976g N-LS concentration at 30 ± 0.1 $^{o}$C]. In the present scenario, the lipid extraction efficiency is 1.6 % w/w lower than the conventional process, which is insignificant. Thus, this study suggests that N-LS aided cell disruption and release of intracellular lipid followed by extraction with chloroform and methanol (1:1 v/v) would be a promising alternative in comparison to conventional chloroform, methanol extraction or mechanical based lipid extraction as they are time and energy consuming. The extracted lipid can also be recovered by replacing chloroform-methanol with other environment friendly solvents such as ethyl acetate, dimethyl carbonate. These studies are underway in our laboratory.

## 3.3 Profile of FAMEs obtained from lipid extracted with N-lauroyl sarcosine treated biomass

The efficiency of lipid conversion to FAMEs is 94.3% (w/w) of total lipids. Lipid mainly contains C16, C18 and C23 fatty acid methyl ester, which is known as biodiesel (Giroud *et al.*, 2013). The cell membrane and lipid bodies are covered with phospholipids. The chloroform and methanol mixture interact with phospholipid (which consists of hydrophobic tail and hydrophilic head) and tears the membrane and releases neutral lipids, mainly containing C 16:0, C 18:0, C 18:1, C 18:2 fatty acids. The Fatty acid profile obtained with N-lauroyl sarcosine assisted lipid extraction and

conventional lipid extraction was presented (Table 2.3.5).The different cell disruption methods like bead vortexing, water bath, grinding with liquid $N_2$, osmotic shock and sonication affect the composition of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and fatty acid methyl esters (FAMEs) (Byreddy et al., 2015). In our case the saturation rate is around 42.5% w/w total lipids (Table 2.3.5), which indicates that the biodiesel will have high oxidation stability than jatropha biodiesel (which is less than 30% weight saturated lipids/ weight of the total lipids). The high oxidation stability tends to increase viscosity and melting point (Kumar Tiwari *et al.*, 2007). It is concluded that there is no significant change in fatty acid profile by using N-lauroyl sarcosine assisted lipid extraction from *Yarrowia lipolytica* SKY-7 and the fatty acids are suitable for biodiesel production.

## 4. CONCLUSION

The low cost and efficient cell disruption method for lipid release from oleaginous yeast *Y.lipolytica* process has been successfully tested. N-lauroyl sarcosine aided cell disruption and lipid release from the cells followed by lipid separation using a lower volume of chloroform and methanol (1:1 v/v) revealed a high lipid extraction efficiency of 95.43% (w/w). The time required to achieve high extraction efficiency was reduced from 12 h in conventional process, i.e. chloroform and methanol assisted cell disruption and lipid recovery, to 8.8 minutes in N-LS aided extraction process. The incubation temperature (30.2$^{o}$ C) and N-LS concentration 48 mg N-LS/g dry biomass were the most important parameters that affected the cell disruption and lipid release.


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# 5. REFERENCES


1. Abraham J & Bhat SG (2008) Permeabilization of baker's yeast with N-lauroyl sarcosine. *J Ind Microbiol Biotechnol* 35(8):799-804.

2. Bligh EG & Dyer WJ (1959) A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Canadian Journal of Biochemistry and Physiology* 37(8):911-917.

3. Bogdan VI, Koklin AE, Krasovsky VG, Lunin VV, Sergeeva YE, Ivashechkin AA & Feofilova EP (2014) Production of fatty acid methyl esters that are the basis for biodiesel fuel from mycelial fungi lipids extracted by supercritical $CO_2$. *Russian Journal of Physical Chemistry B* 8(8):1004-1008.

4. Bonturi N, Matsakas L, Nilsson R, Christakopoulos P, Miranda EA, Berglund KA & Rova U (2015) Single cell oil producing yeasts *Lipomyces starkeyi* and *Rhodosporidium toruloides*: Selection of extraction strategies and biodiesel property prediction. *Energies* 8(6):5040-5052.

5. Byreddy AR, Gupta A, Barrow CJ & Puri M (2015) Comparison of cell disruption methods for improving lipid extraction from thraustochytrid strains. *Marine Drugs* 13(8):5111-5127.

6. Capus A, Monnerat M, Ribeiro LC, de Souza W, Martins JL & Sant'Anna C (2016) Application of high-content image analysis for quantitatively estimating lipid accumulation in oleaginous yeasts with potential for use in biodiesel production. *Bioresource Technology* 203:309-317.

7. Cheng C-H, Du T-B, Pi H-C, Jang S-M, Lin Y-H & Lee H-T (2011) Comparative study of lipid extraction from microalgae by organic solvent and supercritical $CO_2$. *Bioresource Technology* 102(21):10151-10153.

8. Dejoye Tanzi C, Abert Vian M & Chemat F (2013) New procedure for extraction of algal lipids from wet biomass: a green clean and scalable process. *Bioresour Technol* 134:271-275.

9. Folch J, Lees M & Stanley GHS (1957) A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES. *Journal of Biological Chemistry* 226(1):497-509.

10. Garoma T & Janda D (2016) Investigation of the effects of microalgal cell concentration and electroporation, microwave and ultrasonication on lipid extraction efficiency. *Renewable Energy* 86:117-123.

11. Giroud S, Frare C, Strijkstra A, Boerema A, Arnold W & Ruf T (2013) Membrane phospholipid fatty acid composition regulates cardiac SERCA activity in a hibernator, the Syrian hamster (Mesocricetus auratus). *PloS one* 8(5):e63111.

12. Halim R, Gladman B, Danquah MK & Webley PA (2011) Oil extraction from microalgae for biodiesel production. *Bioresource technology* 102(1):178-185.

13. Jin G, Yang F, Hu C, Shen H & Zhao ZK (2012) Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast *Rhodosporidium toruloides*. *Bioresource technology* 111:378-382.

14. Johnson DT & Taconi KA (2007) The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environmental Progress* 26(4):338-348.

15. Kaur P & Satyanarayana T (2010) Improvement in cell-bound phytase activity of *Pichia anomala* by permeabilization and applicability of permeabilized cells in soymilk dephytinization. *J Appl Microbiol* 108(6):2041-2049.

16. Kippert F (1995) A rapid permeabilization procedure for accurate quantitative determination of β-galactosidase activity in yeast cells. *FEMS Microbiology Letters* 128(2):201-206.

17. Kumar P, Barrett DM, Delwiche MJ & Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial and Engineering Chemistry Research* 48(8):3713-3729.

18. Kumar Tiwari A, Kumar A & Raheman H (2007) Biodiesel production from jatropha oil (Jatropha curcas) with high free fatty acids: An optimized process. *Biomass and Bioenergy* 31(8):569-575.

19. Kuttiraja M, Krishna S, Dhouha A & Tyagi RD (2015) A Substrate-Based Approach for the Selection of Oil-Bearing Heterotrophs from Nitrogen-Deficient Soil for Lipid Production. *Applied Biochemistry and Biotechnology* 175(4):1926-1937.

20. Lai YS, Francesco FD, Aguinaga A, Parameswaran P & Rittmann BE (2016) Improving lipid recovery from Scenedesmus wet biomass by surfactant-assisted disruption. *Green Chem.* 18(5):1319-1326.

21. le Maire M, Champeil P & Møller JV (2000) Interaction of membrane proteins and lipids with solubilizing detergents. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1508(1):86-111.

22. Lee JY, Yoo C, Jun SY, Ahn CY & Oh HM (2010) Comparison of several methods for effective lipid extraction from microalgae. *Bioresource technology* 101 Suppl 1:S75-77.

23. Martínez EJ, Raghavan V, González-Andrés F & Gómez X (2015) New Biofuel Alternatives: Integrating Waste Management and Single Cell Oil Production. *International journal of molecular sciences* 16(5):9385-9405.

24. Miranda JR, Passarinho PC & Gouveia L (2012) Pre-treatment optimization of *Scenedesmus obliquus* microalga for bioethanol production. *Bioresource Technology* 104:342-348.

25. Mohajeri E & Noudeh GD (2012) Effect of temperature on the critical micelle concentration and micellization thermodynamic of nonionic surfactants: polyoxyethylene sorbitan fatty acid esters. *Journal of Chemistry* 9(4):2268-2274.

26. Moser B (2011) Biodiesel Production, Properties, and Feedstocks. *Biofuels*, Tomes D, Lakshmanan P & Songstad D (Édit.) Springer New York, 10.1007/978-1-4419-7145-6_15. p 285-347.

27. Patil PD, Gude VG, Mannarswamy A, Deng S, Cooke P, Munson-McGee S, Rhodes I, Lammers P & Nirmalakhandan N (2011) Optimization of direct conversion of wet algae to biodiesel under supercritical methanol conditions. *Bioresour Technol* 102(1):118-122.

28. Sathish A & Sims RC (2012) Biodiesel from mixed culture algae via a wet lipid extraction procedure. *Bioresour Technol* 118:643-647.

29. Seo YH, Lee IG & Han JI (2013) Cultivation and lipid production of yeast Cryptococcus curvatus using pretreated waste active sludge supernatant. *Bioresource Technology* 135:304-308.

30. Vasileva-Tonkova E, Galabova D, Karpenko E & Shulga A (2001) Biosurfactant-rhamnolipid effects on yeast cells. *Letters in applied microbiology* 33(4):280-284.

31. Vicente G, Bautista LF, Rodríguez R, Gutiérrez FJ, Sádaba I, Ruiz-Vázquez RM, Torres-Martínez S & Garre V (2009) Biodiesel production from biomass of an oleaginous fungus. *Biochemical Engineering Journal* 48(1):22-27.

32. Yadav J, Bezawada J, Yan S, Tyagi RD & Surampalli RY (2014) Permeabilization of *Kluyveromyces marxianus* with Mild Detergent for Whey Lactose Hydrolysis and Augmentation of Mixed Culture. *Appl Biochem Biotechnol* 172(6):3207-3222.

33. Yang F, Xiang W, Sun X, Wu H, Li T & Long L (2014) A novel lipid extraction method from wet microalga Picochlorum sp. at room temperature. *Mar Drugs* 12(3):1258-1270.

34. Yoo G, Park W-K, Kim CW, Choi Y-E & Yang J-W (2012) Direct lipid extraction from wet Chlamydomonas reinhardtii biomass using osmotic shock. *Bioresource Technology* 123:717-722.

35. Zhang X, Yan S & Tyagi RD (2015) Strategies of preserving lipids in microorganism after fermentation. *Bioresource Technology* 192:718-725.

36. Zhang X, Yan S, Tyagi RD, Drogui P & Surampalli RY (2014) Ultrasonication assisted lipid extraction from oleaginous microorganisms. *Bioresource Technology* 158:253-261.

**Table 2.3. 1 Box-Behnken experimental design by optimization of factors NLS concentration (mg), Time (min), Temperature (°C) and results of response of variables calculated in the form of extraction efficiency % (w/w).**

| Run | Experimental conditions (factor values) | | | |
|---|---|---|---|---|
| | A | B | C | Extraction efficiency |
| | N-LS concentration (mg) | Temperature (°C) | Time (min) | %w/w |
| 1 | 40 | 35 | 12.5 | 67.5 |
| 2 | 6.25 | 30 | 5 | 61.3 |
| 3 | 6.25 | 25 | 12.5 | 60.3 |
| 4 | 23.12 | 30 | 12.5 | 99.7 |
| 5 | 23.12 | 35 | 5 | 71.5 |
| 6 | 23.12 | 30 | 12.5 | 99.1 |
| 7 | 40 | 30 | 5 | 68.5 |
| 8 | 23.12 | 35 | 20 | 72.5 |
| 9 | 23.12 | 25 | 5 | 66.2 |
| 10 | 6.25 | 35 | 12.5 | 62.4 |
| 11 | 40 | 25 | 12.5 | 64.9 |
| 12 | 23.12 | 30 | 12.5 | 99.7 |
| 13 | 23.12 | 30 | 12.5 | 99.9 |
| 14 | 6.25 | 30 | 20 | 68.3 |
| 15 | 40 | 30 | 20 | 70.7 |
| 16 | 23.12 | 30 | 12.5 | 99.7 |
| 17 | 23.12 | 25 | 20 | 71.2 |

**Table 2.3. 2 Estimated regression coefficients for response**

| **Analysis of variance table** | | | | | |
|---|---|---|---|---|---|
| **Source** | **Sum of Squares** | **df** | **Mean Square** | **F Value** | **p-value Prob > F** |
| Model | 3918.35 | 9 | 435.37 | 1450.90 | < 0.0001 significant |
| A-NLS conc (mg) | 46.56 | 1 | 46.56 | 155.17 | < 0.0001 |
| B-Temparature | 15.96 | 1 | 15.96 | 53.19 | 0.0002 |
| C-Time (Min) | 28.88 | 1 | 28.88 | 96.24 | < 0.0001 |
| AB | 0.063 | 1 | 0.063 | 0.21 | 0.6619 |
| AC | 5.76 | 1 | 5.76 | 19.20 | 0.0032 |
| BC | 4.00 | 1 | 4.00 | 13.33 | 0.0082 |
| $A^2$ | 1600.64 | 1 | 1600.64 | 5334.20 | < 0.0001 |
| $B^2$ | 1125.22 | 1 | 1125.22 | 3749.85 | < 0.0001 |
| $C^2$ | 703.12 | 1 | 703.12 | 2343.18 | < 0.0001 |
| Residual | 2.10 | 7 | 0.30 | | |
| Lack of Fit | 1.73 | 3 | 0.58 | 6.28 | 0.0541 (not significant) |
| Pure Error | 0.37 | 4 | 0.092 | | |
| Cor Total | 3920.46 | 16 | | | |

**Table 2.3. 3 Effect of individual factors on lipid extraction efficiency**

| Factors | (Actual) value | Lower limit | Upper limit | Extraction efficiency % (w/w) |
|---|---|---|---|---|
| N-LS concentration (mg/g dry biomass)* | 23.12 | 6.25 | 40 | 99.1 |
| Temperature ($^{o}$C) | 30 | 25 | 35 | 99.7 |
| Time (min) | 12.5 | 5 | 20 | 99.9 |

*N-LS concentration varied from 6.25 to 40 mg (which is equivalent to 12.5 to 80 mg N-LS/ g dry biomass)

**Table 2.3. 4 Comparison of different methods of lipid extraction from wet biomass.**

| Oleaginous substance | Wet biomass % | Extraction condition | Extraction efficiency (%) | References |
|---|---|---|---|---|
| *Nannochloropsis sp.* | Nr* | Supercritical methanol | 84.15 | (Patil *et al.*, 2011) |
| *R.toruloides* | 94 | Combination of Pretreatment with microwave and recombinant enzyme | 95.4 | (Jin *et al.*, 2012) |
| *Yarrowia lipolytica* | 89 | N-lauroyl sarcosine | 95.4 | This study |
| *N.oculata* | 80 | Simultaneous distillation and extraction process (SDEP) using soxhlet | 90.2 | (Dejoye Tanzi *et al.*, 2013) |
| *C. reinhardtii* | 99.4 | Osmotic shock | 9.06 | (Yoo *et al.*, 2012) |
| *Chlorella* and *Scenedesmus* sp. | 84 | Acid and base hydrolysis | 79 | (Sathish *et al.*, 2012) |
| *Scenedesmus* | Nr* | 3-DAPS* | 100 | (Lai *et al.*, 2016) |
| *Picochlorum* | 66.96 | Ethanol | 32.81 | (Yang *et al.*, 2014) |

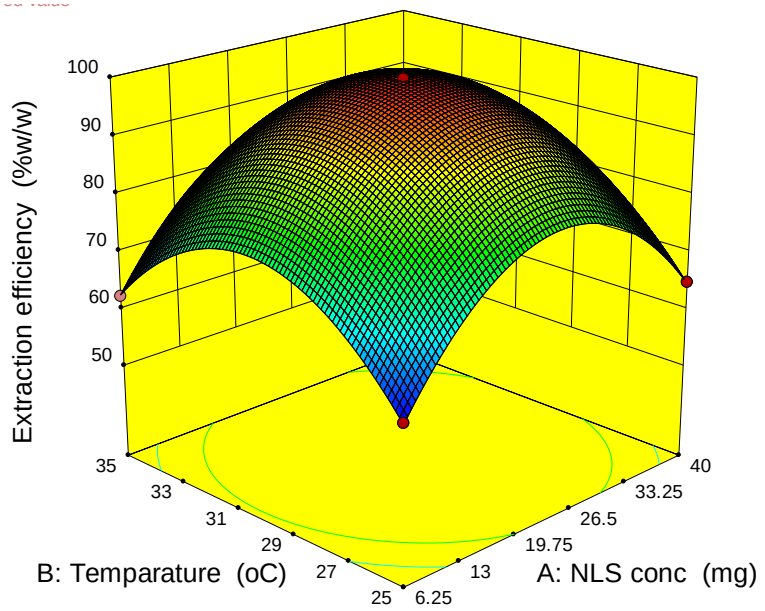
***nd – Not reported**

***3-DAPS – 3-(decyldimethylammonio)- propanesulfonate inner salt**

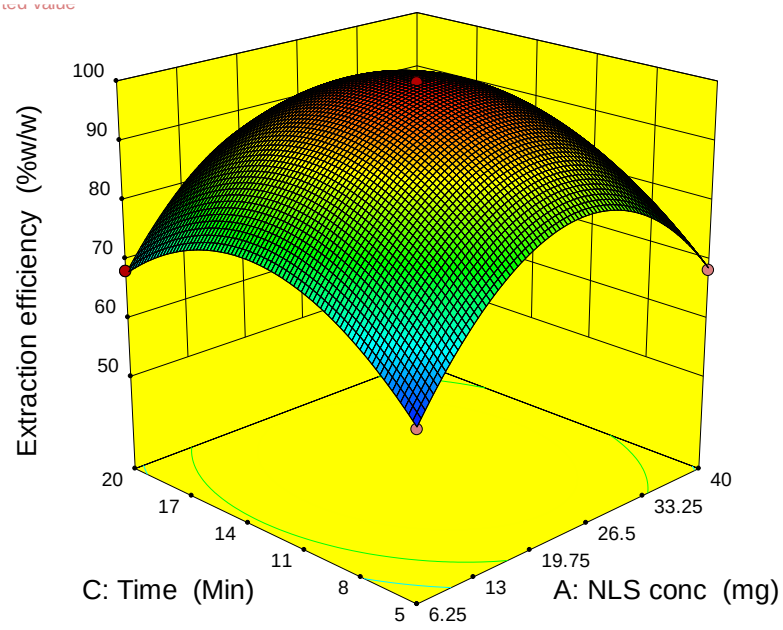
**Table 2.3. 5 Comparison of fatty acid profiles between conventional and N-lauroyl sarcosine assisted lipid extraction processes.**

| Fatty acids | Relative amount of total fatty acid (% w/w) | |
|---|---|---|
| | **Conventional chloroform methanol (2:1 v/v)** | **N-lauroyl sarcosine + chloroform/methanol (1:1 v/v)** |
| Palmitic acid (C16:0) | 17.2 | 16.2 |
| Palmitoleate (C16:1n7) | 0.3 | 0.3 |
| Stearic (C18:0) | 5.1 | 4.5 |
| Oleic (C18:1n9) | 45.6 | 42.5 |
| Vaccenic (C18:1n7) | 1.2 | 1.1 |
| Linoleic (C18:2n6) | 24.7 | 30.2 |
| Linolenate (C18:3n3) | 1.3 | 1.5 |
| C23:0 | 4.6 | 3.7 |

a)


Extraction efficiency (%w/w)
100
90
80
70
60
50
35
33
31
29
27
25
B: Temparature (oC)
6.25
13
19.75
26.5
33.25
40
A: NLS conc (mg)

b)


Extraction efficiency (%w/w)
100
90
80
70
60
50
20
17
14
11
8
5
C: Time (Min)
6.25
13
19.75
26.5
33.25
40
A: NLS conc (mg)

c)

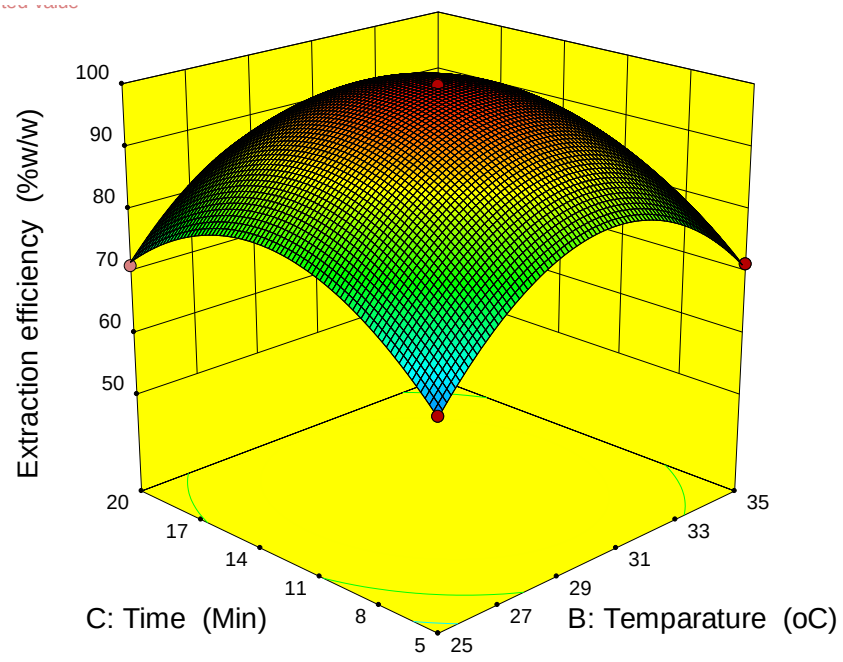



**Figure 2.3. 1 Response surface plot for all design condition. a) Effect of N-LS concentration and temperature with constant time (min), b) Effect of N-LS concentration and time with constant temperature (°C), c) Effect of Temperature and time with constant N-LS concentration (mg)**

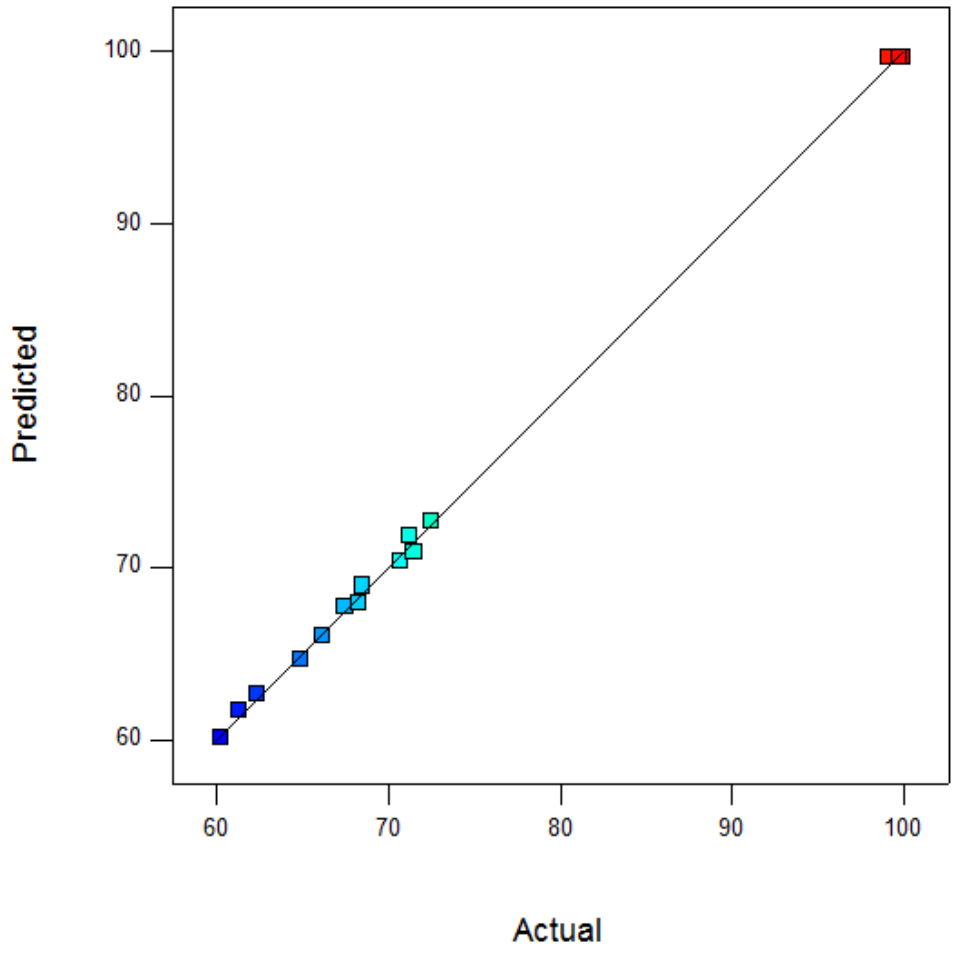



**Figure 2.3. 2 Plot of the experimental and predicted responses.**

# CHAPITRE II

# PARTIE 4

# DETERGENT ASSISTED ULTRASONICATION AIDED *IN SITU* TRANSESTERIFICATION FOR BIODIESEL PRODUCTION FROM OLEAGINOUS YEAST WET BIOMASS

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# RÈSUME

Dans ce travail, la transestérification in situ de la biomasse humide de levure oléagineuse pour la production d'esters méthyliques d'acides gras (FAMEs) en utilisant un catalyseur acide et du méthanol avec ou sans traitement à la N-Lauroyl sarcosine (N-LS) a été réalisée. Le rendement maximum de FAMEs obtenu avec ou sans traitement par N-LS en 24 h de temps de réaction était de 96,1 ± 1,9 et de 71 ± 1,4% en poids, respectivement. Le traitement N-LS de la biomasse suivie ou non d'ultrasonication a donné un rendement maximum de FAMEs de 94,3 ± 1,9% et 82,9 ± 1,8% p / p respectivement. Un rapport molaire méthanol / lipide de 360: 1 et une concentration de catalyseur de 360 mM (64 µL $H_2SO_4$ / g lipide) dans les 5 et 25 minutes de temps de réaction a été utilisé dans ce traitement. La composition de FAMEs obtenue dans la transestérification in situ était similaire à celle des FAMEs obtenus lors d'un procédé classique d'extraction et de transestérification de lipides. Le biodiésel pourra présenter une alternative durable étant donné que ses propriétés (masse volumique, viscosité cinématique, indice de cétane et glycérol total) sont conformes à la norme internationale (ASTM D6751).

