# Conversion of crude glycerol from the biodiesel industry to value added products

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By

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**Abstract**

Crude glycerol is a major by-product of the biodiesel industries. For every 100 kg of biodiesel produced, approximately 10 kg of the byproduct glycerol is generated. With the large increase in biodiesel production, there is a glut in the glycerol produced. Presently crude glycerol is purified to its purer marketable form, burnt as a fuel or mixed with animal feed. However, none of these options contribute considerable revenues to the concerned biodiesel industry. Additionally, some of these routes are not environmentally friendly. It has thus become imperative to find ways to convert crude glycerol to some value-added products.

Bioconversion of crude glycerol to microbial lipids is one possible way to valorize it. However, impurities like methanol, salts and soap present in crude glycerol inhibit the growth of microbes used for such conversions. The research work carried out in this thesis addressed these issues and developed tangible alternatives to overcome these problems.

Initially the possible use of a heterogeneous catalyst Calcium oxide (CaO) attached to support alumina ($Al_2O_3$) for the production of biodiesel was studied. We found that the use of such a catalyst improves the purity of biodiesel and the glycerol produced. Crude glycerol obtained using such insoluble catalysts contained lower levels of impurities and can be converted relatively easily to other useful products. With CaO anchored on $Al_2O_3$ as catalyst, the purity of biodiesel and glycerol were found to be 97.66% and 96.36% respectively. The unanchored heterogeneous catalyst CaO resulted in purities of 96.75% and 92.73% respectively. As the byproduct glycerol containing smaller amount of impurities, the use of anchored heterogeneous catalyst is recommended. The potential use of ash from various sources as a cheap alternative heterogeneous catalyst was also studied. With the use of ash from birch bark and fly ash from wood pellets as catalysts, biodiesel and glycerol with purity in the ranges of 88.06%-99.92% and 78.18%-88.23% respectively were obtained. Since such catalysts are cheap and reusable, their application can reduce expenses and the use of environmentally unsafe compounds.

The crude glycerol used in all experiments was obtained from a biodiesel producer in Ontario (Canada). It was found to contain 44.56 wt.% glycerol and many impurities including 13.86 wt.% methanol, 32.97 wt.% soap and 4.38 wt.%. After the characterization of the sample it was

converted to microbial lipids using an oleaginous yeast *Rhodosporidium toruloides* ATCC 10788. When this strain was grown on crude glycerol, double the biomass (21.16 g/L) and triple the lipid concentration (11.27 g/L) was obtained compared to growth on pure glycerol media. The capacity of this strain to grow on crude glycerol with high levels of impurities and produce large amounts of lipids proves its robustness. Investigation of the effect of individual components on the lipid production ability of this strain showed it to be capable of using soap as a sole carbon source. This was also the reason for enhanced lipid production even in the presence of other impurities present in crude glycerol. The lipids obtained were rich in oleic acid (47.16%), a mono-unsaturated fatty acid (MUFA). Feedstock rich in MUFA are considered suitable for biodiesel production. Thus, the process of conversion of crude glycerol to microbial lipids can be integrated to existing biodiesel plants. This will help in the management of crude glycerol produced during biodiesel production, save transportation and disposal costs and contribute to the revenues of such industries.

The possible applications of microbial lipids obtained from oleaginous (oil producing) microbes depends on its fatty acid composition. Most reports suggest the potential use of microbial lipids for biodiesel production. However, tailoring lipids obtained from these microbes, by changing the fatty acid composition, can make it suitable for use in various biotechnology, pharmaceutical and food industries. The possible use of essential oils from plant sources to change the fatty acid profile of the lipids obtained from *R. toruloides* ATCC 10788 was studied. The addition of seven types of essential oils into the growth medium resulted in lipids with seven different fatty acid profiles. Most of the essential oils tested enhanced the stearic acid content of the lipids. The latter has numerous applications in food, pharmaceuticals and cosmetic industries. Additionally, specific levels of these essential oils in the media changed the metabolic pathways of lipids production resulting in unique fatty acids compositions. For example, use of 2 g/L of orange essential oil in the growth medium produced lipids with fatty acid composition similar to mahua butter which has a number of food, cosmetic and medical applications. Addition of origanum (0.3 g/L) or pine essential oil (3 g/L) into the media improved the oleic acid content of the lipid by 17.90% and 14.35% respectively. This study also provides an insight into the ability of these essential oils to affect the activity of enzymes involved in fatty acid biosynthesis metabolism of *R. toruloides* ATCC 10788. Most of the essential oils used were found to inhibit

Δ9 and Δ12 desaturase enzymes present in the fatty acids biosynthesis metabolic pathways of this strain. This in turn increased the amounts of stearic acids in most of the lipids obtained. Further investigations showed that mono-terpenoids in the essential oils played a major role in bringing about such changes in the fatty acids composition and that the effect of essential oils are specific to the microbial species under study.

Subsequently, the possible use of microbial lipids obtained from crude glycerol for polyol production was studied. Polyol is a precursor to the production of polyurethane foams. In order to produce large quantities of lipids, fermentation of crude glycerol was carried out in a 1L bioreactor. This resulted in 27.48 g/L of biomass and 18.69 g/L of lipids at the end of 168 h. The lipids obtained were then converted to polyol using epoxidation and oxirane ring opening reactions. For comparison, polyols from commonly used vegetable oils (i.e. canola and palm oil) were also produced under similar reaction conditions. The hydroxyl numbers of polyols from canola, palm and microbial oil were found to be 266.86, 222.32 and 230.30 (mg KOH/g of sample) respectively. These values are within the range required for polyurethane production. We subsequently converted the polyols obtained from these oils into rigid and semi-rigid types of polyurethanes. Polyurethanes produced can potentially be used for various commercial applications. In this way, we have been successful in demonstrating the possible use of microbial lipids in biopolymer industries.