## ABSTRACT

*In situ* transesterification of oleaginous yeast wet biomass for fatty acid methyl esters (FAMEs) production using acid catalyst, methanol with or without N-Lauroyl sarcosine (N-LS) treatment was performed. The maximum FAMEs yield obtained with or without N-LS treatment in 24 h reaction time was 96.1 ± 1.9 and 71 ± 1.4 % w/w, respectively. The N-LS treatment of biomass followed by with or without ultrasonication revealed maximum FAMEs yield of 94.3 ± 1.9 % and 82.9 ± 1.8 % w/w using methanol to lipid molar ratio 360:1 and catalyst concentration 360 mM (64 µL $H_2SO_4$/g lipid) within 5 and 25 minutes reaction time, respectively. The FAMEs composition obtained in *in situ* transesterification was similar to that obtained with conventional two step lipid extraction and transesterification process. Biodiesel fuel properties (density, kinematic viscocity, cetane number and total glycerol) were in accordance with international standard (ASTM D6751), which suggests the suitability of biodiesel as a fuel.



**Keywords:** Microbial lipid; N-Lauroyl sarcosine; In situ transesterification; Ultrasonication; Biodiesel

# 1. INTRODUCTION

The global demand towards renewable energy is increasing due to the major problem of greenhouse gas emissions (GHG) (Piemonte *et al.*, 2016). There are two different possible approaches to decrease GHG: a) the use of microalgae to convert $CO_2$ into potential biomass by photosynthesis (ElMekawy *et al.*, 2014) and b) use of the biofuel is another alternative to decrease GHG emissions. Thus, the environment friendly and renewable fuel like biodiesel from oleaginous microorganisms (microalgae, yeast, fungi and bacteria) are found to be attractive in replacing fossil-based fuels (Alvarez *et al.*, 2002, Lunin *et al.*, 2012, Medeiros *et al.*, 2015, Zhang *et al.*, 2014a). However, microbial lipid production using oleaginous microorganism is highly expensive process than first and second generation fuels, which is the most important barrier for commercialization of the process (Rathore *et al.*, 2016).

The biodiesel production process includes microbial oil production, harvesting, lipid extraction and transesterification. The lipid extraction followed by transesterification (two step transesterification) is high energy consumption process (approximately 2.3 to 40.5 Mj/kg) depending upon different cell disruption methods applied to disrupt the oil-bearing cells (Dong *et al.*, 2015, Praveenkumar *et al.*, 2015a, Praveenkumar *et al.*, 2015b). To make the biodiesel production economical, it is a challenge for researchers to decrease the energy consumption for biodiesel production.

*In situ* transesterification (one step process or simultaneous cell disruption and transesterification process) is considered as an emerging alternative to two step transesterification, which has its obvious advantage. *In situ* transesterification involves the direct contact of reactants (methanol, catalyst and co-solvent) with biomass instead of lipids. This could reduce the energy consumption used for extraction of lipids and hence cut down the cost of biodiesel production. In the previous studies, high FAMEs yield up to 92.1% w/w has been accomplished within 20 min reaction time using dry (lyophilized) oleaginous yeast biomass with ultrasonication assisted in-situ transesterification (Zhang *et al.*, 2014b). Another study by Zhang *et al.*,(2016) also reported high FAMEs yield of 95% w/w within 50 min reaction time using dry (lyophilized) sludge biomass with high concentration of sludge solids by ultrasonication assisted in-situ transesterification. However, biodiesel production by *in situ* transesterification of dry (or) lyophilized biomass is a time effective

process, but due to high energy consumption in drying operation, it is not feasible for industrial scale biodiesel production.

*In situ* transesterification using wet biomass is also highly questionable process due to the presence of high water content in the biomass, which may interfere with the transesterification process by enhancing the hydrolysis of FAMEs. It is reported that in the case of alkali based *in situ* transesterification using wet biomass, more than 6% water content reduces the lipid to FAMEs conversion efficiency due to saponification (Suter *et al.*, 1997). Whereas, in case of acid catalyzed *in situ* transesterification using wet algal biomass, more than 20% moisture content affected the lipid to FAMEs conversion efficiency. However, by increasing reactant concentration (sulfuric acid and methanol), the FAMEs yield was improved (Sathish *et al.*, 2012). Nagle *et al.* (1990) also studied the effect of acid and alkali catalysts on conversion of microbial oil to FAMEs and found that acid catalysts resulted in consistently higher yield due to total conversion of free fatty acids (FFA) into FAMEs and there was no soap formation even in the presence of moisture (wet biomass).

Few authors reported the use of surfactants along with the catalysts to enhance the FAMEs yield. The surfactant has high water tolerance and therefore have ability to disrupt the cell wall as well as the phospholipid membrane layer (Brown *et al.*, 2008). Haas *et al.* (2011) described the use of (CTAB) cetyltrimethylammonium bromide (a cationic surfactant) along with an alkali catalyst during *in situ* transesterification of *Jatropha curcas* and observed increased FAME yield as well as a reduction in the catalyst concentration. Sodium dodecyl benzene sulfonate (SDBS) along with the catalyst $H_2SO_4$ also enhanced extraction of FFA and lipids from microalgae. SDBS significantly reduced the catalyst concentration required to convert the oil to FAMEs (Park *et al.*, 2014). N-Lauroyl sarcosine (N-LS), an anionic detergent is nontoxic and biodegradable. It can disrupt the cell wall by formation of micelle at specific concentrations with yeast wet biomass with high moisture content (83.8 %) without effecting fatty acid profile (Yellapu *et al.*, 2016). On the other hand, according to Zhang *et al.,* (2014b) ultrasonication can decrease methanol requirement (*in situ* transesterification) as well as the reaction time. Thus, combining N-LS (N-Lauroyl sarcosine) treatment of oil bearing cells with ultrasonication could further improve the cell disruption and transesterification process. Therefore, the objective of this work is to investigate

the effect of N-LS treatment along with the ultrasonication on oleaginous wet yeast oil bearing biomass for *in situ* transesterification. The different parameters such as ultrasonication time, $H_2SO_4$ (catalyst) concentration and methanol to lipid molar ratios were optimized to enhance the efficiency of conversion of oil to FAMEs. The *in situ* transesterification without ultrasonication as well as without N-LS treatment was also conducted to compare the results.

# 2. MATERIALS AND METHODS

## 2.1 Strain, production and lipid harvesting conditions

*Yarrowia lipolytica* SKY-7, oleaginous yeast (isolated in our lab INRS-ETE Quebec, Canada) (Kuttiraja *et al.*, 2015) was grown in the medium containing 8.5 L starch industry wastewater (SIW) and 500 mL of crude glycerol solution with 11% (w/v) glycerol (byproduct of biodiesel production Quebec, Canada) in a 15L fermenter with working volume 10L (Biogene, Quebec). The fermenter was operated at constant pH 6.8–7.0 and temperature 28 $^{o}$C and dissolved oxygen was maintained above 30% of saturation. After fermentation (72 h), the broth was heated in the fermenter at 80 ± 2$^{o}$C for 10 min to kill cells and to preserve the accumulated lipid inside the cells (Zhang *et al.*, 2015). Thereafter, the biomass was harvested by centrifugation at 8000 rpm for 10 min and the biomass was washed with warm water to remove residual glycerol as well as soap. To estimate dry weight and to perform lipid extraction as well as *in situ* transesterification, 3.1±0.2 g wet biomass (83.8% water) harvested from 25 mL fermented broth was used.

## 2.2 Two-step process (lipid extraction and separation followed by transesterification)

### 2.2.1 Conventional lipid extraction and separation using chloroform and methanol

The standard chloroform and methanol extraction method was used to determine the lipid content of the biomass (Bligh *et al.*, 1959, Folch *et al.*, 1957, Vicente *et al.*, 2009). The lipid extraction was conducted in the same way as discussed in our previous study (Yellapu *et al.*, 2016). The washed

biomass (wet) pellet (3.1 ± 0.2 g having 83.8 moisture content) was mixed with 15 mL solvent mixture of chloroform and methanol (2:1 v/v), and then incubated for 4h in an agitator water bath at 60 $^{o}$C and 100 rpm. The mixture was then centrifuged at 4000 rpm for 10 min. After centrifugation, the mixture was separated in three different layers. The residual biomass was in the middle layer, bottom phase was lipid in chloroform and top layer methanol and water. The bottom layer of chloroform containing lipid was pipetted out and transferred into a pre-weighed glass tube ($L_1$). The rest of the solution (containing cell debris, methanol) was again fortified with 15 mL solvent mixture of chloroform and methanol (2:1 v/v) and again incubated for 4h at 60 $^{o}$C in the agitated water bath. After 4h incubation, the solution was filtered (Fisherbrand™ Qualitative-Grade Filter paper, Particle retention: 5 to10 µm) using vacuum filtration. The filtrate was mixed with previously extracted solution (chloroform solution containing lipid) and the mixed solution was allowed to stand for phase separation. The bottom phase containing lipid in chloroform (the other phase was water and methanol) was collected and subjected to nitrogen sparging until total chloroform evaporated. The samples were further dried in an oven at 60 $^{o}$C until constant weight ($L_2$). The lipid recovery from the biomass was calculated as:

$$CL\ \% = \frac{L2-L1}{DBW} \times 100\% \text{ --------- (1)}$$

The obtained lipid was stored for further transesterification study. Equation (1) CL represents the weight obtained from conventional lipid extraction, $L_1$ expresses the pre-weighed glass tube and $L_2$ denotes the oven dried microbial lipid in a pre-weighed glass tube and DBW denotes dry biomass weight.

### 2.2.2 Lipid transesterification

The lipid obtained from solvent extraction (as described above in section 2.2.1) was first dissolved in hexane (25 mL hexane per gram lipid), then mixed with methanol (methanol to lipid ratio was 6:1 or 0.08 mL methanol per gram lipid) containing sulfuric acid as a catalyst with concentration of 180 mM (4 µL $H_2SO_4$/g lipid) (Zhang *et al.*, 2016). Acid catalyst was used because alkali catalyst will form soap if biomass consists of more than 6% water content (Suter *et al.*, 1997). The mixture was then heated to 60$^{o}$C for 12 h. After the reaction, 50 mL of NaCl solution (5% w/v) per gram of lipid was added and the solution was allowed to stand for 15 min.

FAMEs were extracted in hexane (top) phase. The bottom phase was again treated with hexane (25 mL per gram lipid) to recover the remaining FAMEs. The FAMEs were separated and mixed with the fraction separated earlier. The FAMEs in hexane was washed with 2% sodium bicarbonate solution (10 mL per gram lipid), to remove excess water and the top hexane layer was then dried at 60$^{o}$C in an oven (Halim *et al.*, 2011).

The FAMEs were re-dissolved in hexane (0.01 g FAMEs/10 mL hexane) and analyzed using a Gas Chromatograph linked with FID (GC-FID) (Perkin Elmer, Clarus 500). The dimensions of the column used are 30m x 0.25 mm, with a phase thickness of 0.2 µm. Helium was the carrier gas at a flow rate of 1.18 ml/min with the oven temperature 230 $^{o}$C. Trans esterified sample of 1µl was injected with an automated sample injector and the sample analysis was performed with Agilent chem Station module software from Agilent Technologies. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1,3-Dichlorobenzene was used as internal standard with concentration of 50 ppm.

## 2.3 In situ transesterification (with or without N-LS treatment)

The oil-bearing biomass pellet (3.1±0.2g wet biomass containing 83.8% water) was obtained after centrifuging the fermented and heat-treated broth at 8000 rpm for 10 min. The biomass pellet was washed with hot water. To disrupt the cells and release the lipids, the biomass pellet was treated with N-LS solution (2 mL solution containing 12.5 g/L N-LS, which is equivalent to 48 mg dry N-LS/g dry biomass) for 8 minutes at 30$^{0}$C and 180 rpm in an incubator shaker (Yellapu *et al.*, 2016b). For *in situ* transesterification, sulphuric acid solution in methanol of different strength (180, 360, 540mM) was prepared and added to the reaction mixture (after 8 minutes of N-LS treatment) so as to obtain different molar ratio of methanol to lipid (methanol to lipid molar ratio of 60:1, 120:1 and 240:1). Ten mL of co-solvent (hexane, which aids in the separation of FAMEs) was also added. For control (N-LS non-treated biomass), methanol acid solution and 10 mL hexane was directly added to the washed wet biomass pellet. The reaction mixture was incubated in a water bath at 60$^{o}$C, 100 rpm and 24h. A similar experiment was conducted without adding hexane to check the effect of co-solvent on *in situ* transesterification and concluded that hexane

did not affect the either cell disruption or transesterification process. Six tubes were kept for each reaction parameters and one tube were withdrawn at 2, 4, 6, 10, 16 and 24h to determine FAMEs concentration. After 24h reaction, 5mL hexane was added again in all the tubes to extract FAMEs. For each sample drawn, the reaction mixture was allowed to stand for 15 min for phase separation. The top phase, FAMEs in hexane, was collected and the bottom phase (containing biomass, water, methanol glycerol, catalyst) was again treated with hexane (25mL/g lipid) and allowed for phase separation. The top layer (FAMEs in hexane) was collected and mixed with the previous fraction of FAMEs in hexane. FAMEs in hexane was purified by adding 5% w/v NaCl solution (50 mL NaCl solution per gram lipid) and the solution was allowed to stand for 15 min. FAMEs were extracted in hexane phase (top layer) and the bottom phase was again treated with hexane (25 mL per gram lipid) to recover the residual FAMEs. Further, the FAMEs in hexane were washed with 2% w/v sodium bicarbonate solution (10 mL per gram lipid) to remove the excess water and the top layer containing FAMEs in hexane was then collected and dried at 60$^{o}$C in an oven (Halim *et al.*, 2011). The FAMEs concentration was determined and analyzed with GC-FID as described in section 2.2.2. The FAME yield (%w/w) was calculated based on concentration of FAMEs measured by GC-FID divided by the total lipids concentration × 100%).

## 2.4 Ultrasonication assisted in situ transesterification (with or without N-LS treatment)

All experiments were conducted as outlined in the previous section (section 2.3). However, in this case the reaction mixture was treated with ultrasonication. Ultrasonication was conducted with ultrasonic processor CPX 750 (Cole-parmer Instrument, IL) at 20 kHz. Methanol and $H_2SO_4$ (catalyst) was added to N-LS treated (as outlined in section 2.3) or without N-LS treated wet biomass (3.1±0.2g) along with 25 mL of co-solvent hexane. The ultrasonic horn was directly immersed (5mm) in the solution in a 50 mL glass tube surrounded with ice to control temperature less than 25 $^{o}$C for a desired time. A plastic cover was wrapped around the glass tube in order to minimize the loss of methanol and hexane. The sonication time was varied from 1 to 35 min and on/off cycle was set 50/10 sec respectively, in order to minimize the heat generated. The samples were treated and FAMEs concentration was determined and analyzed as described in sections

2.2.2 and 2.3. All experiments were conducted in triplicate and the calculated standard deviation was less than 5%.

## 2.5 Biodiesel fuel properties

The purified biodiesel obtained from above study was tested for estimation and evaluation of its fuel properties and compared with recent literature, using the prediction models for biodiesel (ASTM standard: D664, D613, D445 and D2075 respectively). The physiochemical characteristics of biodiesel such as cetane number, density, Kinematic viscosity and glycerol concentraion were determined (Kakkad *et al.*, 2015). Biodiesel was further blended with petroleum diesel (5%, 10% and 20% v/v or B-5, B-10 and B-20) and their physico chemical characteristics were also defined.

## 2.6 Statistical Analysis

The data value presented in this article were obtained in triplicate experiments. The statistical analysis was performed using Sigma plot 11.0 (Systat Software, Inc. Chicago, IL, USA), mean values were compared and analyzed using one-way analysis of variance (ANOVA). Differences were considered statistically significant for $p<0.05$.

# 3. RESULTS AND DISCUSSION

## 3.1 Two-step process

The FAMEs yield obtained from two-step (lipid extraction followed by transesterification) transesterification process was found to be 94.6 ± 1.5 % (w/w). The wet biomass used in these experiments consisted of 83.8 % moisture content. According to Laurens *et al.,* ( 2012), the use of alkaline catalyst will form soap by saponification reaction in the presence of 6 % or higher moisture content of biomass. Therefore, in the present study (two-step and *in situ* transesterification), acid catalyst was used to avoid saponification due to high moisture content and conversion of total lipid to FAMEs.

## 3.2 In situ transesterification without N-LS pretreated biomass

The FAMEs yield obtained during *in situ* transesterification at a constant concentration of catalyst and with varying methanol to lipid (lipid in biomass) molar ratio of 60:1 to 360:1 is presented in Figure 2.4.1. The FAMEs yield increased with reaction time in 24h. Methanol concentration and $H_2SO_4$ (Catalyst) concentration affects the oil conversion efficiency to FAMEs (Ehimen *et al.*, 2010). Increasing the $H_2SO_4$ concentration from 180 to 360 mM at a specific methanol to lipid molar ratio (360:1), there is an increase in FAMEs yield from 50.1 ± 1.1 to 71 ± 1.4 % (w/w) (increased up to 1.44 times) (Figure 2.4.1c). However, with further increase in catalyst concentration from 360 to 540 mM, FAMEs yield did not appreciably increase. The reaction time required to reach equilibrium (or maximum FAMEs yield) was decreased from 16 to 10h (Fig.2.5. 1b and 1c), when the catalyst concentration was increased from 180 to 540 mM. An increase in FAMEs yield with an increase in catalyst concentration during *in situ* transesterification was also observed by (Velasquez-Orta *et al.*, 2012). The acid catalyst could involve in reactions other than transesterification, such as hydrolysis of carbohydrates. Therefore, higher concentration of catalyst may be required to achieve high FAMEs yield.

In *in situ* transesterification process, according to equilibrium reaction, 1kg of lipids will produce 1kg of FAMEs. One triglyceride molecule (891 g) requires three methanol molecules (38.04 g) to produce three FAMEs molecules (298 g) and 1 glycerol molecule (92 g). The methanol requirement is very high (360:1 molar ratio, which is equal to 6.4 mL methanol/g lipid) as compared to the two-step transesterification process (0.08 mL/g lipid). FAMEs yield also decreased from 94.6 ± 1.5 (two step) to 50.2 ± 1.1% (w/w) (*in situ*) in 16h reaction time by using similar catalyst concentration (180 mM) (Figure 2.4.1a). According to Zhang *et al.,*(2014b) FAMEs yield obtained was 90.4 ± 1.3 % (w/w) using lyophilized biomass and 360:1 methanol to lipid molar ratio. It explains that high moisture content present in the biomass (lipid content) is a severe obstacle for methanol and catalyst to react with lipids (Laurens *et al.*, 2012). High methanol concentration during *in situ* transesterification process is required to weaken and disrupt the outer cell wall and finally reactants can react with lipids to convert them into FAMEs. According to these results (Figure 2.4.1) an increase in catalyst concentration and methanol to lipid molar ratio is not

a solution to achieve the maximum FAMEs yield. Moreover, consumption of high quantity of methanol will not be economical for biodiesel production.

## 3.3 In situ transesterification with N-LS pre-treated biomass

Biomass (wet) pretreatment with N-LS can disrupt cell wall (Yellapu *et al.*, 2016b). After N-LS treatment, FAMEs yield obtained was 80.3 ± 1.5% w/w with methanol to lipid molar ratio of 60:1 using a 180mM catalyst ($H_2SO_4$) concentration. However, under similar conditions, the FAMEs yield obtained from non-treated biomass was only 12.6 ± 0.4% w/w (Figure 2.4.1 and Figure 2.4.2), which is 6.3 times lower than the yield obtained from N-LS treated biomass. Thus, after N-LS treatment of biomass, the reactants methanol and catalyst ($H_2SO_4$) react easily with lipid in the solution and enhance the lipids conversion efficiency to FAMEs.

An increase in methanol to lipid molar ratio (from 60:1 to 360:1) and catalyst concentration (180 to 540 mM) increased the FAMEs yield (Figure 2.5.2 a,b,c). The maximum FAMEs yield obtained was 96.2 ± 2% (w/w) with 240:1 methanol to lipid molar ratio and 540 mM $H_2SO_4$ concentration (Figure 2.5.2c). However, under similar conditions, the FAMEs yield obtained from non-treated biomass was only 72.2 ± 1.4% (w/w).

*In situ* transesterification with N-LS non-treated biomass using constant methanol to lipid molar ratio (60:1) and by increasing the catalyst concentration of 180, 360 and 540mM, the FAMEs yield obtained was 12.6 ± 0.4, 14 ± 0.3 and 20.3 ± 0.8, respectively (Figure 2.5.1 a,b,c). However, under similar conditions, the FAMEs yield obtained with N-LS treated biomass was 80.3 ± 1.5, 80.9 ± 1.4 and 82.5 ± 1.7, respectively. The increase in FAMEs yield was due to cell wall disruption by N-LS, which lead to a direct contact of lipids with the reactants. Thus, requiring a lower concentration of methanol and catalyst. However, the reaction time to approach the maximum FAMEs yield of 95.3 and 96.2 ± 2% (w/w) was 10h and 16 h, respectively. Thus, due to long reaction time, it may not be practically feasible to use this process for large scale biodiesel production. The similar results were reported by Salam *et al.,* (2016), where pre-treatment of *Nannochloropsis* (wet biomass with 20% moisture content) with sodium dodecyl sulphate (SDS) and *in situ* transesterification resulted in maximum FAMEs yield of 98 % w/w within 24h reaction

time (Salam *et al.*, 2016). Therefore, in this study ultrasonication aided *in situ* transesterification was conducted to decrease reaction time and is presented below.

## 3.3 Ultrasonication assisted in situ transesterification (without N-LS)

Using ultrasonication, the FAMEs yield of 80.2 ± 1.5% w/w (Figure 2.4.3) was obtained with lower requirement of methanol to lipid molar ratio (120:1) and catalyst concentration (180 mM) within a reaction time of 25 min. The similar FAMEs yield of 80.1 ± 1.1% w/w (Figure 2.4.2) was obtained at 10h reaction time using similar reactant conditions [methanol to lipid molar ratio of 120:1 and catalyst ($H_2SO_4$) 180mM concentration] and N-LS assisted cell disruption process without ultrasonication. The ultrasonic wave creates violent shear forces (based on amplitude) upon cell wall, which results in quick cell rupture and resulting in release of lipids. The rapid reaction of methanol with the released lipids for transesterification leads to lower requirement of methanol and shorter reaction time compared to that without ultrasonication.

Increasing the catalyst concentration from 180 to 540 mM at a constant methanol and a lipid molar ratio (240:1), the FAMEs yield increased from 80.2 ± 1.5 to 82.3 ± 0.9% (w/w), which is only 2% (w/w) enhanced (Figure 2.4.3b). Further increase in catalyst ($H_2SO_4$) concentration did not show an appreciable impact on FAMEs yield.

According to Zhang *et al.,*(2014b), high conversion of lipid to FAMEs (92.1% w/w) for lyophilized biomass was achieved with molar ratio of 60:1 using 1% NaOH catalyst within 20 min reaction time. However, in the present study the maximum lipid conversion or FAMEs yield of 82.9 ± 1.6 % w/w was obtained with methanol to lipid molar ratio of 360:1 using 180mM $H_2SO_4$ within 25 min reaction time. Thus, high biomass moisture content requires high concentration of reactants (methanol and catalyst), which is also observed by other researchers (Laurens *et al.*, 2012).

## 3.4 N-LS assisted and ultrasonication aided in situ transesterification

N-LS treatment followed by ultrasonication process leads to a high FAMEs yield of 94.3 ± 1.9% w/w with methanol to lipid molar ratio of 360:1 using 360mM catalyst concentration within 5 min. reaction time (Figure 2.4.4b). However, under similar conditions, the FAMEs yield of 82.9 ±

1.8% w/w (Figure 2.4.4 a,b,c) was obtained within reaction time of 25 min using ultrasonication treated biomass (without N-LS). As per the equilibrium reaction, the methanol requirement is 0.08 mL methanol/g lipid, but in this study 6.4 mL/g methanol gave maximum FAMEs yield. The process ccost can be decreased if the residual methanol with glycerol solution is reutilized for lipid production.