The use of a robust microbial strain capable of growing on crude glycerol and the conversion of the lipids obtained to bioplastics can not only help fix the carbon produced (leading to reduction in GHG emission obtained by its combustion), but also potentially bring revenues to industries that integrate such processes into existing facilities.

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"*Dyauḥ Śāntirantarīkṣan Śanti Pṛithivī Śāntirāpaḥ Śāntiroṣadhayaḥ Vanaspatayaḥ Śāntiviswadevah Śāntibrahma Śāntiḥ Sarva Śāntiḥ Śāntireva Śāntiḥ Sā Mā Śntiredhi*" (From the Vedas; Sukla Yajurveda 36-17).

**This translates to**

"Let there be balance in the space! Let there be balance in the sky! Let there be peace on the earth! Let there be calmness! Let there be growth in the plants! Let there be growth in the tree! Let there be grace in the Gods! Let there be bliss in the Brahman! Let there be balance in everything! Let there be peace and peace! Let such peace be with all of us!"

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*This thesis is dedicated to my father,*
***Late Krishna Prasad Uprety***

## List of Published Works During PhD

### I. Published work related to this thesis

1. **Uprety BK**, Chaiwong W, Ewelike C, Rakshit SK (2016) Biodiesel production using heterogeneous catalysts including wood ash and the importance of enhancing byproduct glycerol purity. Energy Convers Manag 115:191–199.

2. **Uprety BK**, Dalli SS, Rakshit SK (2017) Bioconversion of crude glycerol to microbial lipid using a robust oleaginous yeast *Rhodosporidium toruloides* ATCC 10788 capable of growing in the presence of impurities. Energy Convers Manag 135:117–128.

3. **Uprety BK**, Reddy JV, Dalli SS, Rakshit SK (2017) Utilization of microbial oil obtained from crude glycerol for the production of polyol and its subsequent conversion to polyurethane foams. Bioresour Technol 235:309–315.

4. **Uprety BK**, Rakshit SK (2017) Compositional shift in fatty acid profiles of lipids obtained from oleaginous yeasts upon the addition of essential oil from *Citrus sinensis* L. Appl Biochem Biotechnol. doi: 10.1007/s12010-017-2490-8

### II. Work related to this thesis submitted for publication

5. **Uprety BK**, Rakshit SK (2017) Contribution of specific impurities in crude glycerol towards improved lipid production by *Rhodosporidium toruloides* ATCC 10788 and the ability of this strain to consume soap. J. Biotechnol. (Submitted on: May 4, 2017)

6. **Uprety BK**, Rakshit SK (2017) Use of essential oils from various plants to change the fatty acids profiles of lipids obtained from oleaginous yeasts. J Am Oil Chem Soc. (Submitted on: May 25, 2017)

7. **Uprety BK**, Venkatesagowda B, Rakshit SK (2017) Current prospects on production of microbial lipid and other value added products using crude glycerol obtained from biodiesel industries. BioEnergy Res. (Submitted on: 09 Mar 2017)

### III. Other contributions during PhD

8. Dalli SS, **Uprety BK**, Rakshit SK (2017) Industrial Production of Active Probiotics for Food Enrichment. In: Roos YH, Livney YD (eds) Eng. Foods Bioact. Stab. Deliv. Springer New York, New York, NY, pp 85–118.

9. Dalli SS, **Uprety BK**, Samavi M, Singh R, Rakshit SK (2017) Nanocrystalline Cellulose - Production and Applications. In: Prasad R, Kumar M, Kumar V (eds) Nanotechnology-An Agric. Paradig. Springer.

10. Dalli SS, da Silva SS, **Uprety BK**, Rakshit SK (2017) Enhanced Production of Xylitol from Poplar Wood Hydrolysates Through a Sustainable Process Using Immobilized New Strain *Candida tropicalis* UFMG BX 12-a. Appl Biochem Biotechnol 1–12.

## Abbreviations

| | |
|---|---|
| AE | Amine Equivalent |
| AIER | Acid Ion Exchange Resin |
| AMP | Adenosine Monophosphate |
| ATCC | American Type Culture Collection |
| C16:0 | Palmitic Acid |
| C18:0 | Stearic Acid |
| C18:1 | Oleic Acid |
| C18:2 | Linoleic Acid |
| CG | Crude Glycerol |
| CO | Canola Oil |
| COP | Canola Oil Polyol |
| DBTDL | Dibutyltin Dilaurate |
| DHA | Docosahexaenoic Acid |
| DMEA | Dimethylethanolamine |
| ECO | Epoxidized Canola Oil |
| EMO | Epoxidized Microbial Oil |
| EPA | Eicosapentaenoic Acid |
| EPO | Epoxidized Palm Oil |
| FAAE | Fatty Acid Alkyl Esters |
| FAME | Fatty Acid Methyl Ester |
| FFA | Free Fatty Acid |
| FID | Flame Ionization Detector |
| FTIR | Fourier Transform Infrared Spectroscopy |
| GC | Gas Chromatography |
| Gly | Glycerol |
| HPLC | High Performance Liquid Chromatography |
| IMP | Inosine Monophosphate |
| MO | Microbial Oil |
| MO | Microbial Oil |
| MOP | Microbial Oil Polyol |
| MUFA | Monounsaturated Fatty Acid |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| NC | Not Communicated |
| NCO Index | Isocyanate Index |
| NGOM | Non-glycerol Organic Matter |
| OECD | Organization for Economic Co-operation and Development |
| PG | Pure Glycerol |
| PHB | Poly (3-hydroxybutyrate) |
| PO | Palm Oil |
| POP | Palm Oil Polyol |
| PU | Polyurethane |
| PUFA | Polyunsaturated Fatty Acid |
| RBD | Refine, Bleached and Deodorized |
| RI | Refractive Index |

| | |
|---|---|
| SCO | Single Cell Oil |
| SEM | Scanning Electron Microscopy |
| SEM | Scanning Electron Microscopy |
| SFA | Saturated Fatty Acid |
| TDI | Toluene Di-isocyanate |
| UFA | Unsaturated Fatty Acid |
| XRD | X-ray Diffraction |
| YPG | Yeast extract, Peptones and Glycerol Media |

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# Chapter 1

## General Overview Rationale and Objectives

## 1.1. Introduction

Due to the dramatic rise in energy demand, fluctuating prices of fossil fuels, depletion of its sources and various environmental issues associated with its use, researchers from across the world are constantly looking for alternative sources of energy [1–3]. Biodiesel, chemically known as Fatty Acid Methyl Ester (FAME), is renewable and environmentally friendly alternative to its fossil based counterpart. It can be produced from various sources, including plants oils, animal fats or microbial lipids and blended in different proportion with the fossil based diesel for its use in conventional engines [1]. Many of the developed and developing countries, such as USA, Canada, Brazil and Australia, have made it mandatory to blend biodiesel with fossil based diesel in the range of 2-20% (v/v) [4].

Typical biodiesel production processes involve a transesterification reaction between triglycerides (oils) with methanol in the presence of a catalyst [5]. Significant amount of glycerol is produced as a by-product (or co-product) during this reaction process [1]. The catalyst used for biodiesel production can be acidic, basic or biological in nature. Among these, basic catalysts are most commonly used in many of the biodiesel production industries [6]. Basic catalyst such as sodium hydroxide, potassium hydroxides and calcium oxides are less caustic and requires milder reaction conditions (low temperature and less reaction time) than acidic catalyst such as sulfuric acid and hydrochloric acid. On the other hand, they are cheaper than biological catalyst such as lipases [5, 7]. Even though homogeneous basic catalysts (such as sodium hydroxide) possess various advantages, it cannot be reused. Additionally, if such catalyst are employed for biodiesel production, large quantities of wastewater is generated during the removal and cleaning of catalyst and the products [8]. In order to resolve this issue, the use of heterogeneous basic catalyst has been reported by various authors [1, 9–11]. Heterogeneous catalysts are insoluble in and can be easily separated from the final product making them reusable [12]. Thus, when heterogeneous catalyst such as calcium oxides or magnesium oxides are used, purer biodiesel and crude glycerol by-product are obtained making downstream processing easier. Even though heterogeneous catalyst possesses various benefits over homogeneous catalysts, it will take some time for the industries to adopt it into their production processes. The development of cheaper heterogeneous basic catalysts for biodiesel production would also be of great interest to the prevailing biodiesel industries [1].

During biodiesel production, approximately 10 wt.% of glycerol is produced as a by-product [13]. The glycerol produced during the biodiesel production process is also known as "crude glycerol". With the rise in production of biodiesel across the world, there is also a rise in crude glycerol accumulation [2]. As detailed in published report by OECD 2016, world biodiesel production is expected to reach 37.9 billion liters by the end of 2020 [14, 15]. This will thus leave us with approximately 3.8 billion liters of crude glycerol to be dealt with. From a global perspective, Canada produces a very small percentage of global biodiesel market share [15]. As per the OECD Agricultural Outlook (2016), 32.8 billion liters of biodiesel was produced across the world in 2014 whereas Canada's annual production that year was only 347.5 million liters [15]. However, large production is required to meet the federal biodiesel blending mandate of approximately 2% that varies from province to province. Currently, Canada imports biodiesel from outside to meet such federal mandate [16]. Therefore, the amount of production is expected to increase in the coming years.

As pure glycerol has various applications, purification of crude glycerol to its purer forms is one of the options available to the biodiesel producer. However, due to over production of crude glycerol, the price of pure glycerol has slumped making this route quite uneconomical [2]. Potential use of crude glycerol as a feed for cattle or a low energy fuel has also been explored [17, 18]. But both these options do not add much revenue to the industries either. Additionally, burning of crude glycerol is not a preferred option from an environmental point of view [19]. Thus, many industries have already started treating crude glycerol as a waste [2]. Hence, it has become imperative to produce value added chemicals and products using such a low value commodity.

Conversion of crude glycerol to various value added products have been adopted by various authors [20, 21]. Crude glycerol can be chemically or biologically converted into different components. Some of these includes hydrogen, 1,2 propandiol, butanol, succinic acid, dihydroxyacetone, single cell oils. etc. However, both these conversion processes have been hindered by the impurities present in crude glycerol [20–25]. Crude glycerol generally contains glycerol and other impurities including light solvents (methanol or ethanol, water), soap, residual FAMEs, glycerides, free fatty acids, salts and ash [2, 20]. The ratio of each of these

components in crude glycerol however varies. This is because the types of feedstocks, methods and catalyst used, and the number of downstream processes adopted varies between the biodiesel producers. Among the different components mentioned, methanol, water, soap and salts are the major inhibitors for the conversion of crude glycerol into other useful products [2].

Various microorganisms are capable of accumulating lipids to levels more than 20 wt.% of their dried biomass. Such organisms are known as oleaginous microbes and the lipids obtained from them are called as microbial lipids (single cell oils or microbial oils). Production of microbial lipids from crude glycerol using microalgae, fungi or yeasts (also known as oleaginous microbes) have been previously studied [2, 26]. Bioconversion of crude glycerol into microbial lipids is one of the options to valorize the former. Microbial lipids are chemically similar to vegetable oils. This makes microbial lipids a suitable feedstock for biodiesel production. Most of the previously reported work have focused on utilizing microbial lipid into biodiesel. Conversion of crude glycerol to microbial lipid have an added advantage as the process can be easily integrated to the prevailing biodiesel industries. The microbial lipid produced can be fed back into the biodiesel production plant, thus reducing transportation costs. Besides biodiesel production, use of such lipids to produce various other components would make the overall conversion process (i.e. crude glycerol to microbial lipid) more sustainable [2]. During the bioconversion of crude glycerol into microbial lipids, the impurities present in crude glycerol have been shown to inhibit the growth of microbes used. This subsequently reduces the overall yield of the product [24, 27, 28]. In order to overcome this problem, in certain instances, crude glycerol has been pre-treated before its use for lipid production. Pretreatment of crude glycerol can be suitable for large biodiesel producers but for small and medium scaled industries such pretreatment will not be a possible option as the overall process becomes quite expensive.