The various researchers have reported different FAMEs yield during *in situ* transesterification of oleaginous wet biomass (Table 2.4.1 a,b,c). Different chemicals (such as hydrochloric acid, supercritical methanol and sodium dodecyl sulphate) have been applied for pre-treatment of lipid containing wet biomass (moisture content 20 to 80.4 Wt%). However, low FAMEs yield was obtained after *in situ* transesterification, when high moisture content was present in the biomass (Jazzar *et al.*, 2015, Laurens *et al.*, 2012). Another study by Salam *et al.,*(2016) reported high FAMEs yield of 98% w/w within 24h reaction time, when pre-treated (Sodium dodecyl sulfate) wet biomass with moisture content 20% was used for *in situ* transesterification.

In the present study, the maximum FAMEs yield of 94.3 % w/w was obtained using wet biomass (with 83.8% moisture content) in only 5 min reaction time, which is the highest yield at high moisture content and lowest reaction time (Table 2.4.1 a,b,c). In comparison with the two-step transesterification process, the methanol requirement is high (methanol: lipid ratio 360:1), but the time of reaction decreased from 16 h to 5min using N-LS assisted ultrasonication aided *in situ* transesterification. It indicates that the mild surfactant (N-LS) effectively disrupted the cell wall and released lipids, thereby increasing accessibility of methanol to the internal body lipids and hence further exposed the lipid to direct contact with reactants within a short time. The less reaction time can offset the cost of high reactants volume required at industrial scale biodiesel production. The results obtained in this study are statistically significant for $p<0.05$.

Moreover, pre-treatment cost for cell (wet) wall disruption using N-Lauroyl sarcosine is expected to be low (0.48$ N-LS/kg dry biomass) compared to the conventional process used to release the intracellular lipids without drying and application of organic solvents (chloroform, methanol and isopropanol). In case of the conventional process (centrifuge, drying) high quantity of organic solvents are used for lipid extraction and transesterification, which adds the cost up to >4 $/kg dry

biomass to the whole process (Table 2.4.2). These organic solvents can be recovered by using distillation, but it is highly energy intensive process.

## 3.5 Comparison of FAMEs composition from different transesterification processes

The FAMEs obtained from *in situ* transesterification with (or) without N-LS pretreatment and ultrasonication assisted *in situ* transesterification was presented in Table 2.4.3. Increasing the methanol to lipid molar ratio from 60:1 to 360:1, there is an increase in C16:0, C18:1 and C18:2 during *in situ* transesterification. High methanol to lipid molar ratio (360:1) is required to release the neutral lipid granules, which are surrounded by phospholipids membrane. These neutral lipids are then converted into FAMEs (Giroud *et al.*, 2013). It explains that when the lipids granules are released out of the cell, $H_2SO_4$ and methanol are able to react well with them and improve FAMEs yield. The GC-FID data indicates that microbial FAMEs was mainly composed of oleic acid and linolelaidic acid (82% of total FAMEs). It has been reported that microbial lipids were characterized as highly unsaturated fatty acids content feed stock for biodiesel (Patil *et al.*, 2011). The saturation rate ~48.2 % w/w total lipids (

) indicates that the FAMEs obtained from microbial oil will have very high oxidation stability than the biodiesel obtained from plant oil (*Jatropha*) (Jain *et al.*, 2011). Comparing the fatty acid profiles, the results revealed that N-LS followed by ultrasonic aided *in situ* transesterification did not cause any impact on the FAMEs composition.

## 3.6 Biodiesel fuel properties

The measurement of biodiesel fuel properties is quite complex due to high cost and the requirement of a considerable amount of fuel sample. Therefore, the researchers have developed prediction models and mathematical equations to predict the biodiesel properties from the FAMEs for *Aspergillus candidus*, *Aspergillus terreus* IBB M1 and *Yarrowia lipolytica* strains (Kakkad *et al.*, 2015). In order to certify the biodiesel for commercial sale, well defined standards have been set for FAMEs (ASTM D6751). In the present study, density, cetane number, acid value number, kinematic viscosity and total glycerol percentage were experimentally determined and the results were summarized in Table 2.4.4. The density, cetane number, acid number, kinematic viscosity

and total glycerol percentage of the biodiesel produced by N-LS pretreatment of the biomass followed by ultrasonication assisted in *situ* transesterification was 0.8602 g/cm$^3$ , 65.2, 1.15, 8.08 mm$^2$/s (at 40$^o$C) and 0.095 (%wt), respectively. The fuel properties of pure biodiesel and blended biodiesel (B100, B20, B10 and B5) were almost similar to ASTM standard biodiesel. Moreover, biodiesel obtained from *Yarrowia lipolytica* (N-LS pre-treated and ultrasonication assisted *in situ* transesterification) has fuel properties comparable to the biodiesel obtained from microalgae and fungi (Kakkad *et al.*, 2015, Mostafa *et al.*, 2013). Therefore, the biodiesel obtained by N-LS pretreatment followed by ultrasonication assisted *in situ* transesterification and having properties similar to the standard fuel will be suitable for commercial production and for application as transport fuel as well as electricity generation.

## 4. CONCLUSION

The results obtained in this study revealed that it is feasible to reduce reaction time employing N-LS treatment of wet biomass followed by ultrasonication aided *in situ* transesterification. Maximum FAMEs yield was obtained within 5 min of reaction time using N-LS assisted ultrasonication aided *in situ* transesterification, which is very low as compared to 12 h of reaction time used in the two-step transesterification process. The composition of FAMEs obtained in *in situ* transesterification with (or) without N-LS treatment was similar to that obtained in two step transesterification. The lipid bearing wet biomass (83.8% moisture) conversion to FAMEs using N-LS followed by ultrasonication aided *in situ* transesterification could be a promising approach as it eliminates the use of toxic solvents for lipid extraction and obviates the lyophilization or drying the wet biomass, which helps in reducing the energy consumption for an industrial scale biodiesel production.

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# 5. REFERENCES


1. Alvarez HM & Steinbuchel A (2002) Triacylglycerols in prokaryotic microorganisms. *Appl Microbiol Biotechnol*:367-376.

2. ASTM Biodiesel Specifications. Retrieved October 31, 2016, from http://www.afdc.energy.gov/fuels/biodiesel_specifications.html

3. Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*:911-917.

4. Brown RB & Audet J (2008) Current techniques for single-cell lysis. Journal of The Royal Society Interface 5(Suppl 2):S131-S138.

5. Dong T, Yu L, Gao D, Yu X, Miao C, Zheng Y, Lian J, Li T & Chen S (2015) Direct quantification of fatty acids in wet microalgal and yeast biomass via a rapid *in situ* fatty acid methyl ester derivatization approach. Applied Microbiology Biotechnology:10237-10247.

6. Ehimen EA, Sun ZF & Carrington CG (2010) Variables affecting the *in situ* transesterification of microalgae lipids. Fuel:677-684.

7. ElMekawy A, Hegab HM, Vanbroekhoven K & Pant D (2014) Techno-productive potential of photosynthetic microbial fuel cells through different configurations. Renewable and Sustainable Energy Reviews:617-627.

8. Folch J, Lees M & Stanley GHS (1957) A Simple Method For The Isolation And Purification Of Total Lipides From Animal Tissues. Journal of Biological Chemistry :497-509.

9. Giroud S, Frare C, Strijkstra A, Boerema A, Arnold W & Ruf T (2013) Membrane phospholipid fatty acid composition regulates cardiac SERCA activity in a hibernator, the Syrian hamster (*Mesocricetus auratus*). PloS one.

10. Haas MJ & Wagner K (2011) Simplifying biodiesel production: The direct or in situ transesterification of algal biomass. European Journal of Lipid Science and Technology :1219-1229.

11. Halim R, Gladman B, Danquah MK & Webley PA (2011) Oil extraction from microalgae for biodiesel production. Bioresource technology:178-185.

12. Jain S & Sharma MP (2011) Oxidation stability of blends of Jatropha biodiesel with diesel. Fuel:3014-3020.

13. Jazzar S, Olivares-Carrillo P, de los Ríos AP, Marzouki MN, Acién-Fernández FG, Fernández-Sevilla JM, Molina-Grima E, Smaali I & Quesada-Medina J (2015) Direct supercritical methanolysis of wet and dry unwashed marine microalgae (*Nannochloropsis gaditana*) to biodiesel. Applied Energy:210-219.

14. Kakkad H, Khot M, Zinjarde S & RaviKumar A (2015) Biodiesel Production by Direct In Situ Transesterification of an Oleaginous Tropical Mangrove Fungus Grown on Untreated Agro-Residues and Evaluation of Its Fuel Properties. BioEnergy Research:1788-1799.

15. Kuttiraja M, Krishna S, Dhouha A & Tyagi RD (2015) A Substrate-Based Approach for the Selection of Oil-Bearing Heterotrophs from Nitrogen-Deficient Soil for Lipid Production. Applied Biochemistry and Biotechnology:1926-1937.

16. Laurens LM, Quinn M, Van Wychen S, Templeton DW & Wolfrum EJ (2012) Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification. Anal Bioanal Chemistry:167-178.

17. Lunin VV, Sergeeva YE, Galanina LA, Mysyakina IS, Ivashechkin AA, Bogdan VI & Feofilova EP (2012) Biodiesel fuel production from lipids of filamentous fungi. Applied Biochemistry and Microbiology:46-52.

18. Medeiros DL, Sales EA & Kiperstok A (2015) Energy production from microalgae biomass: carbon footprint and energy balance. Journal of Cleaner Production:493-500.

19. Mostafa SS & El-Gendy NS (2013) Evaluation of fuel properties for microalgae *Spirulina platensis* bio-diesel and its blends with Egyptian petro-diesel. Arabian Journal of Chemistry.

20. Nagle N & Lemke P (1990) Production of methyl ester fuel from microalgae. Applied Biochemistry and Biotechnology :355-361.

21. Park J-Y, Nam B, Choi S-A, Oh Y-K & Lee J-S (2014) Effects of anionic surfactant on extraction of free fatty acid from *Chlorella vulgaris*. Bioresource Technology:620-624.

22. Patil PD, Gude VG, Mannarswamy A, Cooke P, Munson-McGee S, Nirmalakhandan N, Lammers P & Deng S (2011) Optimization of microwave-assisted transesterification of dry algal biomass using response surface methodology. Bioresource Technology:1399-1405.

23. Piemonte V, Di Paola L, Iaquaniello G & Prisciandaro M (2016) Biodiesel production from microalgae: ionic liquid process simulation. Journal of Cleaner Production:62-68.

24. Praveenkumar R, Gwak R, Kang M, Shim TS, Cho S, Lee J, Oh YK, Lee K & Kim B (2015a) Regenerative Astaxanthin Extraction from a Single Microalgal (*Haematococcus pluvialis*) Cell Using a Gold Nano-Scalpel. ACS Appl Material Interfaces :22702-22708.

25. Praveenkumar R, Lee K, Lee J & Oh Y-K (2015b) Breaking dormancy: an energy-efficient means of recovering astaxanthin from microalgae. Green Chemistry:1226-1234.

26. Rathore D, Nizami A-S, Singh A & Pant D (2016) Key issues in estimating energy and greenhouse gas savings of biofuels: challenges and perspectives. Biofuel Research Journal: 380-393.

27. Salam KA, Velasquez-Orta SB & Harvey AP (2016) Surfactant-assisted direct biodiesel production from wet *Nannochloropsis occulata* by *in situ* transesterification/reactive extraction. Biofuel Research Journal:366-371.

28. Sathish A & Sims RC (2012) Biodiesel from mixed culture algae via a wet lipid extraction procedure. Bioresource Technology:643-647.

29. Suter B, Grob K & Pacciarelli B (1997) Determination of fat content and fatty acid composition through 1-min transesterification in the food sample; principles. Zeitschrift für Lebensmitteluntersuchung und -Forschung A:252-258.

30. Velasquez-Orta SB, Lee JGM & Harvey A (2012) Alkaline in situ transesterification of *Chlorella vulgaris*. Fuel:544-550.

31. Vicente G, Bautista LF, Rodríguez R, Gutiérrez FJ, Sádaba I, Ruiz-Vázquez RM, Torres-Martínez S & Garre V (2009) Biodiesel production from biomass of an oleaginous fungus. Biochemical Engineering Journal:22-27.

32. Yellapu SK, Bezawada J, Kaur R, Kuttiraja M & Tyagi RD (2016) Detergent assisted lipid extraction from wet yeast biomass for biodiesel: A response surface methodology approach. Bioresource Technology:667-673.

33. Zhang X, Yan S & Tyagi RD (2015) Strategies of preserving lipids in microorganism after fermentation. Bioresource Technology:718-725.

34. Zhang X, Yan S, Tyagi RD, Drogui P & Surampalli RY (2014a) Ultrasonication assisted lipid extraction from oleaginous microorganisms. Bioresource Technology:253-261.

35. Zhang X, Yan S, Tyagi RD, Drogui P & Surampalli RY (2016) Ultrasonication aided biodiesel production from one-step and two-step transesterification of sludge derived lipid. Energy:401-408.

36. Zhang X, Yan S, Tyagi RD, Surampalli RY & Valero JR (2014b) Ultrasonication aided in-situ transesterification of microbial lipids to biodiesel. Bioresource Technology:175-180.

**Table 2.4. 1 Comparison of FAMEs yield during different in-situ transesterification processes using wet biomass**

| Oleaginous substance | Moisture Wt % | *In-situ* transesterification conditions | FAMEs yield % w/w | References |
|---|---|---|---|---|
| *Chlorella vulgaris* | 78.67 | HCl- catalyst, for 1h at 85$^{o}$C | 43.05 | (Laurens *et al.*, 2012) |
| *Nannochloropsis sp.* | 80.24 | HCl- catalyst, for 1h at 85$^{o}$C | 10.12 | (Laurens *et al.*, 2012) |
| *Yarrowia lipolytica* | 83.8 | $H_2SO_4$ – catalyst, for 10 min, at less than 25$^{o}$C; N-LS[a] | 94.3 | This study |
| *Nannochloropsis occulata* | 20 | SDS[b] and $H_2SO_4$ - catalyst, for 24 h at 60 $^{o}$C | 98.67 | (Salam *et al.*, 2016) |
| *Nannochloropsis sp* | 20 | NaOH – catalyst, for 10 min at 50 $^{o}$C; SCMH[c] | 75 | (Teo *et al.*, 2014) |
| *Nannochloropsis gaditama* | 80 | Supercritical methanol, for 50 min | 47.8 | (Jazzar *et al.*, 2015) |

a- N-Lauroyl sarcosine treated ultrasonication assisted *in-situ* transesterification

b- Sodium dodecyl sulphate

c- Simultaneous cooling and microwave heating

**Table 2.4. 2 Comparison of pre-treatment cost for lipid extraction**

| Method | Biomass harvesting | Pretreatment/Lipid extraction- Cost[a] | Transesterification | References |
|---|---|---|---|---|
| Conventional process | Centrifugation and freeze drying | C/M[b] cost - > 4 $/kg biomass | NaOH catalyst; 12h; 60$^{o}$C | (Halim et al., 2011, Zhang et al., 2014) |
| NUIT[c] | Centrifugation | N-LS cost -0.48 $/kg dry biomass | $H_2SO_4$ catalyst; Sonication 05 min; 25$^{o}$C | This study |
| SDS-IT[d] | Centrifugation and freeze drying | SDS cost - 0.50 $/kg dry biomass | $H_2SO_4$ catalyst; Incubation at 60$^{o}$C, for 24h. | (Salam et al., 2016) |

a- Cost calculated based upon the market rate of chemical in 2016

b- Chloroform and methanol mixture (2:1) if process equipped with distillation and re-utilize solvent.

c- N-larouyl sarcosine (N-LS) pretreatment followed by ultrasonication assisted *in-situ* transesterification

d- Sodium dodecyl sarcosine (SDS) assisted *in-situ* transesterification

**Table 2.4. 3 Comparison of fatty acid profiles of FAMEs produced through two step transesterification and in situ transesterification (with or without N-LS treated biomass); Ultrasonication assisted in-situ transesterification (with or without N-LS treated biomass).**

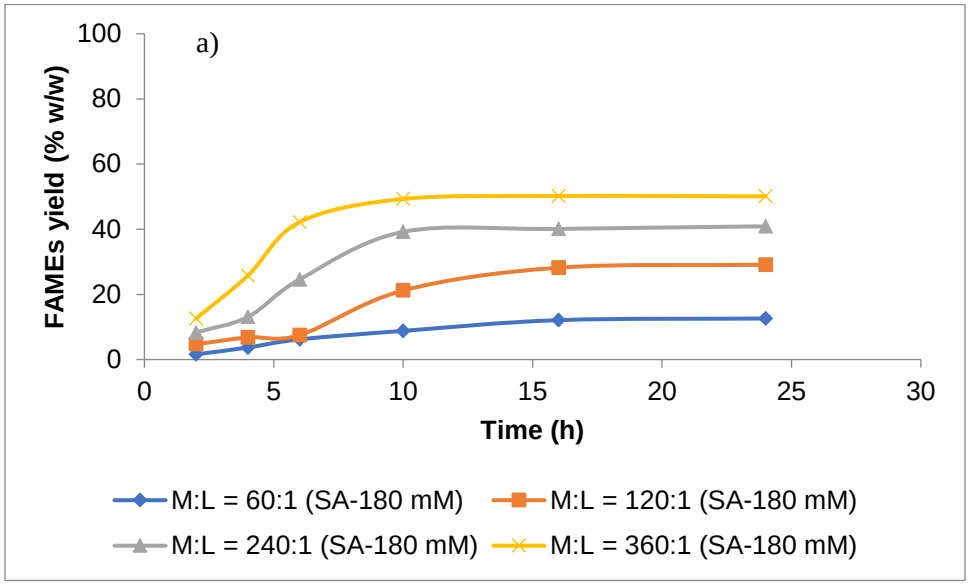
| | Relative amount of total fatty acids (%w/w) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acids | Two step Tranesterification (Microbial oil to FAMEs) | *In-situ* transesterification (Without N-LS treated biomass) | | | | *In-situ* transesterification (N-LS treated biomass) | | | | Ultrasonication aided *in-situ* transesterification (Without N-LS treated biomass) | | | | Ultrasonication aided *in-situ* transesterification (N-LS treated biomass) | | | |
| | | 60:1 | 120:1: | 240:1 | 360:01 | 60:01 | 120:01 | 240:1 | 360:1 | 60:1 | 120:1 | 240:1 | 360:1 | 60:1 | 120:1 | 240:1 | 360:01 |
| C16:0 | 8.5 | 8.1 | 8.9 | 9.3 | 10.1 | 8.7 | 9.1 | 9.7 | 9.9 | 8.9 | 8.8 | 9.5 | 9.8 | 8.7 | 9.1 | 9.3 | 9.6 |
| C16:1 | 1.3 | 0.6 | 0.8 | 1.2 | 0.7 | 0.8 | 0.9 | 1 | 1 | 0.9 | 0.7 | 0.8 | 1.0 | 0.5 | 0.9 | 1.1 | 0.9 |
| C18:0 | 3.5 | 3.9 | 3.8 | 3.3 | 3.2 | 3.2 | 3.1 | 3 | 3.5 | 3.4 | 3.2 | 3.0 | 2.8 | 3.5 | 3.3 | 3.1 | 3.3 |
| C18:1 | 33.1 | 30.8 | 31.7 | 32.9 | 33.3 | 31.2 | 32.2 | 33 | 33.2 | 32.7 | 31.9 | 30.6 | 29.5 | 32.6 | 31.6 | 30.1 | 33 |
| C18:2 | 48.9 | 36.5 | 46.9 | 48.2 | 46.8 | 39.2 | 46.2 | 48.2 | 48.7 | 40.8 | 48.0 | 48.1 | 48.8 | 40.9 | 48.1 | 48.2 | 49 |
| C18:3 | 4.7 | 5.0 | 4.7 | 5.3 | 6.4 | 4.9 | 5.2 | 5.9 | 6.1 | 5.04 | 5.3 | 5.3 | 5.9 | 4.9 | 5.1 | 5.8 | 6.09 |

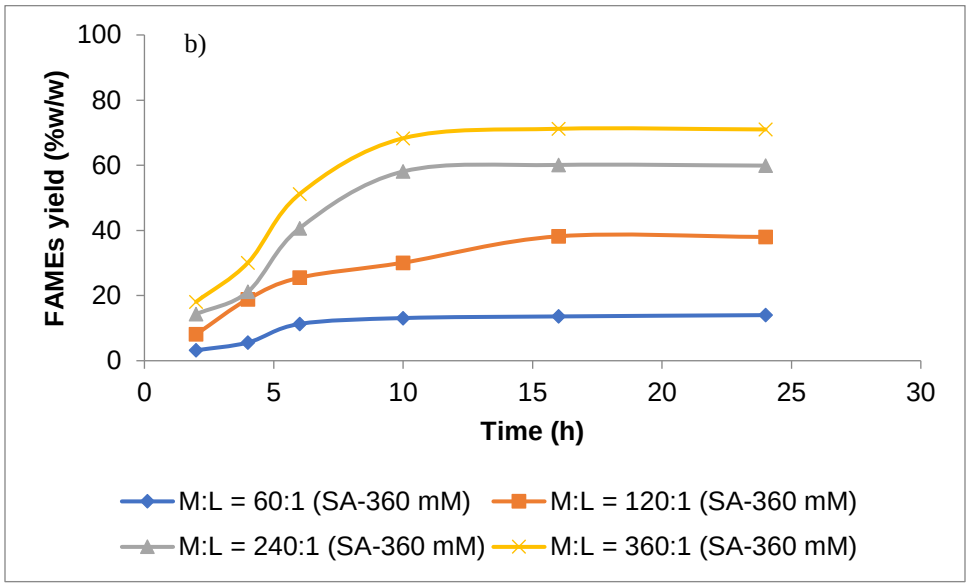
**Table 2.4. 4 Comparison of biodiesel fuel properties**

| | Biodiesel fuel properties | | | | | |
|---|---|---|---|---|---|---|
| | Density g/cm$^3$ (D664) | Cetane number | Acid number | Kinematic viscosity; mm$^2$/s, 40$^o$C (D445) | Total glycerol (% wt) | References |
| B100 | 0.8393 | 47 | 0.50 | 1.9-6.0 | 0.24 | ASTM (D6751-15a) |
| Biodiesel(Microalgae) | 0.8637 | 70 | 0.75 | 12.4 | Nr | (Mostafa *et al.*, 2013) |
| Biodiesel (B-100 [a]) | 0.8602 | 65.2 | 1.15 | 8.08 | 0.095 | This study |
| Biodiesel (B-20[a]) | 0.8452 | 69 | 0.143 | 6.12 | Nd | This study |
| Biodiesel (B-10[a]) | 0.8419 | 67 | 0.075 | 4.99 | Nd | This study |
| Biodiesel (B-5[a]) | 0.8401 | 60 | 0.036 | 4.01 | Nd | This study |

a- Biodiesel obtained from N-lauroyl sarcosine pre-treatment of biomass followed by ultrasonication assisted *in-situ* transesterification

Nd- Not determined; Nr- Not reported


a)
FAMEs yield (% w/w)
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (h)
M:L = 60:1 (SA-180 mM)
M:L = 120:1 (SA-180 mM)
M:L = 240:1 (SA-180 mM)
M:L = 360:1 (SA-180 mM)


b)
FAMEs yield (%w/w)
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (h)
M:L = 60:1 (SA-360 mM)
M:L = 120:1 (SA-360 mM)
M:L = 240:1 (SA-360 mM)
M:L = 360:1 (SA-360 mM)

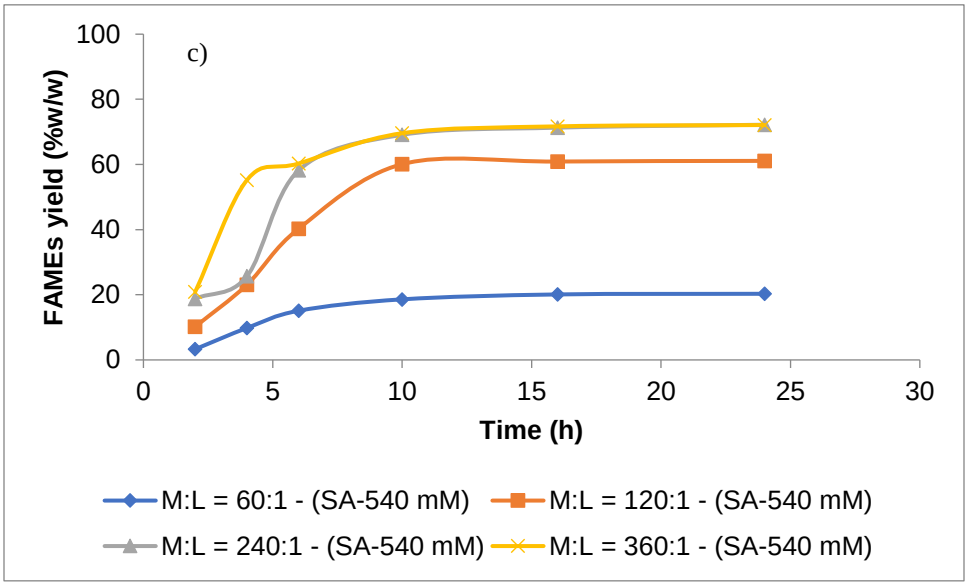
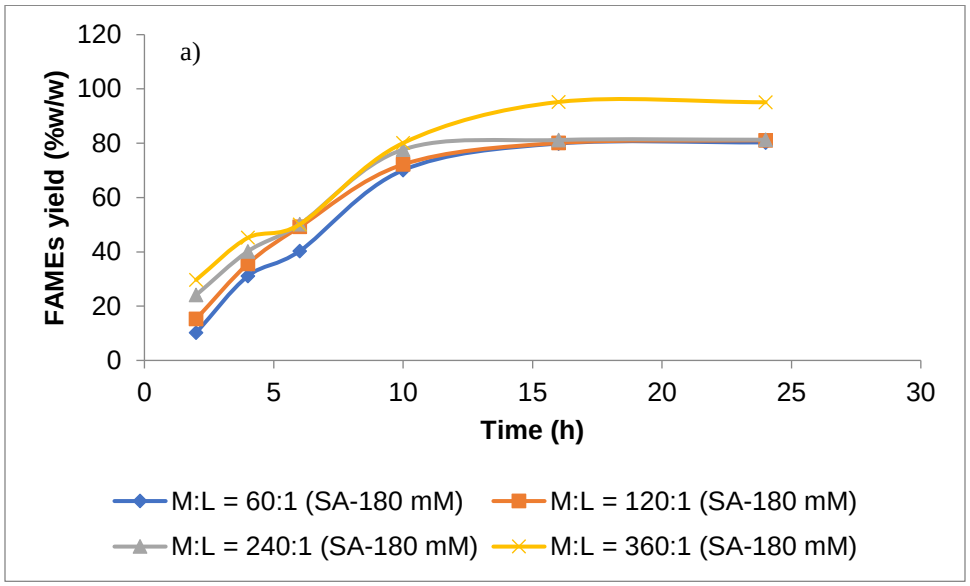
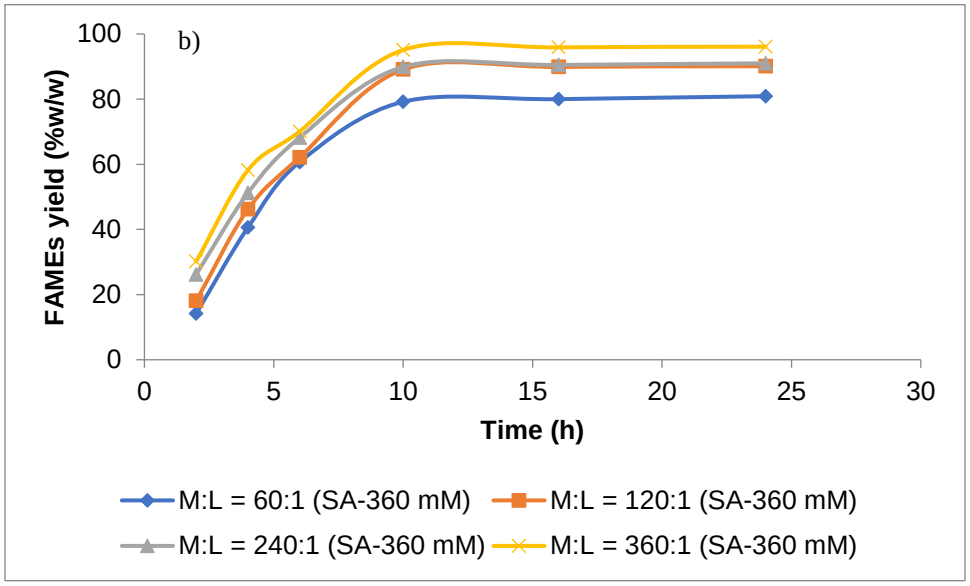



**Figure 2.4. 1 Variation of FAMEs yield with reaction time at different concentration of $H_2SO_4$ [SA (Sulfuric acid) -catalyst] and methanol: lipid (M:L) molar ratio during in situ transesterification of lipid bearing yeast wet biomass: a) SA concentration 180mM $H_2SO_4$ (catalyst); b) SA concentration 360mM $H_2SO_4$ (catalyst); c) SA concentration 540mM $H_2SO_4$ (catalyst). The results are statistically significant and $p<0.05$.**


a)
FAMEs yield (%w/w)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (h)
M:L = 60:1 (SA-180 mM)
M:L = 120:1 (SA-180 mM)
M:L = 240:1 (SA-180 mM)
M:L = 360:1 (SA-180 mM)


b)
FAMEs yield (%w/w)
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (h)
M:L = 60:1 (SA-360 mM)
M:L = 120:1 (SA-360 mM)
M:L = 240:1 (SA-360 mM)
M:L = 360:1 (SA-360 mM)

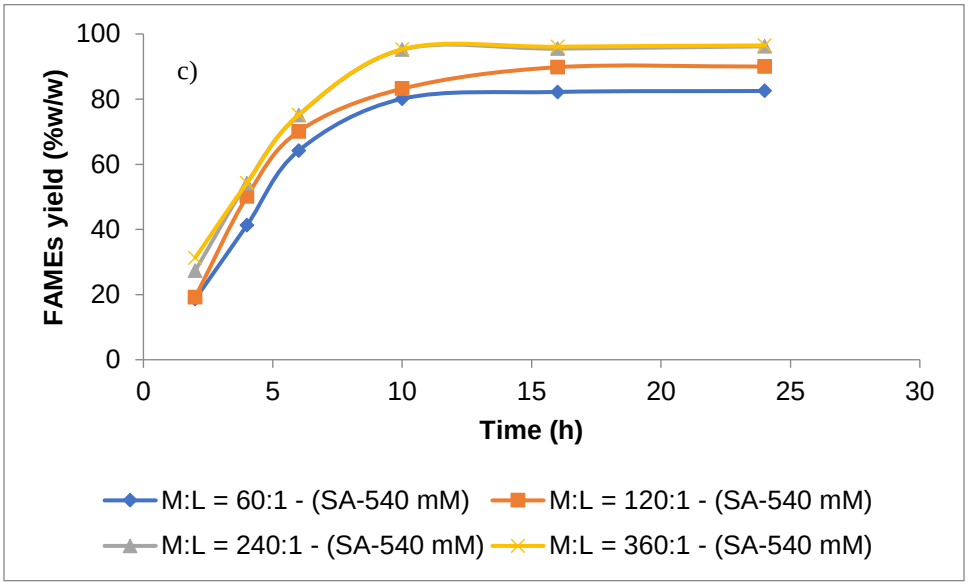
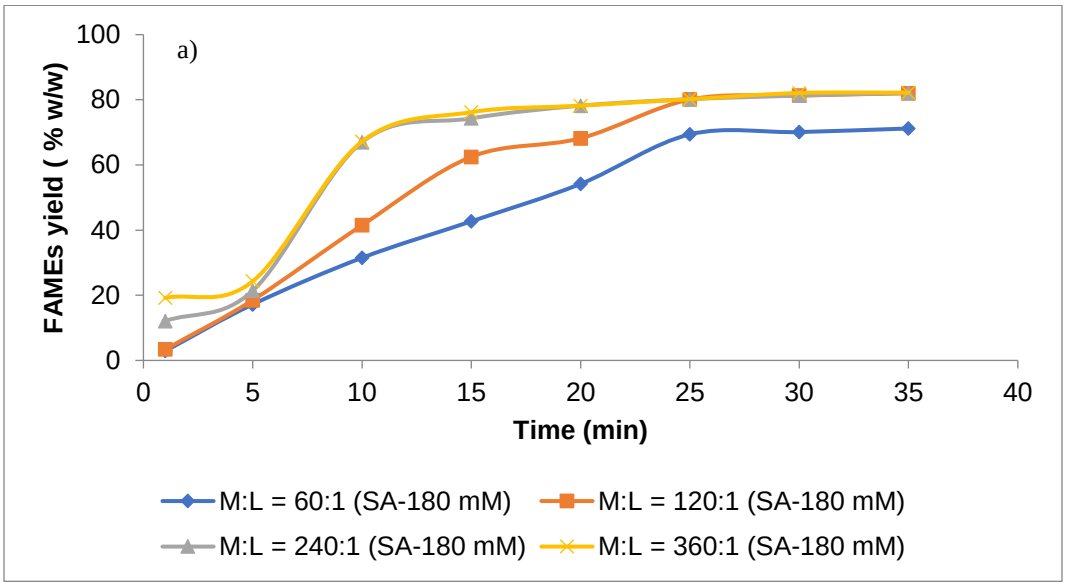
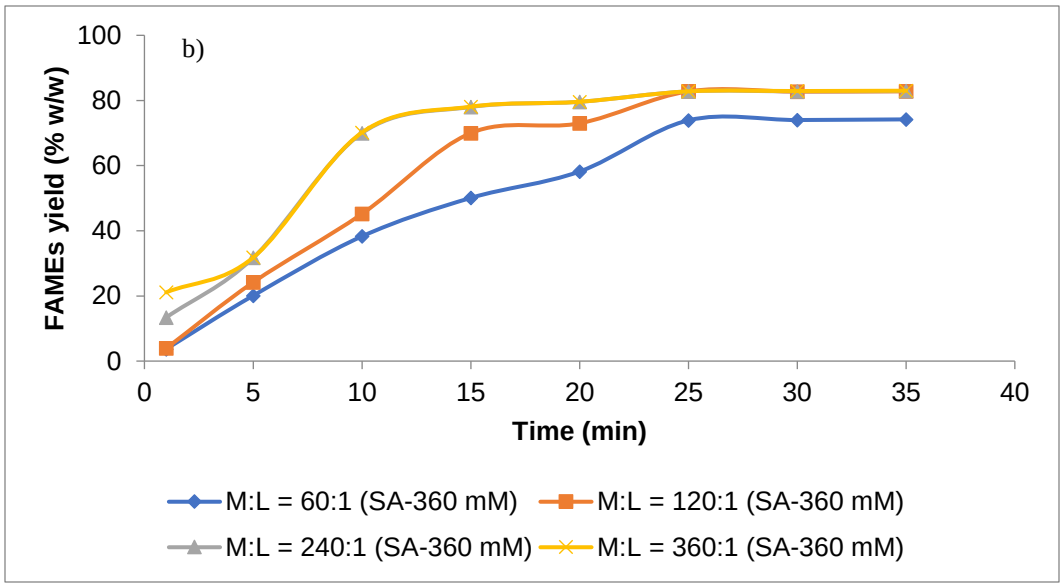



**Figure 2.4. 2 Variation of FAMEs yield with reaction time at different concentration of $H_2SO_4$ [SA(Sulfuric acid) -catalyst] and methanol: lipid (M:L) molar ratio during in situ transesterification of N-Lauroyl sarcosine treated lipid bearing yeast wet biomass: a) SA concentration 180mM $H_2SO_4$ (catalyst); b) SA concentration 360mM $H_2SO_4$ (catalyst); c) SA concentration 540mM $H_2SO_4$ (catalyst). The results are statistically significant and $p<0.05$.**


a)
100
80
60
40
20
0
FAMEs yield ( % w/w)
0
5
10
15
20
25
30
35
40
Time (min)
M:L = 60:1 (SA-180 mM)
M:L = 120:1 (SA-180 mM)
M:L = 240:1 (SA-180 mM)
M:L = 360:1 (SA-180 mM)


b)
100
80
60
40
20
0
FAMEs yield (% w/w)
0
5
10
15
20
25
30
35
40
Time (min)
M:L = 60:1 (SA-360 mM)
M:L = 120:1 (SA-360 mM)
M:L = 240:1 (SA-360 mM)
M:L = 360:1 (SA-360 mM)

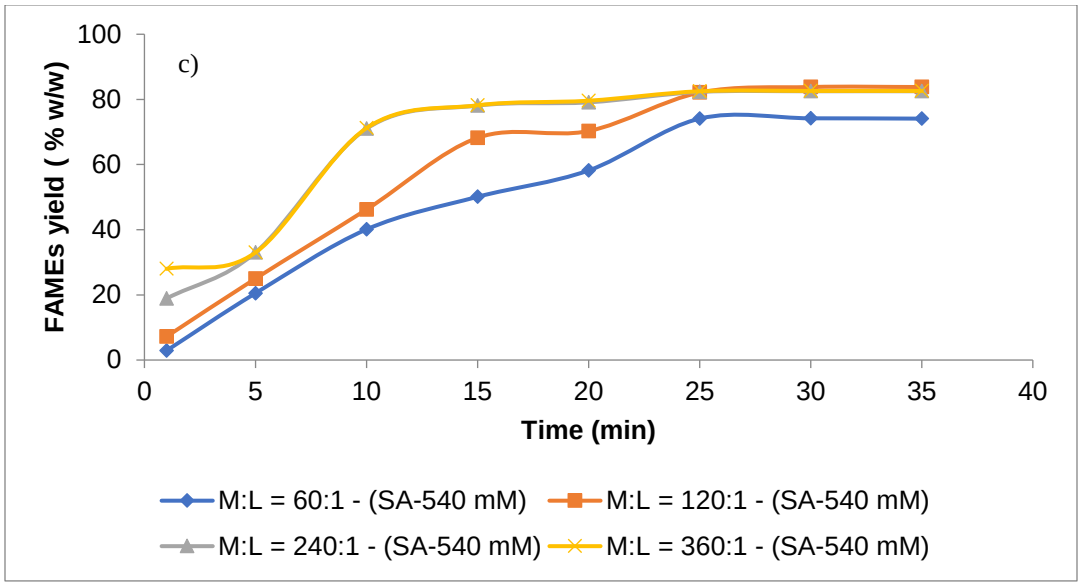
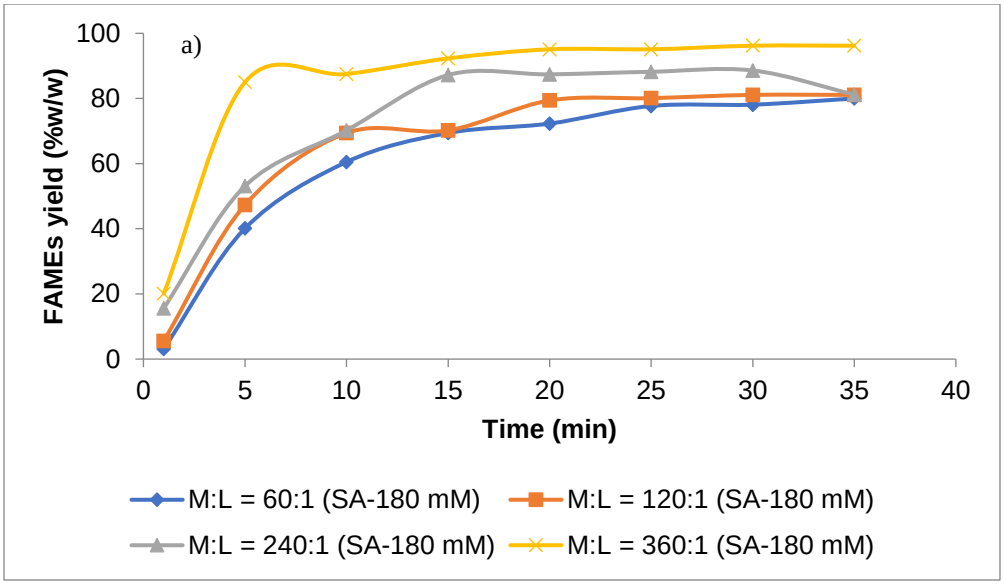
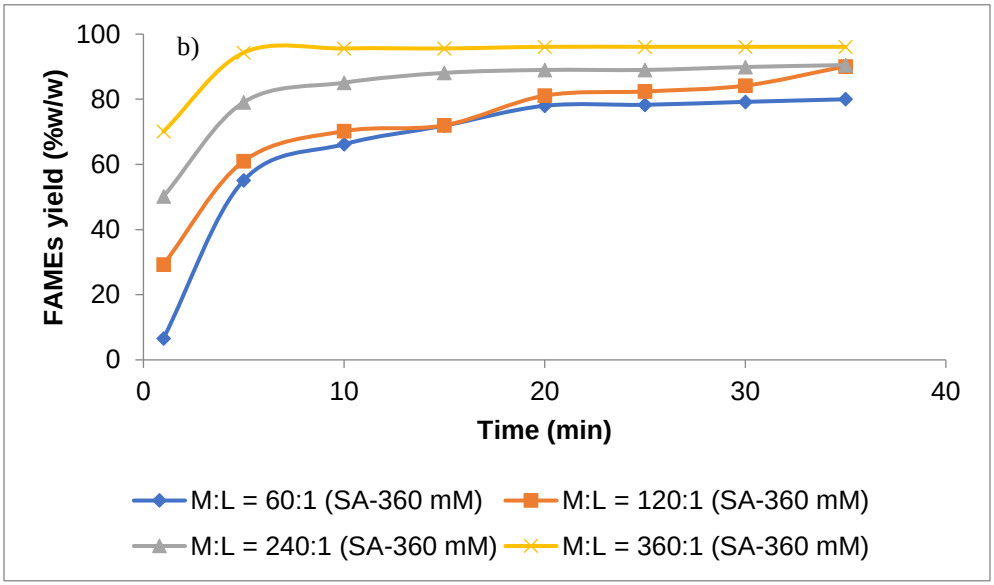



**Figure 2.4. 3 Variation of FAMEs yield with reaction time at different concentration of $H_2SO_4$ [SA (Sulfuric acid) -catalyst] and methanol: lipid (M:L) molar ratio during ultrasonication aided in-situ transesterification of lipid bearing yeast wet biomass: a) SA concentration 180mM $H_2SO_4$ (catalyst); b) SA concentration 360mM $H_2SO_4$ (catalyst); c) SA concentration 540mM $H_2SO_4$ (catalyst). The results are statistically significant and $p<0.05$.**


a)
FAMEs yield (%w/w)
Time (min)
M:L = 60:1 (SA-180 mM)
M:L = 120:1 (SA-180 mM)
M:L = 240:1 (SA-180 mM)
M:L = 360:1 (SA-180 mM)


b)
FAMEs yield (%w/w)
Time (min)
M:L = 60:1 (SA-360 mM)
M:L = 120:1 (SA-360 mM)
M:L = 240:1 (SA-360 mM)
M:L = 360:1 (SA-360 mM)

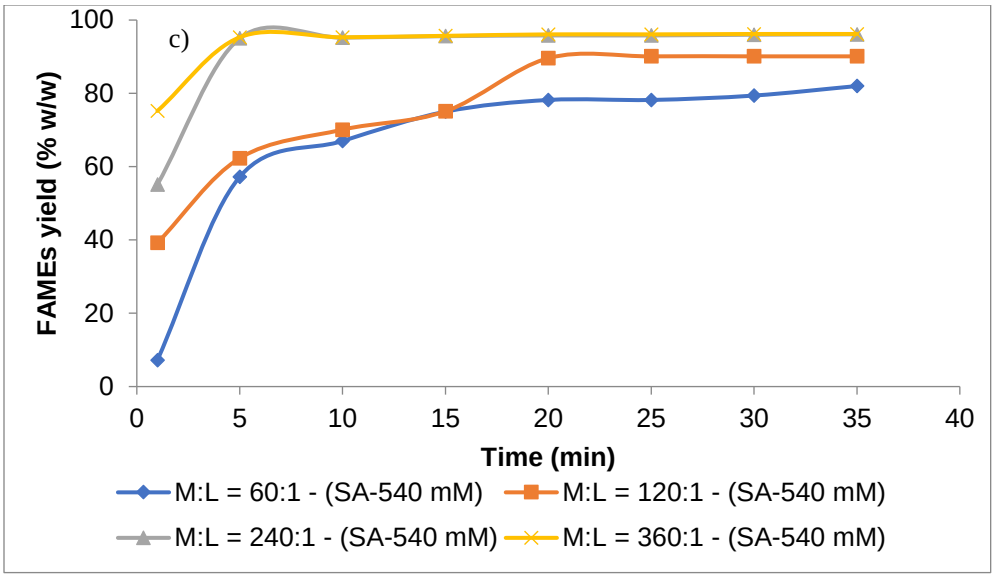



**Figure 2.4. 4 Variation of FAMEs yield with reaction time at different concentration of $H_2SO_4$ [SA (Sulfuric acid) -catalyst] and methanol: lipid (M:L) molar ratio during ultrasonication aided in situ transesterification of N-Lauroyl sarcosine treated lipid bearing yeast wet biomass: a) SA concentration 180mM $H_2SO_4$ (catalyst); b) SA concentration 360mM $H_2SO_4$ (catalyst); c) SA concentration 540mM $H_2SO_4$ (catalyst). The results are statistically significant and $p<0.05$.**

# CHAPITRE II

# PARTIE 5

# FREE NITROUS ACID AND N-LAUROYL SARCOSINE ASSISTED EX-SITU TRANSESTERIFICATION OF MUNICIPAL SLUDGE DERIVED LIPIDS

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**BIORESOURCE TECHNOLOGY (*Submitted 23rd April 2018*)**

# RÈSUME

La levure oléagineuse (*Trichosporon oleaoginous*) a été cultivée dans une boue d'épuration municipale pour produire un lipide microbien. Le bouillon fermenté a été désactivé par traitement thermique et concentré (décanté) en utilisant une combinaison de chlorure de calcium et de substance extrapolymère (39,9 mg EPS / g de biomasse) comme biofloculant. La biomasse de boues décantées a été traitée avec 1) N-LS ou FNA seulement; 2) Traitement séquentiel avec FNA et N-LS pour perturber la paroi cellulaire. Le lipide microbien a été séparé des cellules rompues en utilisant du chloroforme et du méthanol (1: 1) ou du pétrodiesel comme solvant. Le lipide microbien séparé a été transestérifié ex-situ en biodiesel. Le maximum de lipides a été extrait en utilisant 75 mg de N-LS / g de biomasse avec une efficacité d'extraction des lipides de 98,11 ± 1,6 et 95,8 ± 1,1% en poids / poids en utilisant respectivement du chloroforme / méthanol (1: 1) et du pétrole. En cas d'extraction de lipides assistée par FNA, l'efficacité maximale d'extraction des lipides a été de 94,3 ± 1,6 et 90,7 ± 1,1% p / p a été obtenue à 40 mg FNA / g de biomasse en utilisant du chloroforme et du méthanol (1: 1) respectivement. Cependant, au cours de l'extraction de lipides assistée par FNA, l'augmentation de la concentration de FNA au-delà de 10 mg de FNA / g de biomasse a affecté le profil des acides gras. Par conséquent, un traitement séquentiel par FNA et N-LS utilisant une faible concentration de FNA (10 mg FNA / g de biomasse) suivi du N-LS (20 mg de N-LS / g de biomasse) a été utilisé pour une efficacité maximale d'extraction de lipides de 95,1 et 92,3% / w l'utilisation d'hexane et du gasoil de pétrole. Celle-ci n’a affecté guère le profil des acides gras insaturés.

## ABSTRACT

Oleaginous yeast (*Trichosporon oleaginous*) was cultivated in a municipal wastewater sludge to produce microbial lipid. The fermented broth was deactivated by heat treatment and concentrated (settled) by using combination of calcium chloride and extrapolymeric substance (39.9 mg EPS/g biomass) as bioflocculant. The settled sludge biomass was treated with 1) N-LS or FNA only; 2) Sequential treatment with FNA and N-LS to disrupt cell wall. The microbial lipid was separated from the disrupted cells using chloroform and methanol (1:1) or petroleum diesel as solvent. The separated microbial lipid was ex-situ transesterified to biodiesel. The maximum lipid was extracted using 75 mg N-LS/g of biomass with lipid extraction efficiency of 98.11± 1.6 and 95.8 ± 1.1% w/w was achieved using chloroform/methanol (1:1) and petroleum diesel as solvent, respectively. In case of FNA assisted lipid extraction, the maximum lipid extraction efficiency of 94.3 ± 1.6 and 90.7 ± 1.1 %w/w was obtained at 40 mg FNA/g biomass using chloroform and methanol (1:1) and petroleum diesel as a solvent, respectively. However, during FNA assisted lipid extraction, increase in FNA concentration beyond 10 mg FNA/g biomass, affected the fatty acid profile. Therefore, sequential FNA and N-LS treatment using low concentration of FNA (10 mg FNA/g biomass) followed by N-LS (20 mg N-LS /g biomass) was employed and maximum lipid extraction efficiency of 95.1 and 92.3% w/w using hexane and petroleum diesel was obtained without any effect on unsaturated fatty acids profile.



**Keywords:** Sludge biomass, N-lauroyl sarcosine, free nitrous acid, Lipid extraction, Ex-situ transesterification, Biodiesel

# 1. INTRODUCTION

Plant oil (Edible and non-edible) is considered as one of the major feedstock for biodiesel production (Mardhiah et al., 2017). However, biodiesel production using plant oils is limited by the requirement of arable land and social and environment issues regarding the use of food crop for fuel production. (Hajjari et al., 2017). Therefore, an alternative approach involving the use of oleaginous microorganisms cultivated with high lipid content could be used as promising feedstock for biodiesel production.