### 1.2. Rationale for the work

With mandatory blending requirement of biodiesel into petroleum based diesel there has been a very rapid increase in its production in many parts of the world. In Canada, the production was 370.59 million liters in the year 2016 [15]. With a kilogram of glycerol produced per 10 kg of biodiesel produced, there is a huge glut in its production. Heterogeneous catalysts can reduce the generation of environmentally harmful chemicals used during the production of biodiesel.

On the other hand, the crude glycerol produced needs to be converted in an economically feasible manner to useful products. With this background the rational for this study was as follows.

1. Heterogeneous catalysts have advantages over their homogeneous counterparts because they can easily be separated from their final products and reused (i.e. biodiesel and crude glycerol). However, they can still leach into the final products. Thus, the possibility of attaching such heterogeneous catalyst onto supports that reduce leaching of such catalysts into the final product are being developed. Hence, we decided to explore the possibility of using ash from different sources as a cheap alternative renewable catalyst for biodiesel production along with some chemical supports (objective 1).

2. Attempts have and are being made to convert glycerol to value added products. Bioconversion of crude glycerol to microbial lipids is one route to valorize it. However, most of the previously studied microbial strains for such conversions succumbed to the harsh growth conditions posed by the impurities present used in crude glycerol. This in turn reduced the overall yield of the obtained product. Thus, it is required to find, characterize and optimize fermentation of robust microorganisms capable of withstanding these inhibitors and producing large quantities of lipids (objective 2). The effects of individual components present in crude glycerol on the growth and lipid production ability of such a strain have had to be determined. We felt that such a robust strain could have number of beneficial applications.

3. Microbial lipids obtained from oleaginous microbes have frequently been explored for their use as biodiesel feedstock. However, altering the fatty acid composition of obtained lipids can expand the applications of such lipids. Use of essential oils have been shown to alter the fatty acid composition of microbial oil produced. Very few studies have been carried out to understand its effect on oleaginous yeasts. The possibility of using essential oils from different plant sources to alter the chemical composition of lipid can be beneficial. This

was third objective of our study. Tailored lipids with desirable fatty acids composition could be used in food, cosmetic and pharmaceutical industries.

4. Presently, microbial lipids from crude glycerol have been studied for their potential use in food and biodiesel industries. However, its conversion into biopolymer has not been reported previously. We wanted to demonstrate the possible conversion of microbial lipids obtained from crude glycerol to polyol and its subsequent conversion into polyurethane foams (Objective 4). Development of such processes will make the microbial lipid production from cheaper carbon substrate, including crude glycerol, quite sustainable.

### 1.3. Research objectives

Based on the above rationale the overall objective of this work was to produce value added products from crude biodiesel based glycerol. The specific objectives of this study were to:

1. Develop a cheap heterogeneous basic catalyst for biodiesel production.
2. Improve production of microbial lipids using crude glycerol containing high amounts of impurities.
3. Alter the fatty acid metabolic pathway of the oleaginous yeast to obtain lipid with useful fatty acid composition.
4. Produce polyol a precursor to polyurethane foam using microbial lipids produced from crude glycerol.

**References**


1. Uprety BK, Chaiwong W, Ewelike C, Rakshit SK (2016) Biodiesel production using heterogeneous catalysts including wood ash and the importance of enhancing byproduct glycerol purity. Energy Convers Manag 115:191–199. doi: 10.1016/j.enconman.2016.02.032
2. Uprety BK, Dalli SS, Rakshit SK (2017) Bioconversion of crude glycerol to microbial lipid using a robust oleaginous yeast Rhodosporidium toruloides ATCC 10788 capable of growing in the presence of impurities. Energy Convers Manag 135:117–128. doi: 10.1016/j.enconman.2016.12.071
3. Rittmann BE (2008) Opportunities for renewable bioenergy using microorganisms. Biotechnol Bioeng 100:203–212. doi: 10.1002/bit.21875
4. Lane J (2016) Biofuels Mandates Around the World : 2016. BiofuelsDigest 1–10.
5. Zabeti M, Wan Daud WMA, Aroua MK (2009) Activity of solid catalysts for biodiesel production: A review. Fuel Process Technol 90:770–777. doi: 10.1016/j.fuproc.2009.03.010
6. Bharathiraja B, Chakravarthy M, Kumar RR, et al (2014) Biodiesel production using chemical and biological methods – A review of process, catalyst, acyl acceptor, source and process variables. Renew Sustain Energy Rev 38:368–382. doi: 10.1016/j.rser.2014.05.084
7. Freedman B, Pryde EH, Mounts TL (1984) Variables affecting the yields of fatty esters from transesterified vegetable oils. J Am Oil Chem Soc 61:1638–1643. doi: 10.1007/BF02541649
8. Guo F, Fang Z (2011) Biodiesel Production with Solid Catalysts. In: Stoytcheva M (ed) Biodiesel - Feed. Process. Technol. pp 339–358
9. Dossin TF, Reyniers M-F, Marin GB (2006) Kinetics of heterogeneously MgO-catalyzed transesterification. Appl Catal B Environ 62:35–45. doi: 10.1016/j.apcatb.2005.04.005
10. Liu X, He H, Wang Y, et al (2008) Transesterification of soybean oil to biodiesel using CaO as a solid base catalyst. Fuel 87:216–221. doi: 10.1016/j.fuel.2007.04.013
11. Suryaputra W, Winata I, Indraswati N, Ismadji S (2013) Waste capiz (Amusium cristatum) shell as a new heterogeneous catalyst for biodiesel production. Renew