Oleaginous microorganisms can accumulate lipid content up to 80% of its cell dry weight (CDW) in the form of lipid droplets as an energy source. There are several microorganisms, which produce microbial lipids such as *Yarrowia lipolytica*, *Trichosporon oleaginous*, *Mortierella isabellina*, *Lipomyces starkeyi* (Kuttiraja et al., 2015; Rivaldi et al., 2017; Wild et al., 2010; Zhang et al., 2014). However, several researchers recognized that microbial lipid production cost is too high and it is not affordable for biodiesel production (Nigam & Singh, 2011). In microbial lipid cultivation, carbon and nutrients are required, and they count for a significant part of the total microbial lipid cost (Nigam & Singh, 2011). To reduce biodiesel production cost, organic waste including wastewater, agriculture waste, restaurant waste, which are abundant in carbon and nutrients, have been used as raw material in oleaginous microorganism cultivation for lipid production (Chen et al., 2018).

Wastewater sludge is another promising raw material. Zhang et al. (2014) has utilized wastewater sludge as a growth medium for *Trichosporon oleaginous,* and the lipid accumulation reached up to 30% w/w of dry cell biomass. During economic analysis of biodiesel production from microbial lipid using wastewater sludge as raw material, the lipid content was assumed in the range of 30 % to 50% w/w of dry biomass and the final biodiesel unit production cost was 1.08 US $/kg biodiesel (Chen et al., 2018), which is comparable to biodiesel produced using plant oil (1 US $/kg biodiesel). Therefore, wastewater sludge has proved to be promising feedstock for biodiesel production.

After fermentation, biomass harvesting, oil extraction, and transesterification are major steps for biodiesel production. The traditional biomass harvesting is done by using centrifugation, which is an energy-intensive process. There are several methods developed for biomass harvesting based on the type of microorganism such as centrifugation for yeast, filtration for fungi, flocculation, biofiltration and gravity settling for microalgae. But there is lack of knowledge on

oleaginous yeast biomass harvesting with minimal cost. In our previous study, the harvesting of oleaginous yeast biomass was done by using the extra polymeric substance (EPS) as bioflocculant within less than 30 min (Yellapu et al., 2018) and is considered to be simple and low energy intensive process.

Furthermore, biomass drying and cell wall disruption are essential steps for biodiesel production process. Biomass drying by using freeze dryer is not suitable for low-cost products, such as biodiesel. There are several cell wall disruption technologies developed based on the size and type of the cell wall such as 1) physical (ultrasonication, bead beating, homogenization, microwave, etc.,); 2) chemical (Surfactants, acids, base, tritonX100) and supercritical methods 3) Biological (Enzymes) (Yellapu et al., 2018). However, cell wall disruption using existing techniques is limited by high energy requirment. Such as microwave technology, ultrasonication etc.,. Therefore, currently researchers are working on low energy and low-cost cell wall disruption technologies using wet biomass.

Free nitrous acid (FNA, i.e., $HNO_2$), is a weak and monobasic acid in the form of nitrite salts and it has been regarded as a biochemical reagent. FNA and its derivatives such as the nitric oxide radical ($NO\cdot$) and nitrous anhydride ($N_2O_3$) affect protein and polysaccharides degradation (Wu et al., 2018). Hence FNA has been applied in the water and wastewater industry for sludge treatment and biofilm control. However, there have been no reports on the effect of FNA on yeast cellular biomass and specifically on lipid bearing yeast cell wall.

Transesterification is the final step for biodiesel production. But due to the multiple steps involved in biodiesel production using conventional transesterification process, in-situ transesterification (oil extraction and transesterification at the same time) process was investigated. However, there is insufficient literature on oil extraction and transesterification from sludge biomass or lipid bearing biomass grown in sludge. Pastore et al. (2013) reported the lipid or oil separation from secondary sludge using organic solvent (chloroform) and obtained oil content of 17.3% w/w after incubation at 60$^{o}$C for 4 h. However, during oil extraction using solvent, organic matter was also separated along with lipid in the solvent phase, which further affected transesterification efficiency (<70% w/w). Therefore, alternative approach was necessary for cell wall disruption and separation of microbial lipids from sludge biomass without any organic matter. In our earlier study, N-lauroyl sarcosine was used for cell disruption to release lipids and in-situ transesterification using wet yeast biomass and the obtained oil extraction efficiency was >90% with high conversion efficiency to biodiesel (Yellapu et al., 2017). Moreover, N-LS could help in disruption of the cell wall of sludge biomass without mechanical shearing and use of N-LS may

not cause the problem of separation of organic matter from sludge biomass in a solvent along with the lipids.

So far, it is still unclear to produce biodiesel from lipid bearing cells grown in sludge without solubilizing sludge organic matter to the biodiesel. Therefore, in this study, cultivated *T. oleageanous* biomass grown in sludge was settled using bioflocculant (EPS), and further lipid extraction was conducted using N-LS or FNA or sequential FNA process followed by N-LS treatment. The optimum concentration of N-LS or FNA was determined to extract maximum lipid by using chloroform and methanol (1:1) or petroleum diesel as solvent. After that, extracted oil was Ex-situ transesterified and converted to biodiesel and effect of FNA and N-LS on cell wall disruption, and fatty acid profiles were compared.

# 2. MATERIALS AND METHODOLOGY

## 2.1 Municipal sludge medium and cultivation

Secondary wastewater sludge was collected from municipal wastewater treatment plant (Communaute Urbain de Quebec, CAQ), Quebec City, Canada. The sludge was settled for 12h at 4$^{o}$C and the supernatant was discarded. Thereafter, sludge solids were washed by centrifugation at 9000 g for 15 min and final obtained pellet was re-suspended in tap water and solids concentration was determined. The washed and sludge solids (40 g/L) were further diluted (30g/L) to prepare pre-inoculum and production media. In this study, *Trichosporon oleaginous* (ATCC20509) was used for lipid production using washed municipal sludge solids concentration of 30 g/L and pH was adjusted to 11 using 4 N NaOH and sterilized at 121$^{o}$C for 30 min. After cooling, 40 g/L crude glycerol (obtained from local biodiesel industry) was added and pH was adjusted to 6.8 (Zhang et al., 2014). After 72 h fermentation, fermented broth was heat treated to deactivate or to kill lipid bearing cells and it was further used for biomass harvesting and a part was lyophilized to determine total lipids using chloroform and methanol (standard method) (Bligh and dyer 1958; Vicente et al., 2010).

## 2.2 Biomass harvesting – Flocculation

The heat-treated fermented broth obtained by using municipal secondary sludge and crude glycerol as substrate was used throughout the experiments. In this study, 500 mL of heat treated fermented broth was transferred to 1 L glass beaker and jar test (Philips instruments) was performed by slowly adding calcium chloride ($CaCl_2$) and after mixing at 130 rpm for 5 min, pH was adjusted to 9. The $CaCl_2$ was added into the fermented broth until zeta potential was reached

to -9.2mV. The mixing of the fermented broth and calcium chloride was performed at 100rpm. Thereafter, optimized concentration (39.9 mg EPS/g fermented biomass) of EPS was added as per our previous study (Yellapu et al., 2018). The S-EPS (crude) employed in this study was produced by *Cloacibacterium normanense* using wastewater sludge as a raw material (Nouha, 2016). The obtained floc was transferred carefully to measuring cylinder and sludge biomass was allowed to settle for 30 min. Further, settling velocity and flocculation activity was determined as discussed by Yellapu *et al.* (2018). After settling, suspended solids concentration was determined in the settled biomass and it was further used for lipid extraction studies. The cell disruption to release the intracellular lipid was conducted using N-lauroyl sarcosine and/or free nitrous acid. Further transesterification of extracted lipids to fatty acid methyl esters was also performed.

## 2.3 Conventional lipid extraction and transesterification

To determine the total lipids bearing settled yeast biomass grown in sludge (LSYBS), the standard chloroform and methanol extraction procedure with minor modification was used (Bligh & Dyer, 1959). One gram of the lyophilized dry biomass was mixed with 15 mL of chloroform and methanol mixture (2:1 v/v, v/v representing volume by volume ratio, which means every 2 mL of chloroform was mixed with 1 mL of methanol) and then subjected to heat at 60 °C for 4 h. Thereafter, centrifugation (5000 rpm for 15 min) was performed, and the upper top phase containing lipids dissolved in solvent was transferred to a pre-weighed glass vial. The bottom phase containing residual solids was again subjected to lipid extraction using 15 mL chloroform and methanol (2:1 v/v) as discussed above. The final reaction mixture was filtered using Whatmann filter paper and obtained total filtrate (lipids dissolved in solvent) was collected in a pre-weighed screw cap glass tube. After evaporation of the solvent at 60°C, the glass tube was weighed again. The lipid content was calculated as reported by Yellapu *et al.,* (2017). The lipid content of the original sludge (without fermentation) was considered as the control. The experiment was done in triplicates. The extracted lipid was then converted to biodiesel by reaction with methanol (methanol to lipid molar ratio 6:1) in the presence of $H_2SO_4$ (1% v/v methanol) within 12 h reaction time (Yellapu et al., 2017). The biodiesel was further analyzed with Gas Chromatograph linked to Mass Spectroscopy (GC–MS) (Perkin Elmer, Clarus 500). The transesterification efficiency was calculated based on the GC–MS results as discussed in section 2.6.

## 2.4 N-LS or FNA assisted lipid extraction from settled sludge biomass

The LSYBS biomass slurry (5mL, which is equal to 1.1 g dry biomass) was obtained after heat treatment and biomass settling was done with EPS aid. To disrupt the cells and release the intracellular lipids, two different cell wall disruption techniques were applied using N-LS (N-lauroyl sarcosine) or FNA (Free nitrous acid).

i) The biomass slurry was treated with different N-LS (N-lauroyl sarcosine) concentration, and the final concentration of N-LS in the settled biomass slurry was 50, 75, 100, 125 and 200 mg N-LS/g dry biomass. The incubation was done at $30^0$C and 180 rpm in an incubator shaker for 20 minutes. After the reaction, 15 mL of chloroform and methanol (1:1 v/v) was added to the reaction mixture and vortexed for 5 min. After that, the total mixture was filtered using 0.45µm Whatman filter paper with vacuum pressure. The obtained filtrate consists of two phases, top phase with water and methanol layer and bottom phase with microbial lipid in chloroform phase. The bottom phase consisting of microbial lipid in chloroform phase was separated and transferred to clean pre-weighed glass tube ($CM_{W1}$) and dried in hot air oven at 60°C until a constant weight ($CM_{W2}$). CLw was total lipid extracted by using standard chloroform and methanol. The lipid extraction efficiency was calculated as follows.

Lipid extraction efficiency = $(CM_{W2} – CM_{W1}) / CL_{W\,X}$ 100 % ----------(1)

In another set of experiments, the petroleum diesel was used as a solvent to separate lipid from the reaction mixture. The petroleum diesel (50mL/g lipid) was added to N-LS treated mixture and kept for incubation at 70°C for 10 minutes (Optimized conditions in our laboratory) to enhance emulsification of microbial lipids (present in N-LS treated solution) to petroleum diesel. The screw-capped glass tubes containing the reaction mixture were tightly closed. After incubation, the mixture in tubes was kept for phase separation until clear top phase (approximately in 30 min) was obtained. The top phase consists of microbial lipid in petroleum diesel and the bottom phase with residual defatted biomass, water and other intracellular products. The microbial lipid present in petroleum diesel ($L_{pd}$) was directly used for transesterification without drying. The transesterified microbial lipid to FAMEs (Fatty acid methyl esters) was analyzed using FT-IR and obtained FAMEs were used to calculate the weight of the microbial lipid separated using petroleum diesel.

The total lipid obtained by using petroleum diesel was calculated according to Equation (2):

Lipid recovery efficiency of petroleum diesel (% w/w) = $L_{pd}$ / $L_c$ × 100 % -------(2)

Where, $L_{pd}$ is the weight of lipid (g) present in (5 mL of settled biomass, which is equal to 1.1 g dry biomass) 15 mL petroleum diesel. $L_c$ is the dry weight of lipid (Lc = 0.55 g) obtained using dry cells and standard chloroform and methanol method (Bligh and dyer 1958) using 1.1 g dry biomass (or 5 mL of settled biomass 220 g/L)

ii) Another cell wall disruption technique used in this study was using FNA (Free nitrous acid). The settled biomass slurry was treated with different concentration of sodium nitrite ($NaNO_2$) and final concentration of sodium nitrite converted to FNA in settled biomass slurry was 0.020, 0.040, 0.060 and 0.08 g FNA/g dry biomass. The pH was kept approximately constant during the whole pre-treatment at 5.0 ± 0.2 by manually adding 0.5 M HCl. The concentration of FNA was varied by controlling the nitrite concentration at pH 5 as described. The FNA concentration was calculated using the following formula.

$C_{FNA} = C_{NO2^-N} / (Ka \times 10^{pH})$

Where $C_{FNA}$ and $C_{NO2^-N}$ is the concentration of FNA and nitrite dissolved in the solution and $Ka = e^{2300\ (273.15+T)}$ at an operation temperature T (°C) (25°C in this study).

The incubation was done at 25°C and 180 rpm in an incubator shaker for 20 minutes and, lipid in the reaction mixture was separated using chloroform and methanol or petroleum diesel as discussed in section 2.4.

Once lipid extraction was done using chloroform and methanol (1:1 v/v) or petroleum diesel, immediately, transesterification was conducted to know the effect of N-LS or FNA concentration on the fatty acid profile. Depending upon the impact of reactants (N-LS or FNA) on fatty acid profile, sequential treatment using low concentration of FNA (High concentration of FNA can react with unsaturated fatty acids and convert them into saturated fatty acids, which can decrease the quality of biodiesel) and varying concentration of N-LS was performed as below.

## 2.5 Sequential free nitrous acid and N-lauroyl sarcosine treatment for lipid extraction

The optimized concentrations of reactants (FNA) was selected based upon the effect of reactants on fatty acid profiles. For each test, 5 mL (consists 1.1 g dry biomass) of LSYBS biomass slurry was treated for lipid extraction with the selected concentration of reactants, depended upon their effect on FAMEs profile. The sequential process was followed by using two different steps: 1) The

FNA (10 mg FNA/g dry sludge biomass) was added to reaction tube containing 5mL of settled fermented biomass and incubated for 30 min with agitation of 200 rpm at room temperature (25$^{o}$C).

After the reaction, the second step, optimization of N-LS concentration (to find the minimum concentration required to breakdown the phospholipid membrane around lipid bodies) was performed. 2) The reaction mixture was treated with different N-LS (N-lauroyl sarcosine) concentration, and the final concentration of N-LS in reaction mixture was 0.05, 0.010, 0.015 and 0.020 g N-LS/g sludge biomass. After the reaction, the lipid was separated from the reaction mixture with chloroform and methanol or petroleum diesel as discussed in above section 2.4.

## 2.6 Ex-situ transesterification of recovered lipids

The transesterification was conducted for different samples: 1) Dried microbial lipids recovered using chloroform and methanol (N-LS or FNA treated or combination of FNA and N-LS to disrupt the cells); 2) Microbial lipids (N-LS or FNA or combination of FNA and N-LS disrupted cells) present in petroleum diesel (Ex-situ transesterification).

The dried microbial biomass lipids recovered using chloroform and methanol were dissolved in hexane (20 mg lipid/mL hexane) for transesterification process. However, microbial lipids recovered by using petroleum diesel were used as such for further process. The transesterification process was conducted by using sodium hydroxide as a catalyst dissolved in methanol (6:1 methanol to lipid molar ratio or 0.4 mL methanol per gram lipid) solution, and the final concentration of catalyst to lipid was 1% v/w (1mL $H_2SO_4$/100g lipid). The mixture was then heated to 70$^{o}$C for 2h. After the reaction, the mixture was cooled to room temperature, and 10mL water (mL/g lipid) was added. After mixing, the mixture was kept for phase separation for 30 min. The top phase contained biodiesel in hexane (control), or biodiesel (BD) in petroleum diesel (PD) and the bottom phase contained a residual catalyst, residual methanol, and glycerol. The biodiesel in hexane (control) was further purified as discussed in our previous study (Yellapu et al., 2016). Moreover, the biodiesel present in petroleum diesel was washed with 2% w/w sodium bicarbonate (to remove excess water) and allowed to stand for phase separation until a clear solution was obtained in the top phase. The top phase contained biodiesel in petroleum diesel, and bottom phase contained residual water with sodium bicarbonate. The biodiesel present in petroleum diesel was directly quantified by using Fourier transform infrared spectrometer (FT-IR) to determine the percentage of biodiesel present in the petroleum diesel. In case of control or N-

LS disrupted cells (lipids recovered by chloroform and methanol as presented in section 2.3), the biodiesel concentration was determined by using GC-MS**.** The lipid conversion efficiency to biodiesel (% w/w) was calculated based on the concentration of biodiesel measured by GC-MS divided by the total lipid concentration × 100%. All experiments were conducted in triplicate, and the standard deviation was less than 5%.

The lipid conversion to biodiesel present in petroleum diesel was calculated using Eq (3)

Lipid conversion efficiency % (w/w) = $B_{pd}$ (or) $B_c$ / 0.4 g × 100 % ---------- (3)

Where $B_{pd}$ is grams of biodiesel in petroleum diesel. $B_c$ is biodiesel concentration obtained from control experiment.

## 2.7 Quantification of biodiesel using FT-IR

The biodiesel present in petroleum diesel was quantified using (FT-IR) Fourier transform infrared spectrometer (NicoletTM is 50FT-IR, Thermoscientific Inc) equipped with standard KBx beam splitter with DTGS detector. The FT-IR scanning for sample analysis was conducted as per ASTM D7371.The 50 µL of sample containing biodiesel in petroleum diesel obtained from transesterification was placed in a smart iTx-Diamond. The spectra was collected in 40 seconds (16 scans and 4 cm-1 resolution). The data was collected using OMNICTM spectroscopy software, and the chemometrics was performed using TQ AnalystTM. The standard was prepared by using different concentration (B-1 to 100 % v/v) of pure biodiesel (obtained from trans-esterified sunflower oil). The Quantification of biodiesel was performed in triplicate, and the standard deviation was less than 5%. The biodiesel percentage was calculated based on the height and area of the peak obtained in standard calibration graph.

# 3. RESULTS AND DISCUSSION

## 3.1 Lipid production, Biomass settling and lipid extraction

The biomass concentration obtained after 72h fermentation was 42 g/L and after heat treatment, the biomass concentration decreased to 40 g/L. It explains that some of the suspended organic matter was solubilized during heat treatment. Shair *et al.,* (2015) investigated that the optimal zeta potential to achieve efficient flocculation activity should be between +10, 0, -10 mV. Therefore, in this study, once zeta potential of fermented broth (lipid bearing sludge biomass - LSYBS biomass)

decreased to less than -10mV, bio flocculant (EPS) was added slowly as optimized in our earlier study (Yellapu *et al.,* 2017). After addition of EPS in the fermented broth, initially small flocs were formed when mixed at 130rpm and after slow mixing at 80rpm, floc size increased. The solution was transferred to measuring cylinder to determine settling velocity of biomass, which was found to be 1.28 mm/s and flocculation activity of 94.2% was recorded after 15 min of settling. The final biomass concentration of settled slurry was 210 g/L for 40g/L of initial biomass concentration (Table 2.5.1). After biomass settling, lipid content of the settled solids as well as for the initial sludge solids (without fermentation) was determined using conventional chloroform and methanol (2:1 v/v) extraction method. The lipid content for settled biomass and initial sludge solids was 55 ± 1.4% (w/w) and 5.21 ± 0.1% (w/w) respectively. The lipid content obtained by standard methods considered as the total lipid content of biomass (Yellapu et al., 2017; Zhang et al., 2016).

## 3.2 Optimization of N-Lauroyl sarcosine (N-LS) concentration for lipid extraction

In our earlier study, it was observed that 40 mg N-LS/g dry biomass was required for complete cell wall disruption of wet biomass (without sludge) at reaction temperature of 30$^{o}$C and incubation time of 10 min (Yellapu et al., 2016). Moreover, the lipid bearing sludge biomass is a complex matrix containing lipid bearing biomass and other solids. Therefore, N-LS concentration was further optimized between 50 to 200 mg/g biomass for cell wall disruption using settled sludge biomass. It was observed that an increase in N-LS concentration, increased the lipid extraction efficiency and the maximum lipid extraction efficiency of 98.11± 1.6 and 95.8 ± 1.1% w/w was achieved at 75 mg N-LS/g dry biomass in case of chloroform/methanol (1:1) and petroleum diesel as a solvent, respectively (Figure 2.5.1). Further increase in N-LS concentration, decreased the lipid extraction efficiency. It has been reported that the cell wall disruption is caused by formation of micelle at critical concentration of N-LS (Yellapu et al., 2015). Zhang et al., (2016) also studied oil extraction using sludge biomass in chloroform and methanol (1:1) with ultrasonication reactor and maximum lipid extraction efficiency of 108% w/w was obtained at 20 min. The lipid extraction efficiency of >100% was due to the fact that ultrasonication caused the dissociation of the impurities associated with sludge and after dissociation, the impurities were extracted along with lipids. The study suggested that ultrasonication could not be suitable for lipid extraction from complex matrix such as sludge and an extra step will have to be added to further purify the lipids by dissolving the lipids in organic solvent.

However, in this study, treatment with N-LS caused disruption of cell wall without breakdown of sludge solids and the lipids extracted in the N-LS reaction mixture Once N-LS breaks the cell wall and lipid bodies, lipids will be dispersed in the form of oil droplets in the N-LS reaction mixture solution) were further separated into solvent (chloroform and methanol or petroleum diesel) interphase within 5 min. The lower contact time (<5 min) between NLS reaction mixture (consisting of lipids, cell debris and sludge solids) and solvent (chloroform and methanol or petroleum diesel) led to elimination of sludge solids dissolution along with the lipids. Therefore, N-lauroyl sarcosine assisted lipid extraction can be successfully employed for lipid extraction from sludge biomass as compared to the other extraction methods.

## 3.3 Optimization of free nitrous acid for lipid extraction

Free nitrous acid (FNA) assisted cell wall disruption is another alternative approach to extract lipid. As presented in Figure 2.5.2 with increase in FNA concentration from 10 to 80 mg FNA/ g biomass, there was an increase in lipid extraction efficiency and maximum lipid extraction efficiency of 94.3 ± 1.6 and 90.7 ± 1.1 %w/w was obtained at 40 mg FNA/g biomass using chloroform and methanol (1:1) and petroleum diesel as a solvent, respectively. However, with further increase in FNA concentration, the lipid extraction efficiency was constant. The lipid extraction efficiency was almost similar in case of hexane or petroleum diesel as a solvent. Research on reactive nitrogen species have shown that FNA and its derivatives such as nitric oxide (NO.) and nitrous anhydride ($N_2O_3$) are affective for degradation of proteins and polysaccharides (Dedon & Tannenbaum, 2004), suggesting that FNA can disrupt the cell wall and use of hydrophobic solvents (chloroform or n-hexane) can recover the lipids without any further incubation. Therefore, it can be concluded that low FNA concentration (compared to NLS) was able to disrupt the cell wall, however, increasing FNA concentration, may also have negative impact on fatty acid profile (discussed latter). Therefore, to ascertain if there was an impact of FNA on microbial lipids composition, further experiments were performed by using low concentration of FNA followed by N-LS treatment.

## 3.4 Sequential lipid extraction using free nitrous acid and N-lauroyl sarcosine

The lipid extraction efficiency of sludge biomass using low concentration of free nitrous acid (10 mg FNA/g biomass) was 58.2 and 54.7 % w/w with hexane or petroleum diesel (Table 2.5.2). Whereas sequential lipid extraction with free nitrous acid (10 mg FNA/g biomass) and N-lauroyl

sarcosine concentration varying from 5 to 40 mg/g biomass increased the lipid extraction efficiency from 58.2 to 95.1 and 54.7 to 92.3% w/w using hexane or petroleum diesel. The maximum lipid extraction efficiency was obtained at N-LS concentration of 20 mg/g biomass. Experimental results and microscopic observations revealed that the reason behind the increased lipid extraction efficiency by sequential treatment using FNA followed by N-LS treatment was degradation of cell membrane and cell wall by FNA treatment without associated lipid release. Once free nitrous acid disrupted the cell wall around the yeast biomass, in the next step of treatment, N-LS formed a micelle around the lipid bodies inside the microbial cell and broke the phospholipid membrane surrounding lipid bodies and the neutral lipids were released , which were recovered by the solvent (Dong et al., 2016).

The cost of chemicals required for cell wall disruption using only N-LS treatment and sequential treatment with FNA followed by N-LS has been compared. The low concentration of reactants (FNA- 10mg/g biomass and NLS- 20mg/g biomass) was required for sequential treatment compared to only N-LS treatment, where high concentration of N-LS (75mg/g biomass). The bulk price of sodium nitrate and N-lauroyl sarcosine was 450 and 2000 $/ ton (https://www.alibaba.com/) and sodium nitrite was almost 75% cheaper than FNA. Therefore, sequential treatment using FNA followed by N-LS treatment is substantial approach for lipid extraction due to the fact that low concentration of chemicals is required for cell wall disruption of lipid bearing microbial biomass grown in sludge.