Energy 50:795–799. doi: 10.1016/j.renene.2012.08.060

12. Lam MK, Lee KT (2011) Production of Biodiesel Using Palm Oil. In: Pandey A, Larroche C, Ricke SC, et al (eds) Biofuels Altern. Feed. Convers. Process., 1st ed. Academic Press, p 629
13. Uprety BK, Reddy JV, Dalli SS, Rakshit SK (2017) Utilization of microbial oil obtained from crude glycerol for the production of polyol and its subsequent conversion to polyurethane foams. Bioresour Technol. doi: 10.1016/j.biortech.2017.03.126
14. Fox 2016 (2017) World biodiesel production/consumption to rise 14% by 2020: OECD/FAO. In: S&P Glob. Platts. http://www.platts.com/latest-news/agriculture/london/world-biodiesel-productionconsumption-to-rise-26485632. Accessed 18 Feb 2017
15. OECD C (2016) OECD-FAO Agricultural Outlook 2016-2025. In: Stat Technol. http://stats.oecd.org/viewhtml.aspx?datasetcode=HIGH_AGLINK_2016&lang=en#. Accessed 18 Feb 2017
16. Dessureault D (2016) Canada Biofuels Annual 2016.
17. Mclea L, Ball MEE, Kilpatrick D, Elliott C (2011) The effect of glycerol inclusion on broiler performance and nutrient digestibility. Br Poult Sci 52:368–375. doi: 10.1080/00071668.2011.584520
18. Yang F, Hanna M a, Sun R (2012) Value-added uses for crude glycerol-a byproduct of biodiesel production. Biotechnol Biofuels 5:13. doi: 10.1186/1754-6834-5-13
19. Hassannia J (2011) Crude Glycerol Combustion System- Introduction and Commercialization Plan.
20. Kong PS, Aroua MK, Daud WMAW (2016) Conversion of crude and pure glycerol into derivatives: A feasibility evaluation. Renew Sustain Energy Rev 63:533–555. doi: 10.1016/j.rser.2016.05.054
21. Garlapati VK, Shankar U, Budhiraja A (2016) Bioconversion technologies of crude glycerol to value added industrial products. Biotechnol Reports 9:9–14. doi: 10.1016/j.btre.2015.11.002
22. Ilham Z, Saka S (2016) Esterification of glycerol from biodiesel production to glycerol carbonate in non-catalytic supercritical dimethyl carbonate. Springerplus 923.
23. Wegenhart BL, Liu S, Thom M, et al (2012) Solvent-Free Methods for Making Acetals

Derived from Glycerol and Furfural and Their Use as a Biodiesel Fuel Component. ACS Catal 2:2524–2530.

24. Pyle DJ, Garcia RA, Wen Z (2008) Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: Effects of impurities on DHA production and algal biomass composition. J Agric Food Chem 56:3933–3939. doi: 10.1021/jf800602s
25. Mothes G, Schnorpfeil C, Ackermann JU (2007) Production of PHB from crude glycerol. Eng Life Sci 7:475–479. doi: 10.1002/elsc.200620210
26. Meng X, Yang J, Xu X, et al (2009) Biodiesel production from oleaginous microorganisms. Renew Energy 34:1–5.
27. Ardi MS, Aroua MK, Hashim NA (2015) Progress, prospect and challenges in glycerol purification process: A review. Renew Sustain Energy Rev 42:1164–1173.
28. Tang S, Boehme L, Lam H, Zhang Z (2009) Pichia pastoris fermentation for phytase production using crude glycerol from biodiesel production as the sole carbon source. Biochem Eng J 43:157–162.

# Chapter 2

## Literature Review: "Current prospects on production of microbial lipid and other value added products using crude glycerol obtained from biodiesel industries"

In this chapter a literature review of the work carried out in this thesis is presented. In the introduction to each of the subsequent chapters, additional literature directly related to the specific objectives are included.

This chapter has been submitted to **BioEnergy Research (IF 2.4) ***

* **Uprety BK**, Venkatesagowda B, Rakshit SK (2017) Current prospects on production of microbial lipid and other value added products using crude glycerol obtained from biodiesel industries. BioEnergy Res. (Submitted on: 09 Mar 2017)

## 2.1. Biodiesel and its production

Biodiesel is one of the most promising alternatives to fossil based diesel. It is generally produced by the transesterification of triglycerides using an alcohol and in the presence of catalyst [1]. The typical reaction involved in biodiesel production process is shown in Fig. 2.1.

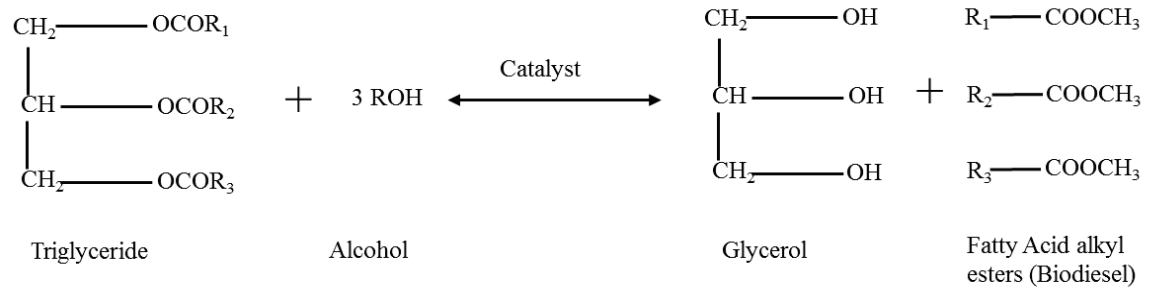


Fig 2.1. Transesterification of triglyceride into biodiesel

Biodiesel is also known as Fatty Acid Alkyl Esters or FAAE, chemically [2]. The alkyl group is decided by the acyl acceptor used for the transesterification reaction of triglycerides (TAGs). Since methanol is commonly used in the prevailing biodiesel production plants, biodiesel is also known as Fatty Acid Methyl Ester or FAME [3, 4]. Biodiesel can be blended in various ratios with fossil based diesel and can be used even in conventional engines [5]. Its popularity is mainly due to reduced emission of toxic gases upon its use and the renewable nature of the feedstocks required for its production [3].

Feedstocks for biodiesel production includes plant oils, waste cooking oils, animal fats and microbial lipids [6]. Biodiesel production using vegetable oils including canola, rapeseed, soybean, palm, corn and olive have been reported by Joshi et al. (2009), Zhang and You (2015), Alkabbash et al. (2009), Mata et al. (2012), Sanchez and Vasudevan (2006) respectively [7–11]. Biodiesel production from plant oils require use of agricultural lands for cultivation of oil seed crops. Moreover, used of vegetable oils for such purposes could lead to a spiraling rise in edible oil prices [1]. Table 2.1 lists varieties of vegetable oils used for biodiesel production and their unit price as of 2016.

Table 2.1. Cost comparison of vegetable oils commonly used for biodiesel production. Values were obtained from [12] but in case of canola oil the value was obtained from [13]. The price available in Canadian Dollars was converted into USD (1 CAD = 0.74 USD)

| Oil Sources | Dec 2005 Cost/ Metric ton (USD) | Dec 2016 Cost/Metric ton (USD) |
|---|---|---|
| Soybean | 466.00 | 800.26 |
| Palm | 368.90 | 711.76 |
| Rapeseed | 769.72 | 917.34 |
| Peanut | 947.00 | 1507.50 |
| Coconut | 553.00 | 1683.75 |
| Canola | 410.24 | 751.75 |

Similarly, production of animal fat oils requires large amounts of feed for producing the animals and need of large areas to cultivate them. Thus, use of oils obtained from varieties of fungi, yeast and algae have been explored as an alternative feedstock for its production [14].

Global biodiesel production has increased from approximately 797 million liters in 2000 [15] to 32.8 billion liters in 2014 and is expected to reach 37.9 billion liters by 2020 [16, 17]. In 2015, USA was the leading biodiesel producer followed by Brazil and Germany [18, 19]. As per the OECD Agricultural Outlook (2016), 142.61 million liters of biodiesel was produced across Canada in 2010 and increased to 347.5 million liters in 2014 [20]. The amount of biodiesel produced in Canada in year 2014 was still only ~1% of the total biodiesel produced across the world. From a global perspective, Canada produces a very small percentage of global biodiesel market share [20]. However, large production is required to meet the federal biodiesel blending mandate of approximately 2% that varies from province to province. Currently, Canada imports biodiesel from outside to meet such federal mandate [21]. Therefore, the amount of production is expected to increase in the coming years.

## 2.2. Heterogeneous catalyst used in biodiesel industries

Generally, the catalysts used for transesterification reaction can be either acidic or basic in nature. Acid catalysts such as sulfuric acid, hydrochloric acid and solid acids (such as silica-bonded N-propyl sulfamic acid, $SO_4/SnO_2$, S-$ZrO_2$ sulfated zirconia, etc.) are used for biodiesel production. Similarly, sodium hydroxide and potassium hydroxide are commonly employed as basic catalysts in many biodiesel industries [3]. Acid catalysts are suitable for the transesterification of low-grade oils containing water and free fatty acids, while base catalysts are commonly used for the transesterification of triglyceride feedstocks with low amounts of water and free fatty acids content [22]. Acid catalyzed transesterification reactions are corrosive, quite slow and requires high molar ratio of alcohol to oil and reaction temperatures [23, 24]. The use of bases are preferred over acids as catalysts, as the former are comparatively faster and requires less reaction temperature [25]. Additionally, the use of base reduces the amount of alcohol used during biodiesel production process [1, 24]. Basic catalysts such as sodium hydroxide or potassium hydroxide are known as homogeneous catalyst as they form a single phase with the reactants [26, 27]. Even though use of homogeneous catalysts possesses several advantages, they are not reusable in nature. Additionally, large quantity of water is required to remove or neutralize such catalyst at the end of reaction. This in turn generate large quantities of wastewater [28, 29]. In order to address these issues, use of heterogeneous basic catalysts such as CaO and MgO have been reported by previous workers [30, 31]. We have recently reported the use of wood ash as a cheap heterogeneous basic catalyst for the biodiesel production [32]. Drawbacks of using heterogeneous catalysts for biodiesel production, include mass transfer limitations, low active sites available for catalytic reaction and leaching of the catalyst into the reaction media [32]. To overcome such problems, use of various types of catalyst supports (mainly alumina, silica, zinc oxide and zirconium oxide) have been reported by various authors [3, 32–35]. Anchoring of heterogeneous catalyst to these supports have shown to reduce diffusion limitation and leaching and increase the catalytic activity of the used catalyst [36].

Besides acids and bases, enzymes (biocatalysts) have also been used as catalysts [37]. Lipases obtained from various microbial sources have been explored for their ability to convert vegetable oils into biodiesel [38]. Enzyme catalysts requires milder conditions compared to acid catalysts and possess better resistance to free fatty acids and water present in oil feedstocks

when compared to basic catalysts [2, 39]. Additionally, purity of biodiesel and glycerol obtained using such catalysts have shown to be better compared to acid or base catalysts [2]. Because of this, fewer numbers of washings are required to purify the obtained products. Hence, use of enzyme catalyst is an environmentally friendly process as reduced amount of wastewater is generated from biodiesel [40]. However, the activity of enzyme during the transesterification process is inhibited by alcohol and the glycerol by-product. This subsequently will reduce the overall biodiesel yield [41]. Moreover, use of enzyme to produce biodiesel is still an expensive process due to high costs of the enzymes required [38, 42].