## 3.5 Transesterification

The fatty acids profile of biodiesel produced from lipids (extracted by using different methods) was almost similar with hexane and petroleum diesel as a co-solvent. The lipid extracted from original secondary watewater sludge or LSYBS biomass mainly consists of C16 and C18, which are also main components in vegetable oils and animal fats. In addition, N-LS assisted lipid extraction gave similar fatty acids profile as lipids obtained from conventional lipid extraction using settled yeast biomass grown in sludge (Table 2.5.3). The fatty acid profile of free nitrous acid assisted lipid extraction revealed that with increase in FNA concentration, the unsaturated fatty acids concentration such as C16:1 was decreased from 14 to 4 % w/w and C18:1, C18:2 and C18:3 were decreased by less than 0.5% w/w compared to N-LS assisted lipid extraction (75 mg N-LS/g biomass). However, concentration of saturated fatty acids such as C14:0, C15:0, C16:0, C17:0, C18:0 increased slightly with increase in FNA concentration. It means that FNA has direct impact on triacyl glycerides and it is converting unsaturated fatty acids to saturated fatty acids. It has

been reported that active groups of FNA-ionized system such as NO (nitric oxide) and $NO_2$ (nitrogen dioxide) can induce lipid peroxidation (Wu et al., 2018, Bai et al., 2014). The reaction between NO and $O_2$ (superoxide) generates ONOO (peroxynitrite), which is a powerful oxidant that exhibits complex chemistry with biological molecules such as unsaturated fatty acids. Therefore, it is very likely that the reason for the negative effect of high FNA concentrations on unsaturated fatty acids recovery is caused by damage or oxidation of unsaturated fatty acids by NO and $NO_2$.

These results are further validated by using sequential treatment with low concentration of FNA (10 mg FNA/g biomass) and N-LS assisted lipid extraction and further transesterification of oil to FAMEs. Using low concentration of FNA and with increase in N-LS concentration, there was increase in C16:1, C18:1, C18:2 and C18:3 and maximum lipid extraction efficiency was also achieved. It has been suggested that to enhance oxidation stability of FAMEs (biodiesel), the high percentage of unsaturated fatty acid is required to minimize engine ignition problem and for storage as well.

## 3.6 Biodiesel and metal contaminants

Table 2.5.4, represents the concentration of metals present in sludge and biodiesel obtained from lipid bearing yeast biomass grown in sludge (settled biomass). The concentration of metals leached during extraction of oil and conversion to biodiesel with chloroform and methanol (1:1) or petroleum diesel as a solvent. The concentration of metals presents in biodiesel obtained after transesterification using hexane or petroleum diesel as follows: P>Ca>Al>S>Fe>Ba>Cr. The final obtained biodiesel was contaminated with metals, and it may cause a problem for engine ignition. As per biodiesel ASTM D6751, the metals concentration needs to be less than or equal to ASTM biodiesel norms (sulfur- 0.05mg/L; calcium – 5 mg/L; magnesium – 5 mg/L; phosphorus – 0.01 mg/L; sodium – 5 mg/L). Therefore, to use biodiesel obtained from sludge in a commercial and automobile sector, research work on further purification is going on to remove contaminants by using dry washing method.

## 4. CONCLUSION

N-LS assisted lipid extraction using chloroform and methanol (1:1) or petroleum diesel gave almost similar lipid extraction efficiency but in case of FNA assisted lipid extraction, maximum lipid recovery was obtained using chloroform and methanol (1:1). However, high FNA concentration directly affected the fatty acid profile. Therefore, sequential lipid extraction using low concentration of FNA followed by N-LS was employed, and it gave maximum lipid extraction efficiency of 95% (w/w) without affecting fatty acid profile. Therefore, this approach can be beneficial for lipid extraction and further biodiesel production using lipid bearing yeast biomass grown in sludge.

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# 5. REFERENCES


Bligh, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*, **37**(8), 911-917.

Chen, J., Tyagi, R.D., Li, J., Zhang, X., Drogui, P., Sun, F. 2018. Economic assessment of biodiesel production from wastewater sludge. *Bioresource Technology*, **253**, 41-48.

Dedon, P.C., Tannenbaum, S.R. 2004. Reactive nitrogen species in the chemical biology of inflammation. *Archives of Biochemistry and Biophysics*, **423**(1), 12-22.

Dong, T., Knoshaug, E.P., Pienkos, P.T., Laurens, L.M. 2016. Lipid recovery from wet oleaginous microbial biomass for biofuel production: a critical review. *Applied Energy*, **177**, 879-895.

Hajjari, M., Tabatabaei, M., Aghbashlo, M., Ghanavati, H. 2017. A review on the prospects of sustainable biodiesel production: a global scenario with an emphasis on waste-oil biodiesel utilization. *Renewable and Sustainable Energy Reviews*, **72**, 445-464.

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. *Applied biochemistry and biotechnology*, **175**(4), 1926-1937.

Mardhiah, H.H., Ong, H.C., Masjuki, H., Lim, S., Lee, H. 2017. A review on latest developments and future prospects of heterogeneous catalyst in biodiesel production from non-edible oils. *Renewable and sustainable energy reviews*, **67**, 1225-1236.

Nigam, P.S., Singh, A. 2011. Production of liquid biofuels from renewable resources. *Progress in energy and combustion science*, **37**(1), 52-68.

Nouha, K. 2016. Characterization of Extracellular Polymeric Substances (Eps) Produced by Cloacibacterium normanense Isolated from Wastewater Sludge for Sludge Settling and Dewatering. *Journal of Civil & Environmental Engineering*, **05**(06).

Pastore, C., Lopez, A., Lotito, V., Mascolo, G. 2013. Biodiesel from dewatered wastewater sludge: A two-step process for a more advantageous production. *Chemosphere*, **92**(6), 667-673.

Rivaldi, J.D., Carvalho, A.K.F., da Conceição, L.R.V., de Castro, H.F. 2017. Assessing the potential of fatty acids produced by filamentous fungi as feedstock for biodiesel production. *Preparative Biochemistry and Biotechnology*, **47**(10), 970-976.

Wild, R., Patil, S., Popović, M., Zappi, M., Dufreche, S., Bajpai, R. 2010. Lipids from Lipomyces starkeyi. *Food Technology and Biotechnology*, **48**(3), 329-335.

Wu, J., Yang, Q., Luo, W., Sun, J., Xu, Q., Chen, F., Zhao, J., Yi, K., Wang, X., Wang, D., Li, X., Zeng, G. 2018. Role of free nitrous acid in the pretreatment of waste activated sludge: Extracellular polymeric substances disruption or cells lysis? *Chemical Engineering Journal*, **336**, 28-37.

Yellapu, S.K., Bezawada, J., Kaur, R., Kuttiraja, M., Tyagi, R.D. 2016. Detergent assisted lipid extraction from wet yeast biomass for biodiesel: A response surface methodology approach. *Bioresour Technol*, **218**, 667-673.

Yellapu, S.K., Kaur, R., Kumar, L.R., Tiwari, B., Zhang, X., Tyagi, R.D. 2018. Recent developments of downstream processing for microbial lipids and conversion to biodiesel. *Bioresource technology*.

Yellapu, S.K., Kaur, R., Tyagi, R.D. 2017. Detergent assisted ultrasonication aided in situ transesterification for biodiesel production from oleaginous yeast wet biomass. *Bioresource technology*, **224**, 365-372.

Zhang, X., Yan, S., Tyagi, R.D., Drogui, P., Surampalli, R.Y. 2016. Ultrasonication aided biodiesel production from one-step and two-step transesterification of sludge derived lipid. *Energy*, **94**, 401-408.

Zhang, X., Yan, S., Tyagi, R.D., Surampalli, R.Y., Valéro, J.R. 2014. Lipid production from Trichosporon oleaginosus cultivated with pre-treated secondary wastewater sludge. *Fuel*, **134**, 274-282.

**Table 2.5. 1 Oleaginous biomass settling obtained from sludge medium using extra polymeric substance (EPS) as a bio flocculant**

| Factors | Values |
|---|---|
| Initial biomass conc. (g/L) | 40 |
| After biomass settling (g/L) | 210 |
| Settling velocity (mm/s) | 1.28 |
| Flocculation activity (%) | 94.2 |
| Settling time (min) | 15 |

**Table 2.5. 2 Sequential lipid extraction using free nitrous acid and N-lauroyl sarcosine**

| Reaction number | FNA (mg/g sludge biomass) | N-LS (mg/g sludge biomass) | Lipid extraction efficiency % (w/w) | |
|---|---|---|---|---|
| | | | Chloroform/methanol (1:1) | Petroleum diesel |
| 1 | 10 | 0 | 58.2 | 54.7 |
| 2 | | 5 | 63.3 | 60.1 |
| 3 | | 10 | 69.9 | 72.1 |
| 4 | | 15 | 84.2 | 80.8 |
| 5 | | 20 | 95.3 | 92.4 |
| 6 | | 40 | 95.1 | 92.3 |

**Table 2.5. 3 Comparison of fatty acid profiles of biodiesel produced from the lipid of *Trichosporon oleaginosus* using chloroform and methanol (or) petroleum diesel**

| | Relative amount of fatty acids % (w/w) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Free nitrous acid | | | | | Sequential of FNA* and N-LS | | | | |
| **Fatty acids** | **Original sludge** | **Sludge biomass** | **N-LS (75mg/g biomass)** | **10** | **20** | **40** | **60** | **80** | **5** | **10** | **15** | **20** | **40** |
| **C14:0** | 0.52 | 1.8 | 1.52 | <0.5 | 1.12 | 1.09 | 1.42 | <0.9 | <0.5 | 1.52 | 1.52 | 1.51 | 1.52 |
| **C15:0** | 0.74 | 3.12 | 3.44 | 3.01 | 2.92 | 3.71 | 3.46 | 3.98 | 3.46 | 2.98 | 3.01 | 3.23 | 3.31 |
| **C16:0** | 31.82 | 28.21 | 28 | 22.11 | 33.52 | 36.11 | 40.11 | 39.11 | 22.11 | 25.71 | 27.03 | 27.89 | 28.9 |
| **C16:1** | 2.17 | 19.01 | 19.46 | 14.03 | 8.07 | 4.23 | 4.01 | 4.13 | 14.03 | 15.79 | 17.21 | 20.11 | 20.16 |
| **C17:0** | 4.41 | 2.22 | 1.89 | 0.71 | 1.46 | 2.93 | 3.21 | 3.31 | 0.71 | <0.5 | 1.42 | 1.71 | 1.89 |
| **C18:0** | 12.23 | 12.33 | 11.98 | <0.5 | 20.89 | 24.76 | 28.89 | 30.89 | <0.5 | 5.21 | 9.89 | 11.33 | 11.42 |
| **C18:1** | 32.31 | 22.98 | 21.77 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | 8.49 | 18.77 | 21.82 | 21.01 |
| **C18:2** | 6.11 | 2.45 | 2.13 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | 2.09 | 2.42 | 1.89 |
| **C18:3** | 5.08 | 1.01 | 0.99 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.6 | 0.72 | 1.03 | 1.08 |
| **C20:1** | 1.12 | 1.45 | 1.41 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.6 | 0.93 | 1.31 | 1.56 |

- FNA concentration (10 mg FNA/g biomass)

**Table 2.5. 4 Comparison of metals in sludge solids and biodiesel obtained from oleaginous biomass cultivated in municipal sludge**

| Metals | Municipal secondary wastewater sludge solids (g/kg) | Biodiesel (sludge oleaginous biomass) |
|---|---|---|
| Al | 47.3 | 15.1 |
| Ba | 99.7 | 0.6 |
| Ca | 192 | 18.1 |
| Cr | 43.1 | 0.6 |
| Cu | 0.076 | <0.01 |
| Fe | 10.3 | 1.7 |
| P | 92.1 | 35.2 |
| S | 62.3 | 14.6 |

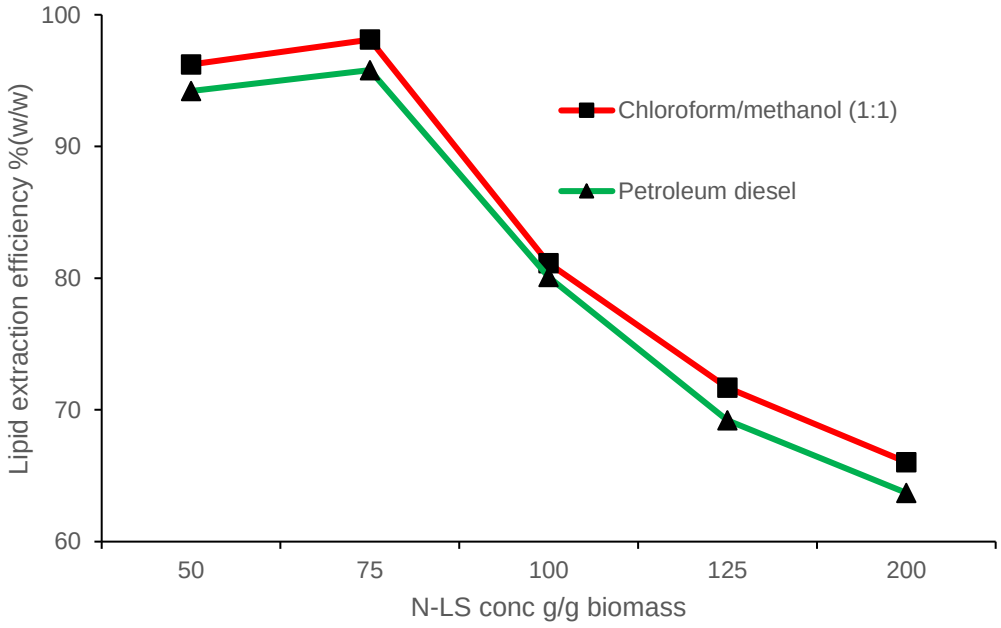


**Figure 2.5. 1 N-Lauroyl sarcosine assisted lipid extraction from sludge biomass with hexane (or) petroleum diesel as solvent; standard deviation was less than 5%.**

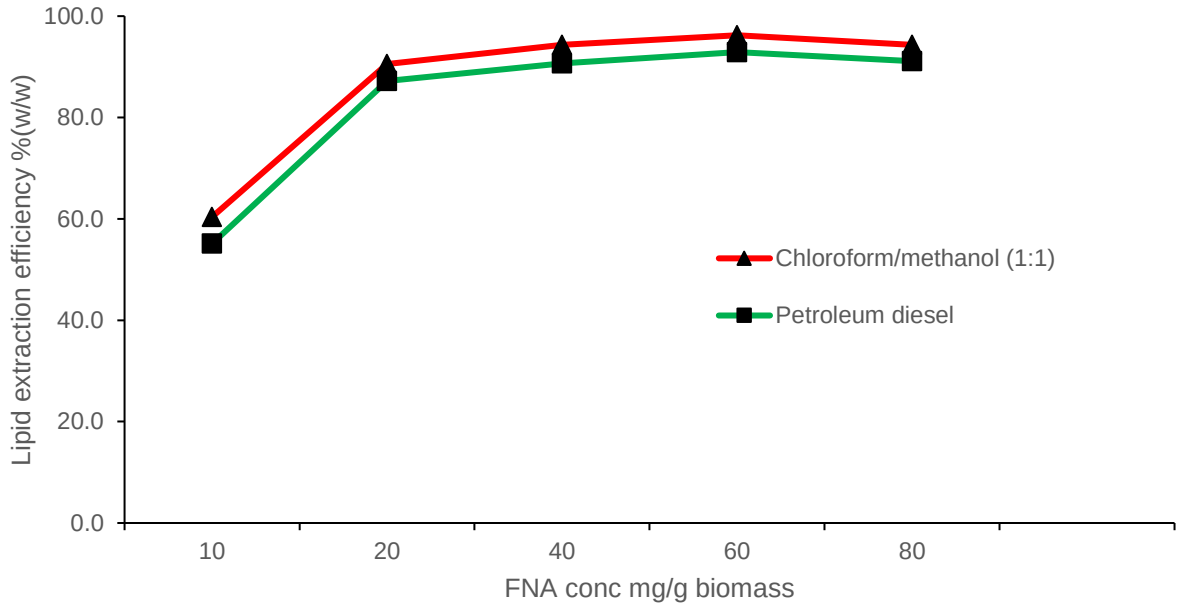


**Figure 2.5. 2 Free nitrous acid assisted cell wall disruption using hexane or petroleum diesel**

# CHAPTER II

# PATRIE 6

# OIL EXTRACTION FROM PRIMARY, SECONDARY WASTEWATER SLUDGE, SCUM, AND CONVERSION TO BIODIESEL

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**BIOFUELS (*Submitted 10th March 2018*)**

# RÈSUMÈ

Cette étude a pour objectif d’étudier l'extraction de l'huile à partir de boues primaires, secondaires et de l’écume provenant de l'usine de traitement des eaux usées municipales. Les divers paramètres tels que le volume de solvant, la température, l'agitation et la teneur en humidité ont été optimisés pour maximiser l'efficacité d'extraction d'huile à partir de l'écume lyophilisée. L'efficacité de l'extraction de l'huile en utilisant l'hexane et le diesel pétrolier comme solvant, a été comparée pour chaque paramètre. Les paramètres physiques optimaux pour l'extraction à l'aide d'écume lyophilisée étaient de 75 g de solides / L de solvant, 60 ° C, 300 tr / min pendant 30 min et 100 et 94,3% P / P d'huile d'hexane et de diesel. Les mêmes paramètres ont également été utilisés pour l’extraction l'huile des boues primaires et secondaires séchées. Une efficacité de 95,2 et 94,7% poids / poids a été obtenue en utilisant l'hexane et le diesel pétrolier respectivement. En outre, les résultats de la transestérification ex-situ ont montré que l'écume contient une concentration plus élevée de lipide neutre que celle des boues primaires et secondaires. En conclusion, l'écume est une matière première potentielle pour la production de biodiesel mais il requière plus d’études à l'avenir.

## ABSTRACT

This study is to investigate extraction of oil from primary, secondary and scum sludge obtained from the municipal wastewater treatment plant. The various parameters such as solvent volume, temperature, agitation and moisture content were optimized to maximize the oil extraction efficiency using freeze-dried scum. The oil extraction efficiency was compared for each parameter using hexane and petroleum diesel as a solvent. The optimum physical parameters for oil extraction using freeze-dried scum were 75 g solids/L solvent, temperature 60$^{o}$C, agitation 300 rpm for 30 min and maximum oil extraction efficiency of 100 and 94.3% w/w was obtained using hexane and petroleum diesel respectively. The optimized parameters for freeze-dried scum were further used to separate oil from dried primary and secondary sludge and oil extraction efficiency of 95.2 and 94.7 % w/w was obtained using hexane and petroleum diesel respectively. The obtained results using 1g of scum were further validated by using 1Kg of scum under optimized conditions and oil extraction efficiency of 94.1 and 92.3 % w/w was obtained by using hexane and petroleum diesel respectively. Furthermore, the ex-situ transesterification was performed, and results showed that scum had a higher neutral lipid than that of primary and secondary sludge. In brief, scum is a potential feedstock for the production of biodiesel, and more work is needed to be done in the future.



**Keywords**: Wastewater sludge, scum, oil extraction, petroleum diesel, Ex-situ transesterification, biodiesel.

# 1. INTRODUCTION

Alternative technologies for biodiesel production are getting improvised from last five years of research. Biodiesel is one of the substitutes for petroleum diesel, and it is the best candidate compared to all energy sources (Ashnani et al., 2014). Biodiesel has been directly used for automobiles without any further engine modification, but in certain countries, biodiesel is blended with petroleum diesel in various levels (B2%, B3%, B4%, B5% and B10%) (Abedin et al., 2016). Consequently, with an increase in biodiesel utilization, there was a decrease in greenhouse gas (GHG) emissions as less fossil fuel diesel was consumed (Abedin et al., 2016).

Due to an increase of metropolitan and cosmopolitan cities, there was an increase in wastewater treatment plants all over Canada. There are approximately 2,303 municipal wastewater treatment facilities serving 36 million people (CCME 2014). In Canada, a significant amount of dewatered sludge solids is disposed of using landfills, incinerating, underground disposal and lagoons. The management of the sludge solids causes environmental pollution and economic stress on wastewater treatment plants. The cost of sludge management is one of the critical challenges for the wastewater industry. Therefore, an extensive approach is necessary to decrease the sludge management cost and GHG emissions (Demirbas et al., 2017).

The possibility of using municipal sludge as feedstock for biodiesel production is a critical approach to decrease the sludge management cost. Moreover, there is a significant availability of sludge with high oil content in the developed countries (Wang et al., 2016). The amount of oil present in sludge varies and is strongly dependent upon the population and urbanization of the city (Wang et al., 2015). In theory, scum sludge mainly composed of fats, oil, and grease, (FOG) which are rich in free fatty acids (Marufuzzaman et al., 2014) and consists of high lipid content (Bi et al., 2015; Sangaletti-Gerhard et al., 2015). Thus, scum sludge is more beneficial for biodiesel production than primary or secondary sludge. Wang et al. (2016) used freeze dried scum solids for oil extraction using hexane as an organic solvent, and 91.2% (w/w) of oil was extracted. Further, *In-situ* transesterification to obtain biodiesel from scum sludge accounted for 57.5 to 64.1% of unsaturated fatty acid esters. Utilization of organic solvents can increase the biodiesel production cost. Therefore, several studies used hexane as a co-solvent to separate oil from the sludge solids. Consequently, an innovative and economic process is necessary to produce biodiesel from the sludge or scum biosolids.

Therefore, the primary objective of this study is to develop a sustainable approach for wastewater sludge or scum as a feedstock for biodiesel production without the use of any toxic organic solvent. In this study, petroleum diesel was used as a co-solvent and results were compared using a standard solvent (hexane) to separate oil and effect of different physical factors (temperature, agitation, time, solids concentration, solids moisture content and solvent recycling) on oil extraction efficiency were studied. The separated oil in the solvent was directly transesterified to biodiesel.

On the other hand, the challenge of this research is towards the development of alternative routes for sludge management in wastewater treatment plant and to introduce a novel solvent (petroleum diesel) to extract oil from sludge, which can be further trans esterified and directly used as a biofuel without any further blending.

# 2. MATERIALS AND METHODS

## 2.1 Sampling and characterization of wastewater sludge and scum

The primary sludge, secondary sludge and scum were collected from a municipal wastewater treatment plant located in Quebec City, Canada. The primary and secondary sludge was filtered using standard mesh filter (800mm) to remove rocks, fibers and hair from the sludge and filtrate were stored in airtight container at 4$^{o}$C. After 24h, sludge solids were settled to the bottom of the tank, and top layer water was removed slowly. The settled solids (of both primary and secondary sludge) and wet scum were transferred to freeze-thaw trays and freeze-dried for four days to remove the total moisture. After freeze-drying, samples were used for oil extraction studies. The freeze-dried sludge solids were characterized to know the metals concentration, oil concentration % (w/w) and free fatty acids (FFA) % (w/w) (Table 2.6.1). The chemicals and organic solvents used in this study were purchased from standard companies, and petroleum diesel was obtained from a local gas station, Quebec City, Canada.

## 2.2 Oil extraction from different sludge using hexane (or) petroleum diesel

The freeze-dried sludge solids (primary sludge, secondary sludge, and scum) were crushed using a mortar and pestle into a powder and dissolved in hexane (or) petroleum diesel to extract oil from

different sludge or scum powder. One gm of each different sludge or scum powder was weighed in different screw-capped glass tubes, and 15 mL of hexane or petroleum diesel was added and well mixed. The solvent and sludge powder mixture were incubated for four h at 70$^{o}$C with 400 rotation per minute (rpm) agitation in an orbital shaking incubator (Thermoscientific Inc., Canada). After incubation, the total mixture was filtered using a standard filter with mesh size 125mm. In case of hexane as a co-solvent, the filtrate was transferred into a pre-weighed glass tube (W1) and oven dried at 60$^{o}$C until constant weight (W2) was obtained. The oil content % (w/w) of the different freeze-dried sludge and scum was calculated as:

$$CO\ \% = \frac{W2-W1}{1} \times 100\% \quad \text{--------- (1)}$$

In Equation (1), CO% represents the % of oil content obtained by conventional oil extraction method using hexane as solvent. $W_1$ represents the weight of pre-weighed glass tube, and $W_2$ denotes the weight of oven dried oil weight in a pre-weighed glass tube, and 1 denotes the dry scum or sludge powder weight in gram. The extracted oil was stored for further transesterification study.

However, in case of petroleum diesel as a co-solvent, the filtered oil present in petroleum diesel ($O_{pd}$) was directly used for transesterification without drying. The transesterified oil to FAMEs (Fatty acid methyl esters) was analyzed using FT-IR and obtained FAMEs were used to calculate the weight of the sludge oil separated using petroleum diesel.

The total oil obtained by using petroleum diesel was calculated according to equation (2):

$$\text{Oil extraction efficiency of petroleum diesel (\% w/w)} = O_{pd} / O_c \times 100\ \% \text{ -------} \quad (2)$$

Whereas, $O_{pd}$ is the weight of oil (g) present in 15 mL petroleum diesel. $O_c$ is the dry weight of oil (Oc = 0.23g) obtained by using 1g of dried sludge powder with hexane as solvent.