### 2.3. Crude glycerol from biodiesel industry

During biodiesel production, approximately 10% wt. of glycerol is produced as a by-product. For every 100 kg of biodiesel produced, approximately 10 kg of glycerol is obtained. The glycerol obtained during biodiesel production process contains many impurities and thus called crude glycerol [43, 32]. Pure glycerol finds its use in varieties of sectors including food, pharma and cosmetics industries. Thus, purification of crude glycerol into its pure form has been one of the most common routes to manage the large amount of crude glycerol by-product [37]. Use of a single or a combination of techniques to purify crude glycerol have been reported previously. Some techniques include acidification followed by neutralization and solvent extraction, distillation under reduced pressure, membrane separation technology, ion-exchange adsorption and nano-cavitation technology [23, 44]. However, with considerable increase in biodiesel production, there is a rise in glycerol production too [45–47]. As discussed in section 2.1, the amount of biodiesel produced across the world is expected to reach approximately 38 billion liters by 2020 [16]. This will generate approximately 3.8 billion liters of crude glycerol. Due to a glut of crude glycerol in the market, the price of pure glycerol has plummeted over the last few years. This has made crude glycerol purification into its purer form quite uneconomical. Before the expansion of biodiesel production in USA, the price of pure and crude glycerol was $0.70 per pound and $0.25 per pound respectively. By 2007, the amount of pure glycerol slumped down to $0.30 per pound and that of crude glycerol to $0.05 per pound [48]. By mid-2014, the market price of pure glycerol and crude glycerol were $0.41/lb and $0.11/lb respectively [49, 50]. Even though there is improvement in market prices of these two commodities recently, the cost is expected to go down in future with increased biodiesel

production across the world [17]. Most biodiesel industries are currently facing a problem to manage the glycerol produced in a cheap and environmentally friendly way. In this regard it is worth mentioning that some of the biodiesel companies have already started treating crude glycerol as waste [51, 52]. Hence, it has become quite important to vigorously develop ways to use crude glycerol to produce value added chemicals and products.

### 2.4. Impurities in crude glycerol

Generally, crude glycerol contains glycerol, light solvent (methanol or ethanol, water), soap, small amount of FAME, glycerides, free fatty acids, salts and ash [50]. The amount of each of these components in any crude glycerol however varies. Chemical composition of crude glycerol reported by some researchers is tabulated in Table 2.2. Valerio et al. (2015) reported the glycerol content in crude glycerol from biodiesel to be as low as 15.4 wt. % whereas Hansen et al. (2009) found out the glycerol concentration in crude glycerol (obtained from 7 biodiesel producers across Australia) to be in the range of 38 to 96 wt.%. Similarly, methanol, soap, water, FAMEs and free fatty acids (FFAs) can vary between 0.-22.7, 0-36.1, 0-28.7, 0-30.9 and 0-3.6 wt.% respectively. The difference in compositions are mainly due to variation in feedstock, catalyst, methods and downstream processing among biodiesel producers [43].

Table 2.2. Typical composition of crude glycerol reported in literature

| **Components (wt.%)** | **Hu et al. (2012)** [55] | **Hansen et al. (2009)** [54] | **Xiao et al. (2013)** [56] | **Valerio et al. (2015)** [53] | **Chen and Walker (2011)** [57] | **Liang et al. (2010)** [46] |
|---|---|---|---|---|---|---|
| Glycerol | 22.9-63.0 | 38.4-96.5 | 31.5-74.5 | 15.4-30.2 | 62 | 48.7 |
| Methanol | 6.2-12.6 | <0.01-13.94 | - | 14.4-15.5 | 22.6 | 22.7 |
| Water | 1.0-28.7 | 0.0-16.1 | - | 0.9-5.1 | 8.0 | 25.6 |
| Soap | BDL- 31.4 | - | 19.8-36.1 | 22.4-29.1 | - | 3.0 |
| FAMEs | BDL- 28.8 | - | 0.6-29.3 | 22.4-30.9 | - | - |
| Glycerides | BDL- 7.0 | - | 0.5-4.3 | - | - | - |
| FFAs | BDL- 3.0 | - | 0.0-3.6 | 0.5-2.1 | - | - |
| Ash | 2.7-5.7 | 0.0-29.4 | - | 3.5-4.2 | 2.4 | - |

BDL: Below Detection Level

In addition to the major components shown in Table 2.2, crude glycerol also contains small quantity of metals such as Na, Ca, Mg, P and S. Concentration of sodium metals depends on the amount sodium hydroxide catalyst used during the biodiesel production. Thus, its amount in crude glycerol can in some cases exceed 1%, whereas other metals are mostly present in the range of 4-163 ppm [58].

From an environmental point of view, disposal of crude glycerol in an unmanaged way could lead to various problems. Many large-scale biodiesel producers recover excess unreacted methanol and reuse it. However, due to the low cost of methanol, most of the medium and small sized biodiesel industries finds it uneconomical to recover the methanol [59]. If crude glycerol containing high levels of methanol is released to the environment, it can be toxic to various flora and fauna present available in the ecosystem as methanol poses inhibitory activity on their growth. Contamination of drinking water resources with methanol can be very toxic to humans as well [48, 60]. Most of the produced crude glycerol are generally basic in nature as it contains large amount of sodium hydroxide catalyst used during the biodiesel production. The release of such basic compound into the environment without any treatment harms the biotic community of the surrounding [60]. Contamination of water systems with such crude glycerol can produce offensive odors as microorganisms present in nature takes a lot of time to recycle alkaline water. Additionally, other components such as soap and heavy metals also have detrimental effects on the health of human beings and animals. Hence, it is essential to develop methods which can make use of glycerol for the production of value added products.