## 2.3 Optimization of oil extraction parameters using dry scum

The physical parameters for oil extraction were optimized using freeze-dried scum powder. After that, the optimized parameters for freeze-dried scum were further used to separate oil from dried primary and secondary sludge.

### 2.3.1 Effect of temperature

The dried scum samples of one gram were weighed in different screw-capped glass tubes and mixed with 15 mL hexane or petroleum diesel. The tubes were incubated at different temperatures (30, 40, 50, 60, 70 and 80$^{o}$C) for 4h at the agitation of 400rpm. After the reaction, the samples were filtered and analyzed as discussed in *section 2.2.*

### 2.3.2 Effect of agitation

The dried scum samples of each one gram were weighed in different screw-capped test tubes and were mixed with 15 mL hexane (or) petroleum diesel. The test tubes were incubated at above optimized temperature of 60$^{o}$C in an orbital shaker incubator (Thermo Scientific Inc.) with different agitation speed of 100 to 400 rotation per minute (rpm) for 4h. After the reaction, samples were filtered and analyzed as discussed in *section 2.2.*

### 2.3.3 Effect of extraction time

The dried scum samples of each one gram were weighed in different screw-capped glass tubes and mixed with 15 mL hexane or petroleum diesel. The tubes were incubated at 60$^{o}$C temperature for a different incubation time of 30 to 240 min with an optimized agitation speed of 300rpm. After the reaction, samples were filtered and analyzed as discussed in *section 2.2.*

### 2.3.4 Effect of moisture content

The dried scum samples of each one gm were weighed in different screw-capped glass tubes, and moisture content of 0, 20, 40 and 60 % wt was adjusted with tap water. In another tube, scum without drying (72.9% moisture content) was added in equal weight to 1g dry scum. The hexane or petroleum diesel of 15 mL/1g dry scum was added, and total mixture was incubated at 60$^{o}$C using orbital shaking incubator for 1h with an agitation speed of 300 rpm. After the reaction, the total mixture was filtered using 125mm mesh size filter, and total filtrate volume was estimated for solvent recovery %.

Solvent recovery % (v/v) = Filtrate solvent volume / Initial solvent volume x 100

The filtrate was analyzed for oil content % wt as discussed in *section 2.2*.

### 2.3.5 Effect of sludge solids concentration

The different dried scum powdered samples (0.375, 0.75, 1.125, 1.5, 1.87 and 2.25g) were weighed in each screw-capped glass tubes with constant solvent volume (15 mL) and final scum solids concentration in the glass tube was 25, 50, 75, 100, 125 and 150g solids/L solvent, respectively. The hexane or petroleum diesel was added to each glass tube and incubated at 60$^{o}$C for 4h with agitation at 300 rpm. After cooling to room temperature, the total reaction mixture was filtered and analyzed as discussed in *section 2.2*.

## 2.4 Recyclability of hexane and petroleum diesel as a solvent for oil extraction

The dried scum and wet scum (moisture content 72.3% w/w) samples of one gram and 3.5 g (which was equal to 1 g dry weight) respectively were weighed in four different screw-capped glass tubes in two sets and further mixed with 15 mL hexane or petroleum diesel. The reaction was performed under optimized conditions, and tubes were incubated at 60$^{o}$C temperature for an incubation time of 60 min with an agitation speed of 300rpm. After the reaction, the samples were filtered using 125mm mesh size filter, and volume of filtrate (oil in petroleum diesel or oil in hexane) was measured for solvent recovery %. After that, one gram of dried scum and 3.5g of wet scum was again added into the different filtrate (oil in a solvent obtained by using dry or wet scum) and incubated under optimized conditions. The samples were further filtered, and volume of filtrate was measured. The same process was repeated two more times (total recycled- three times) to increase the oil concentration, and this process is known as solvent recycling. The solvent recovery % and oil concentration in the solvent were calculated after each cycle as discussed in section 2.2 and 2.3.4.

## 2.5 Oil extraction from primary and secondary sludge solids using optimized parameters

The optimized parameters used for oil extraction from dry scum solids were used to extract oil from freeze-dried primary and secondary sludge samples. One gram of dried sludge was weighed in screw-capped glass tubes and homogeneously mixed with 15 mL of hexane or petroleum diesel and kept at 60$^{o}$C for 60 minutes in orbital shaking incubator with 300 rpm agitation. After the reaction, the total mixture was filtered using a standard filter of mesh size 125mm. The filtrate was analyzed for oil extraction efficiency % w/w as discussed in *section 2.2.*

## 2.6 Validation of oil extraction using one kilogram of scum

The experiment was conducted using a 15L plastic bucket (oil extraction reactor) equipped with an agitator, temperature control Jacket with knob (Figure 2.6.3). One kilogram of scum (moisture content 72.3%) was added to oil extraction reactor, and solids concentration was adjusted to 75g scum solids/L solvent (hexane or petroleum diesel) and closed with a tight lid and container was sealed. After that, optimum parameters such as temperature 60$^{o}$C, agitation 300 rpm were controlled, and the reaction was terminated in 60 min. After oil extraction, the complete mixture was filtered using 800 mm standard filter. The filtrate was collected in a beaker, and it was allowed to settle for two h, and clear phase separation was observed in two phases. The top phase consists of scum oil dissolved in solvent and bottom phase consists of water with some residual solids. Top phase was again recycled (0, 1 and 2 times) to extract oil repeatedly and to know saturation level of oil to be dissolved (or extracted) insolvent and similar steps were followed as explained above. After each cycle, the volume of the solvent (hexane or petroleum diesel) was measured, and transesterification was conducted to determine oil to FAMEs (fatty acid methyl esters) conversion efficiency.

## 2.7 Acid Transesterification and biodiesel metal contaminants

The oil samples obtained using hexane or petroleum diesel were transesterified using acid as a catalyst. Acid catalyst was used for transesterification due to high free fatty acid (>3% w/w) content in the extracted oil. The FAMEs obtained from oil separated using hexane as a solvent were analyzed with GC-FID as discussed in our earlier study (Yellapu et al., 2017). However, in case of oil separated using petroleum diesel as a solvent, the obtained FAMEs were analyzed with FT-IR as discussed by Yellapu *et al.,* (2018). The obtained FAMEs were characterized for metal analysis using ICP-AES axial vista.

# 3. RESULTS AND DISCUSSION

The characterization of primary sludge, secondary sludge and scum are presented in Table 2.6.1. The free fatty acid content of the oil extracted from primary, secondary sludge and scum was 13.9, 14.2 and 12.5 % w/w, respectively. The oil content varied in three different sludge due to the process of wastewater treatment. The primary sludge solids mainly consist of macromolecular lipids, fibers, and rocks. However, secondary sludge composed of suspended solids with microbial cells produced during aerobic biological treatment of wastewater treatment. The scum

was a combined mixture of, fats, oil, and grease (FOG); cellulose fibers, hairs and other light solids (Demirbas et al., 2017). Therefore, in the present study, the maximum oil content was observed in scum sludge using conventional oil extraction with hexane as a solvent (Table 2.6.1).

Several researchers have reported the presence of different oil content in the primary, secondary sludge, and scum and it may be due to the urbanization and industrialization of metropolitan cities (Yapıcıoğlu & Demir, 2017). Oil can be separated from sludge or any other matter (biomass) with polar and non-polar solvents. While organic solvents (hexane) are expensive, therefore an alternative and low-cost solvent such as petroleum diesel were used to separate oil from different dried sludge and scum powders. The oil content (%) obtained from sludge and scum using petroleum diesel and hexane was almost similar (Figure 2.6.1). The oil extraction efficiency obtained using hexane as a solvent was considered as 100%. The oil extraction efficiency using petroleum diesel as a solvent for primary sludge, secondary sludge and scum were 97, 98.1 and 97.8 % w/w., respectively. A similar variation of oil extraction results was obtained by Wang et al. (2016) using a combination of different solvents such as methanol, hexane, and acetone and maximum oil content of 33% w/w were obtained from scum solids using hexane as a solvent.

The physical nature of oil on sludge matrices was similar in case of primary, secondary sludge, and scum. Therefore, scum dried powder was used to optimize physical parameters (temperature, time, agitation and solvent volume) for oil recovery and optimized parameters were further used for extraction of oil from primary and secondary sludge.

## 3.1 Effects of physical and chemical parameters for oil extraction

### 3.1.1 Effect of temperature

The oil extraction efficiency curve, illustrating the effect of different temperatures on freeze-dried scum with hexane or petroleum diesel as a solvent is presented in Figure 2.6.2a. The oil, fats, and grease bounded to scum solids were solubilized based upon physical characteristics and nature of the oil. The solubility of oil embedded in the scum solids increased with increase in temperature from 30 to 80$^{o}$C with constant reaction time of 4h and 400 rpm agitation, and maximum oil extraction efficiency of 98.63 ± 1.1 % (w/w) was obtained at 60$^{o}$C. Mu et al. (2016) reported that fats, oils, and grease (FOG) are liquefied at a temperature higher than 40$^{o}$C. However, the temperature higher than 80$^{o}$C can cause solubilization of organic matter into the solvent (hindering the oil measurement), and it may also decrease transesterification efficiency.

Samaram et al. (2015) investigated the effect of temperature on the kinetics of oil extraction from corn germ and reported that increase in the temperature enhances the capacity of solvents to dissolve the oil because of thermal energy that overcomes the cohesive and adhesive interactions. Furthermore, the collision theory states that two molecules will only react if they have enough activation energy (Diphare & Muzenda, 2013). When the mixture is heated, the energy levels of the molecules increases and when the molecules are in their excited state, there will be more collisions between the molecules. As a result, the rate of reaction or decomposition increases. Moreover, the energy input regarding heat or temperature provides the required energy to break the intermolecular forces of attraction between molecules resulting in easy solubility in organic solvents.

### 3.1.2 Effect of agitation

The effect of agitation speed on oil separation using hexane or petroleum diesel is presented in Figure 2.6.2b. Agitation increases the eddy diffusion and the transfer of FOG from the scum to the solvent (Diphare & Muzenda, 2013). The oil extraction efficiency increased from 64.16 to 98.98% (w/w) with an increase in agitation speed. The maximum oil extraction efficiency of 98.29% was obtained at 300 rpm with a constant optimum temperature of 60$^{o}$C within 4h reaction time. However, for agitation speed higher than 300 rpm, there was no significant increase in the oil recovery. The dependence of oil extraction efficiency on agitation shows that mass transfer plays a vital role in extraction.

Pilusa et al. (2013) reported that the role of agitation is to break down the grease molecules to liberate oil molecules as well as to increase its active surface area to interact with the solvent. Pilusa et al. (2013) further explained that once the bonds holding the scum matrix and oil are broken with the aid of agitation, oil floats in the mixture. Kadi and Fellag (2001) studied the effect of stirrer speed on oil extraction from olive foot cake using hexane as a solvent. The oil content of 6.9 to 17.7% (w/w) was extracted at agitation speed varying from 600 to 1000 rpm. It explains agitation is one of the crucial factors for oil separation from the scum or sludge solids.

### 3.1.3 Effect of time

Extraction time is an important parameter in the design and operation of extraction processes. The oil extraction efficiency increased with increase in incubation time (Figure 2.6.2c). The maximum oil extraction efficiency of 98.97 % (w/w) was achieved within 60 min incubation time at a constant temperature of 60$^{o}$C with agitation of 300 rpm.

The increase in extraction time above 60 min did not show any improvement in oil extraction. Various researchers have been identified oil extraction from pulverized sludge solids at different reaction time in different studies and the change in reaction time could be further dependent on specific factors such as; a) reaction volume, b) solvent type, c) condition of solids (wet/dry) and d) physical parameters (Ibrahim & Hamza, 2017). Hence, optimization of extraction time is necessary for every oil extraction process.

### 3.1.4 Effect of moisture content

The effect of moisture content on oil extraction efficiency using hexane or petroleum diesel is presented in Figure 2.6.2d. The oil extraction efficiency was almost similar to an increase in moisture content from 10 to 72.3% w/w using hexane and petroleum diesel as a solvent. The oil extraction efficiency of 98.9 and 97.2 % (w/w) was attained at 10 and 72.3% moisture content using hexane as solvent. Similarly, the oil extraction efficiency of 98.6 and 95.9 % (w/w) was obtained using petroleum diesel as a solvent with optimum parameters such as the temperature of 60$^{o}$C, agitation 300rpm and time 60 min. However, the color of the filtrate (oil dissolved in a solvent) was lighter with an increase in moisture content from 10 to 72.3% w/w. After reaction of solvent with scum solids containing different moisture content, the solvent recovery percentage increased from 80 to 96%v/v with an increase in moisture content. It explains that freeze-dried scum solids react with solvent during oil extraction and absorbs more solvent. The absorbing capacity of the solvent to the sludge solids decreases with increase in moisture content of the solids. Therefore, solvent recovery (%) was higher with an increase in moisture content of the scum solids.

### 3.1.5 Effect of scum solids concentration

The oil extraction efficiency using a different concentration of freeze-dried scum solids with hexane or petroleum diesel is presented in Figure 2.6.2e. The maximum oil recovery was obtained by using 25 to 75 g scum solids/L solvent. After that, with an increase in solids concentration from 100 to 150 g solids/L solvent at optimum values of temperature 60$^{o}$C, 60 min, and agitation 300rpm, the decrease in oil recovery from 93.5 to 90.8 % w/w was observed using hexane as solvent. A similar scenario was found in case of petroleum diesel as a co-solvent. It may be due to the impact of solids concentration on the energy transfer between the surfaces of the particles. With higher solids concentration, the energy requirement for liquid-liquid separation would be lower. Hence, at the low solids concentration, the interaction between solvent and oil will be higher

(Zhang et al., 2014). Therefore, the optimum solids concentration of scum to achieve maximum oil extraction efficiency was 75 g solids/L solvent.

## 3.2 Recyclability of hexane and petroleum diesel

The solvent (hexane or petroleum diesel) can dissolve oil from sludge or scum solids. Reuse of the same solvent (filtrate) to dissolve oil from scum or sludge repeatedly is known as solvent recycling. Table 2.6.2 represents the effect of solvent recycling on oil extraction efficiency as well as solvent recovery % using hexane or petroleum diesel as a solvent with dry and wet scum (72.3% w/w moisture content). The oil concentration in solvent was increasing proportionally using hexane or petroleum diesel and oil concentration of 18.18, 33.3 and 59.4 g oil/L solvent was observed in case of dry scum, whereas by using wet scum, oil concentration was 19.4, 39.3 and 64.2 g oil/L during 0, 1 and 2 recycle, respectively. The oil extraction efficiency % (w/w) and solvent recovery % (v/v) were almost similar for hexane and petroleum diesel. However, wet and dry scum drastically affected the solvent recovery % v/v and oil extraction efficiency % w/w at each recycle time. The solvent recovery and oil extraction efficiency decreased by 12-14% v/v and 13-19% v/v, respectively with increasing recycle numbers of 0, 1 and 2 using dry scum and hexane or petroleum diesel as a solvent. However, in case of wet scum, the solvent recovery and oil extraction efficiency decreased by 3 -12% w/w and 4 - 9 % w/w respectively with increasing recycle numbers of 0, 1 and 2 using hexane or petroleum diesel as a solvent. These results represent that the solvent was not absorbed in the wet scum solids as non-polar nature of the solvent prevents the absorption along with moisture or water which is polar. Therefore, use of wet scum for oil recovery can reduce the solvent contamination in scum solids.

## 3.3 Oil extraction from primary and secondary sludge solids using optimized parameters

The parameters optimized for scum solids were used to separate oil from freeze-dried solids of primary sludge and secondary sludge. The solvent hexane and petroleum diesel under optimized physical parameters (temperature – 60$^{o}$C, reaction time – 60 min, agitation – 300 rpm and 75 g solids/L solvent) gave oil extraction efficiency of 97.3 and 96.1 % w/w (with primary sludge), respectively. In case of secondary sludge, the obtained oil extraction efficiency was 95.2 and 96.3 % w/w using hexane or petroleum diesel, respectively. The maximum oil extraction efficiency obtained using primary and secondary sludge was almost similar to that obtained by using scum

solids under optimized conditions. It means that the optimized parameters can be employed for oil extraction using a different type of sludge solids.

## 3.4 Validation of oil extraction using one kilogram of scum

The optimized parameters were validated using one kilogram of scum with moisture content 73.8%. In this study, the results were almost similar to that obtained by using 1 g of scum with hexane or petroleum diesel as solvents and oil extraction efficiency of 94.1 and 92.3% w/w, respectively, was obtained. The recycling capability of hexane, and petroleum diesel was validated, and the loss of solvent increased, and solvent recovery % decreased with each recycle of 0 to 2 Figure 2.6.4a After each recycle, the relative oil concentration in the solvent increased using hexane or petroleum diesel (Figure 2.6.4). However, maximum oil concentration of 63.6 g/L was obtained using petroleum diesel. The relative oil concentration using hexane (57.5 g/L) was lower as compared to petroleum diesel as a solvent. Relative oil concentration and solvent recovery % are interdependent. When a number of recycling increased, the hexane recovery percentage decreased more as compared to petroleum diesel, and by that way, the relative oil concentration also decreased in case of hexane. The decrease in oil concentration can be explained based upon the physical property of solvent such as boiling point. The boiling point of hexane is 60$^{o}$C, and petroleum diesel is 160 to 230$^{o}$C, and in this experiment, the optimum temperature for oil extraction was 60$^{o}$C. Therefore, during the reaction (oil extraction) hexane was in boiling stage and it may evaporate, and therefore solvent recovery % decreased during each cycle, which further affected the relative oil concentration. Therefore, petroleum diesel was found to be an effective solvent for oil extraction from scum.

## 3.5 Ex-situ Transesterification and biodiesel metal contaminants

The oil recovered from primary sludge, secondary sludge and scum was directly transesterified without any further drying (with solvent). The oil to FAMEs conversion efficiency of scum using hexane and petroleum diesel as a co-solvent was 93.3 ± 1.9 and 95.2 ± 1.1 % w/w, respectively. More or less similar conversion efficiency was obtained using primary sludge and secondary sludge. Figure 2.6.5 represents that oil extracted from primary and secondary sludge was mainly converted to C14 and C16, whereas scum oil was converted to C18:1 and C18:2. It explains neutral lipids are maximum in scum solids as compared to primary or secondary sludge. The FAMEs obtained from high neutral lipids are very high combustible for engine ignition.

Table 2.6.3 represents the concentration of metals present in different (primary sludge, secondary sludge, and scum) sludge or scum solids (g/kg) and final product biodiesel (mg/L). The concentration of metals leached during extraction of oil and conversion to biodiesel for primary, secondary and scum sludge was different with hexane or petroleum diesel as a solvent. The concentration of metals presents in biodiesel obtained by ex-situ transesterification using hexane is as follows: a) Biodiesel obtained from primary sludge – Al > Ca >P > S > Fe > Cu; Biodiesel obtained from secondary sludge - Al > Ca >P > S > Fe > Cu; Biodiesel obtained from scum - Al > Ca >P > S > Fe > Cu. The metals were present in low concentration in the biodiesel, converted by using extracted oil by using petroleum diesel as solvent as compared to biodiesel trans esterified by using oil extracted by using hexane. The final obtained biodiesel was contaminated with metals, and it may cause a problem for engine ignition. As per biodiesel ASTM D6751, the metals concentration need to be less than or equal to ASTM biodiesel norms (sulfur- 0.05mg/L; calcium – 5 mg/L; magnesium – 5 mg/L; phosphorus – 0.01 mg/L; sodium – 5 mg/L) for biodiesel produced by using primary, secondary and scum solids. Therefore, to use biodiesel obtained from sludge in a commercial and automobile sector, research work on further purification is going on remove contaminants by using dry washing methods with ion exchange resins such as Lewatit®, DW-R10, and SEPLITE®.

## 4. CONCLUSION

The primary sludge, secondary sludge, and scum were used for oil extraction, and the obtained oil was further transesterified to biodiesel. The physical parameters significantly affected the oil extraction efficiency from different sludge solids and scum using hexane and petroleum diesel as a solvent. The optimized parameters for maximum oil extraction efficiency were temperature 60$^{o}$C, agitation 300 rpm, time 60 min and 75 g/L solids concentration. The oil extraction efficiency obtained using hexane, and petroleum diesel was almost similar in all cases. The moisture content in solids affected the solvent recovery percentage, but there is no impact on oil extraction efficiency. In ex-situ transesterification without oil drying gave >90% w/w FAMES conversion. The presence of metals affected the quality of biodiesel. However, further research towards the purification of biodiesel to remove contaminants could be beneficial to establish the scum oil as substantial feedstock for biodiesel production.

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# 5. REFERENCES


Abedin, M., Kalam, M., Masjuki, H., Sabri, M., Rahman, S.A., Sanjid, A., Fattah, I.R. 2016. Production of biodiesel from a non-edible source and study of its combustion, and emission characteristics: A comparative study with B5. *Renewable Energy*, **88**, 20-29.

Ashnani, M.H.M., Johari, A., Hashim, H., Hasani, E. 2014. A source of renewable energy in Malaysia, why biodiesel *Renewable and Sustainable Energy Reviews*, **35**, 244-257.

Bi, C.-h., Min, M., Nie, Y., Xie, Q.-l., Lu, Q., Deng, X.-y., Anderson, E., Li, D., Chen, P., Ruan, R. 2015. Process development for scum to biodiesel conversion. *Bioresource Technology*, **185**, 185-193.

Canada-wide strategy for the management of municipal wastewater effluent, 2014. Canada council of ministers of the environment, PN 1522.

Demirbas, A., Edris, G., Alalayah, W.M. 2017. Sludge production from municipal wastewater treatment in sewage treatment plant. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, 1-8.

Diphare, M., Muzenda, E. 2013. The effect of extraction conditions on oil yield from waste lubricating grease.

Ibrahim, S., Hamza, E. 2017. Optimization of Lipid Extraction from Municipal Scum Sludge for Biodiesel Production Using Statistical Approach. *International Journal of Renewable Energy Development*, **6**(2).

Kadi, H., Fellag, H. 2001. Modeling of oil extraction from olive foot cake using hexane. *Grasas y Aceites*, **52**(6), 369-372.

Marufuzzaman, M., Ekşioğlu, S.D., Hernandez, R. 2014. Environmentally Friendly Supply Chain Planning and Design for Biodiesel Production via Wastewater Sludge. *Transportation Science*, **48**(4), 555-574.

Mu, D., Addy, M., Anderson, E., Chen, P., Ruan, R. 2016. A life cycle assessment and economic analysis of the Scum-to-Biodiesel technology in wastewater treatment plants. *Bioresource technology*, **204**, 89-97.

Pilusa, T.J., Muzenda, E., Shukla, M. 2013. Thermo-chemical extraction of fuel oil from waste lubricating grease. *Waste management*, **33**(6), 1509-1515.

Samaram, S., Mirhosseini, H., Tan, C.P., Ghazali, H.M., Bordbar, S., Serjouie, A. 2015. Optimisation of ultrasound-assisted extraction of oil from papaya seed by response surface methodology: Oil recovery, radical scavenging antioxidant activity, and oxidation stability. *Food Chemistry*, **172**, 7-17.

Sangaletti-Gerhard, N., Cea, M., Risco, V., Navia, R. 2015. In situ biodiesel production from greasy sewage sludge using acid and enzymatic catalysts. *Bioresource Technology*, **179**, 63-70.

Wang, Y., Feng, S., Bai, X., Zhao, J., Xia, S. 2016. Scum sludge as a potential feedstock for biodiesel production from wastewater treatment plants. *Waste Management*, **47**, 91-97.

Wang, Z., Zhang, L., Li, J., Zhong, H. 2015. Disposal of Oily Sludge. *Petroleum Science and Technology*, **33**(9), 1045-1052.

Yapıcıoğlu, P., Demir, Ö. 2017. Life Cycle Assessment of Sewage Sludge Treatment-An Overview. *Harran Üniversitesi Mühendislik Dergisi*, **2**(2).

Yellapu, S.K., Kaur, R., Tyagi, R.D. 2017. Detergent assisted ultrasonication aided in situ transesterification for biodiesel production from oleaginous yeast wet biomass. *Bioresource technology*, **224**, 365-372.

Zhang, X., Yan, S., Tyagi, R.D., Drogui, P., Surampalli, R.Y. 2014. Ultrasonication assisted lipid extraction from oleaginous microorganisms. *Bioresource Technology*, **158**, 253-261.

**Table 2.6. 1 Characterization of different sludge solids and scum**

| | Primary sludge | Secondary sludge | Scum |
|---|---|---|---|
| Oil content % wt | 7.28 | 5.31 | 29.3 |
| FFA content % wt | 13.9 | 14.2 | 12.5 |
| Oil color | Dark brown | Dark brown | Dark brown |
| Density | 1.092 | 1.102 | 1.189 |

**Table 2.6. 2 Comparison of solvent recycle using wet and dry scum with hexane or petroleum diesel as solvent**

| | Recycle (No. of times) | Solvent | Solvent recovery % (v/v) | Oil extraction efficiency % (w/w) |
|---|---|---|---|---|
| Scum (Dry) | 0 | Hexane | 94.1 | 98.9 |
| | 1 | | 87.4 | 94.2 |
| | 2 | | 80.3 | 79.1 |
| Scum (Moisture) | 0 | | 99.3 | 97.2 |
| | 1 | | 96.9 | 95.5. |
| | 2 | | 95.1 | 88.1 |
| Scum (Dry) | 0 | Petroleum diesel | 95.2 | 98.6 |
| | 1 | | 89.2 | 95.2 |
| | 2 | | 83.3 | 85.5 |
| Scum (Moisture) | 0 | | 99.1 | 95.9 |
| | 1 | | 97.3 | 93.2 |
| | 2 | | 96.2 | 91.1 |

**Table 2.6. 3 Characterization of metals in sludge and scum solids and biodiesel obtained using sludge or scum oil**

| Metals | Solids (g/kg) | | | Biodiesel (mg/L)-Hexane | | | Biodiesel (mg/L)- Petroleum diesel | | |
|---|---|---|---|---|---|---|---|---|---|
| | P. sludge[1] | S. sludge[2] | Scum | P. sludge[1] | S. sludge[2] | Scum | P. sludge[1] | S. sludge[2] | Scum |
| Al | 16.3 | 4.53 | 0.82 | 1.01 | 0.318 | 0.04 | 0.98 | 0.282 | 0.031 |
| Ca | 10.7 | 4.63 | 4.36 | 0.727 | 0.32 | 0.28 | 0.61 | 0.31 | 0.17 |
| Cu | 0.9 | 0.62 | 1.03 | 0.051 | 0.038 | 0.052 | 0.043 | 0.028 | 0.027 |
| Fe | 1.3 | 0.8 | 2.7 | 0.083 | 0.041 | 0.189 | 0.071 | 0.034 | 0.142 |
| P | 5.22 | 2.66 | 1.11 | 0.281 | 0.13 | 0.05 | 0.201 | 0.08 | 0.037 |
| S | 1.28 | 0.6 | 0.8 | 0.072 | 0.031 | 0.042 | 0.061 | 0.024 | 0.038 |

1. Primary sludge
2. Secondary sludge

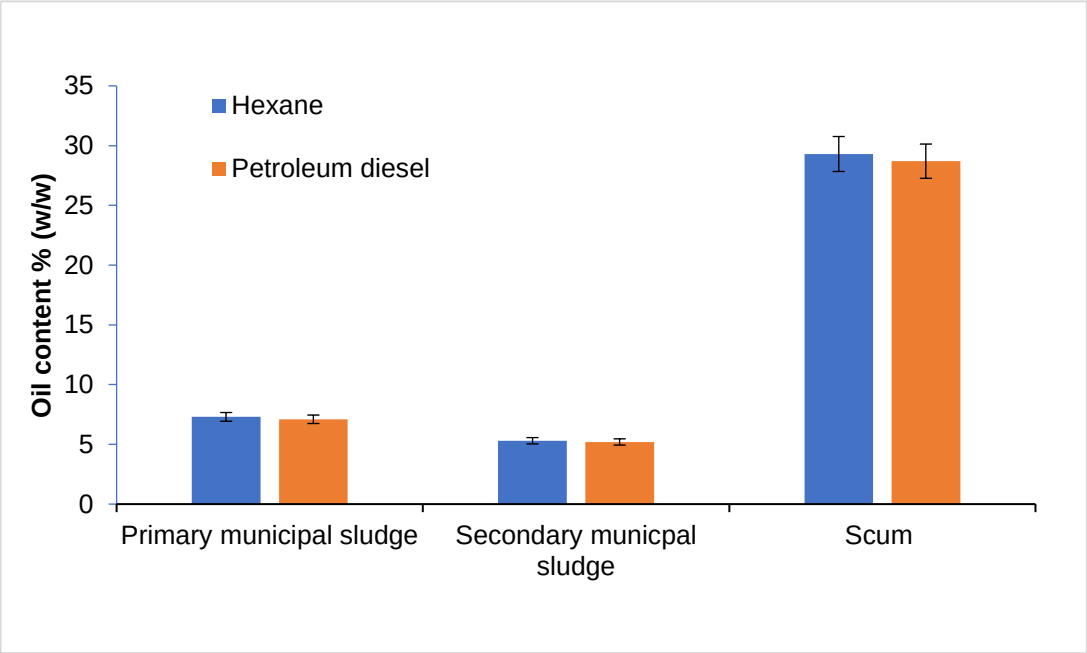
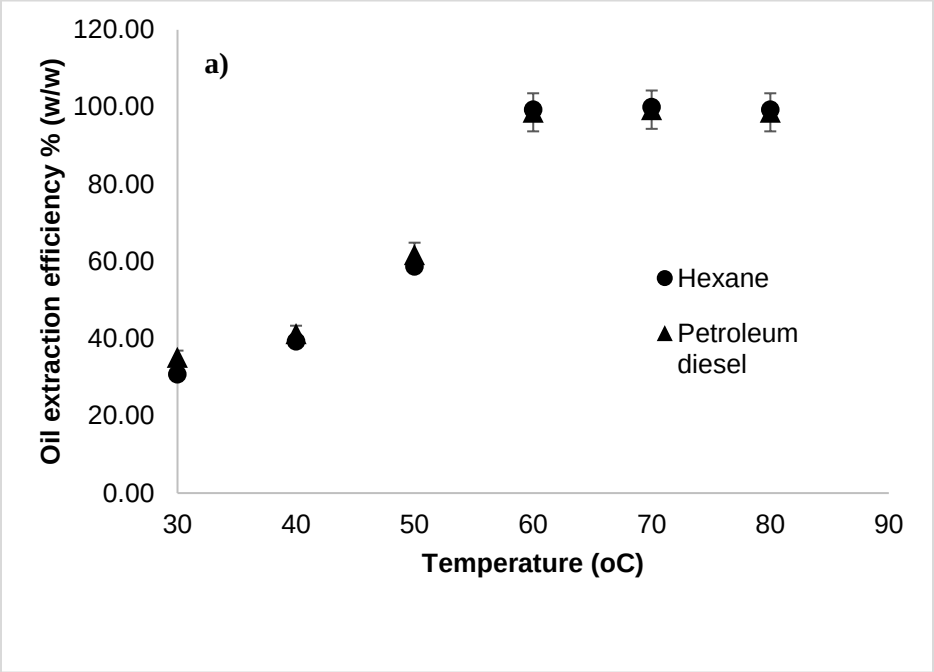
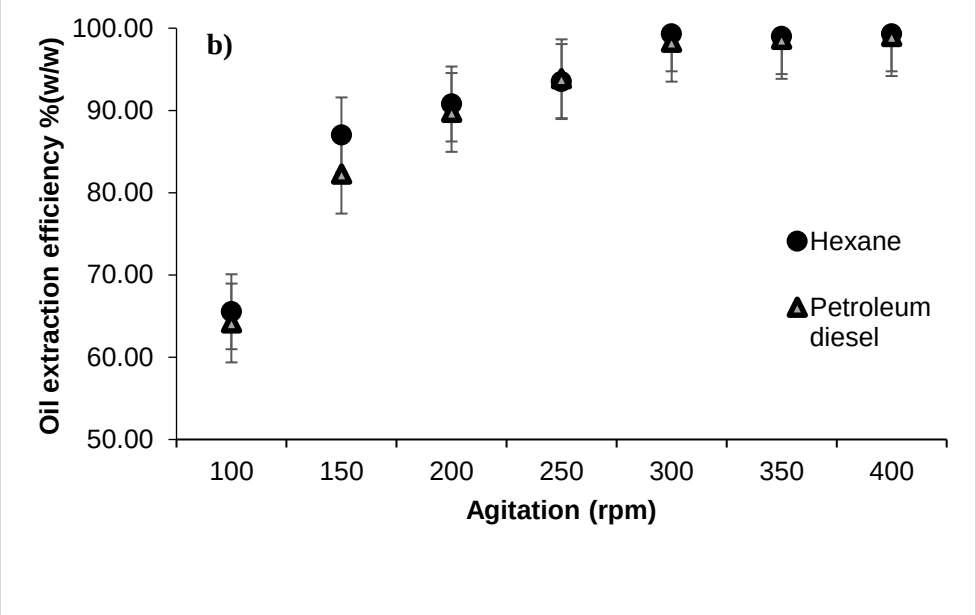
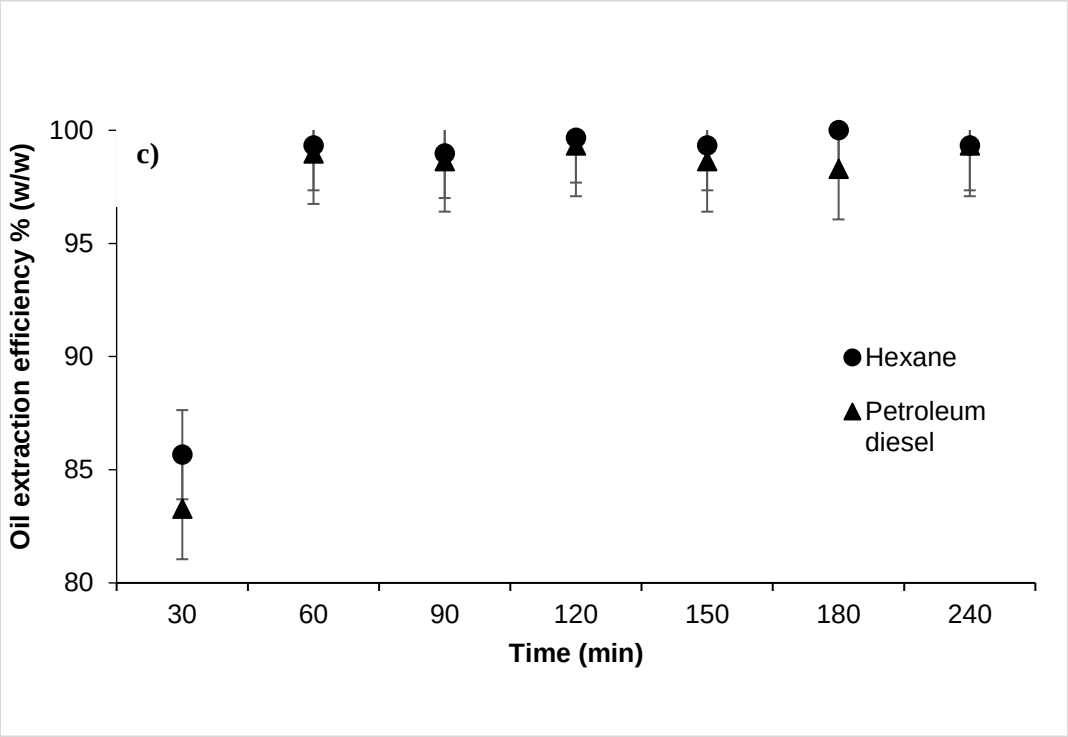
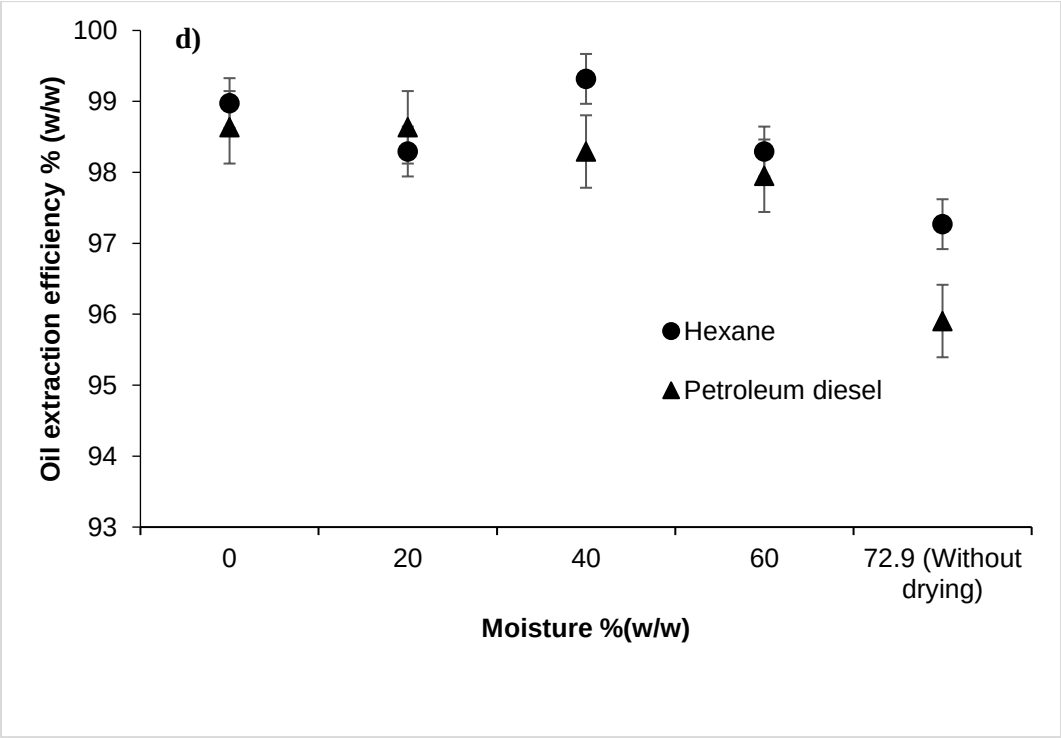



**Figure 2.6. 1 Comparison of oil extracted from primary sludge, secondary sludge and scum using hexane or petroleum diesel.**


a)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Oil extraction efficiency % (w/w)
30
40
50
60
70
80
90
Temperature (oC)
Hexane
Petroleum diesel


b)
100.00
90.00
80.00
70.00
60.00
50.00
Oil extraction efficiency %(w/w)
100
150
200
250
300
350
400
Agitation (rpm)
Hexane
Petroleum diesel


c)
Oil extraction efficiency % (w/w)
100
95
90
85
80
30
60
90
120
150
180
240
Time (min)
Hexane
Petroleum diesel


d)
100
99
98
97
96
95
94
93
Oil extraction efficiency % (w/w)
Hexane
Petroleum diesel
0
20
40
60
72.9 (Without drying)
Moisture %(w/w)

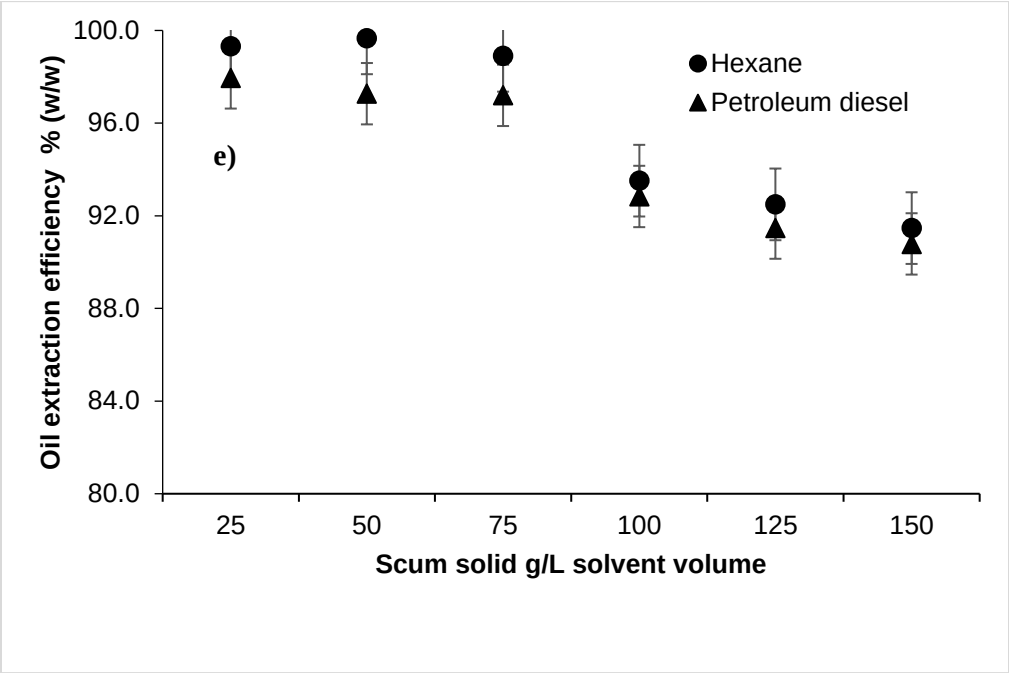



**Figure 2.6. 2 Effect of different factors on oil recovery from scum using hexane or petroleum diesel as solvents a) Temperature b) agitation c) Time d) Moisture content e) scum solids concentration**

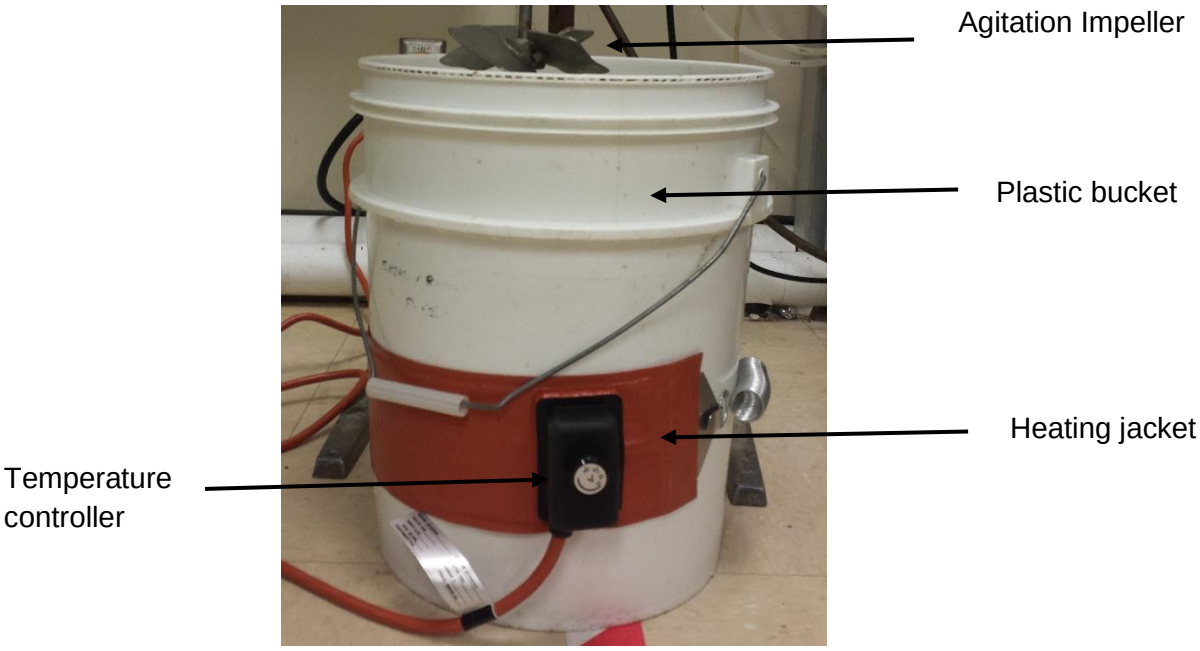



**Figure 2.6. 3 Reactor model**

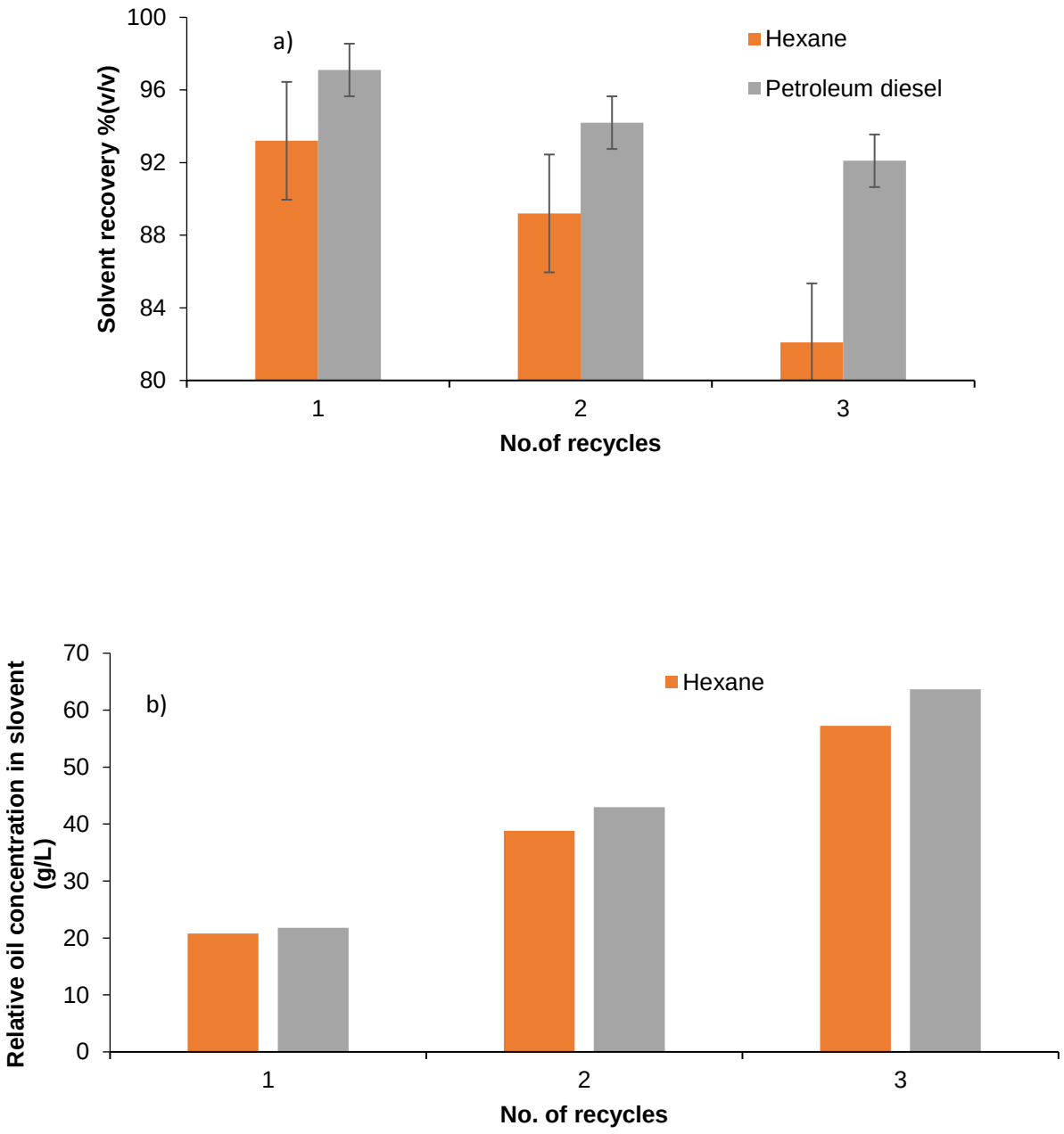



**Figure 2.6. 4 Validation study using one-kilogram scum with hexane (H) and petroleum diesel (PD) a) solvent recovery percentage b) relative oil extraction and oil loss during solvent recycle.**

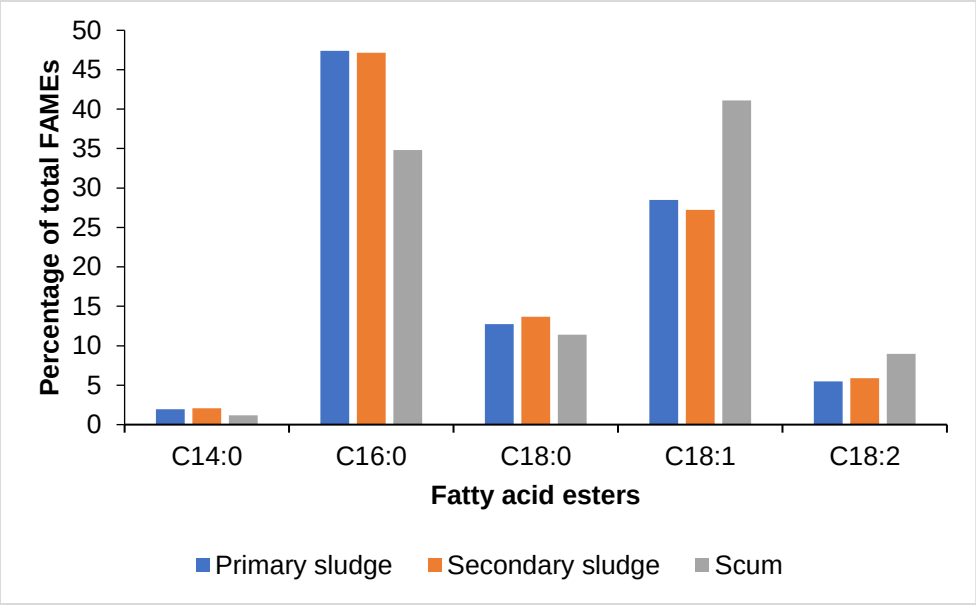



**Figure 2.6. 5 Comparison of fatty acid profiles of biodiesel obtained from primary sludge, secondary sludge and scum oil**