### 2.5. Conversion of crude glycerol into value added products

Presently, crude glycerol obtained from biodiesel industries is mainly used as an animal feedstock or burnt as a low energy fuel [48]. Even though crude glycerol contains varieties of impurities, it has found to be a good energy source. Thompson and He (2006) carried out the nutrition content of crude glycerol and found to have protein, fats and carbohydrates in the range of 0.06–0.44%, 1–13%, 75–83% respectively [61]. Inclusion of certain amount of crude glycerol in the diets of ruminants and non-ruminants have thus shown to have beneficial effects. Crude glycerol samples can have a digestible energy values in the range of 1600-1650 kcal/lb with metabolizing energy values in the range of 1500-1580 kcal/lb [62]. Therefore, addition of

crude glycerol up to 6.7% in the diets of broilers have shown to improve the feed conversion ratio [63]. Similarly, feeding crude glycerol up to 15% to cattle significantly improved their weight and feed efficiency [48]. However, certain impurities present in crude glycerol can cause various negative effects in these animals. For example, presence of potassium impurities in crude glycerol has caused wet litter problems in broilers [64]. Presence of higher concentrations of methanol is toxic to animals. Thus, crude glycerol with lower amounts of impurities can only be used as an animal feedstock and must be studied in detail before its inclusion in animal feeds [62]. However, producing crude glycerol with low levels of impurities is not easy and economically feasible as multiple purifications steps are required.

Energy obtained by burning crude glycerol can be used to run the biodiesel process or another co-located systems. Glycerol is very viscous in nature, has high auto-ignition temperature and low heating value [59]. This makes burning crude glycerol technically complex, energy inefficient and economically unsuitable. Moreover, incomplete combustion of glycerol also releases highly toxic aldehydes (such as acrolein) thus making such process unsafe for humans and for the environment [65].

Due to problems discussed in the above paragraphs, many researchers have studied the conversion of crude glycerol into various value added chemicals and products [66]. The routes used to convert crude glycerol into useful chemicals or products can be generally categorized as (i) Chemical conversion routes and (ii) Biological conversion routes.

### 2.5.1. Chemical conversion routes

Different researchers have discussed chemical methods to convert crude glycerol into different value added compounds [53, 67–71]. Some of the common chemicals that have been obtained by this route include hydrogen, 1,2 propandiol, acrolein, dihydroacetone (DHA), glyceric acid, triacetin, monoglycerides, etc. [50].

Product yields of over 80% using chemical route have been previously reported. For instance, during the chemical conversion of crude glycerol to hydrogen or 1,2 propandiol overall yield of 88% and 90% respectively were achieved [67, 68]. However, most of these chemical processes

make use of expensive catalysts, faces the problems of catalyst deactivation, use of high pressures, temperatures and harmful chemicals [50, 69, 70, 72]. Different impurities (mainly methanol and water) present in crude glycerol have been shown to reduce the efficiency of catalyst used [70]. During the production of dioxlane and dioxane from crude glycerol, Wegenhart et al. (2012) reported the clumping of catalyst used (MCM-41) and decrease in its catalytic activity due to absorption of water present in crude glycerol [73]. Some of the impurities present in crude glycerol has also found to react with the synthesized final compound to produce undesirable by-products. For instance, Ilham and Saka (2016) found that during the production of glycerol carbonate from crude glycerol, glycerol carbonate (final product) reacted with water and salts present in crude glycerol to form glycidol. Formation of this undesirable by-products reduced the overall yield of glycerol carbonate from crude glycerol [74]. Table 2.3 shows varieties of compounds that can be potentially obtained from crude glycerol using various chemical pathways. It includes the yield and associated problems during such the conversion processes.

Table 2.3. Value added compounds obtained from crude glycerol using chemical processes and the related yields, conversion and associated problems associated

| Products | Crude glycerol composition (wt.%) | Method used | Operating conditions | Performance | General problems associated with the used method | References |
|---|---|---|---|---|---|---|
| Hydrogen | Glycerol 70-90<br>Methanol <15<br>Water <15<br>Inorganic salts < 5<br>Polyglycerol impurities < 5 | Steam reforming process | 525-600 °C; 1.09 h; Ni/Co catalyst | Y: 88% | • Heavy components in CG promote coke formation reducing the hydrogen yield<br>• High temperature required | [67] |
| 1,2 propandiol | Glycerol 80<br>FAMEs 20 | Hydrogenolysis | 240 $^0$C; 10 h; 40 bar; Cu: Zn:Cr:Zr mixed metal oxide | Y: 90% | • Not communicated<br>• In general, this route makes use of expensive catalysts and the salts present in CG typically poisons the catalyst used [75] | [68] |
| Acrolein | Glycerol 91.7<br>Methanol 3.8<br>Soap ~3.3<br>$Na^+$ 6470 mg/L<br>$Ca^{2+}$ 113 mg/L<br>$K^+$ 19.1 mg/L<br>$Al^{3+}$ 4 mg/L<br>$Mg^{2+}$ 2.4 mg/L | Dehydration | 335 $^0$C; 41 s; 172 bar; 45mM $H_2SO_4$ | Y: >80% | • Alkali residue present in CG neutralizes the acid used. Thus, high amount of acid is required | [69] |
| Dihydroxyacetone (DHA) | Glycerol 68.5<br>Sulphur 2.5<br>Phosphorous 0.2<br>Potassium 3.4<br>Sodium 0.1<br>Water ~ 18.8 | Oxidation | 60 $^0$C; 24 h; 1 atm; 1M NaOH; 600 rpm; $Au/TiO_2$ catalyst | C: 4.7%<br>S: 25% | • Deactivation of catalyst due to impurities in the crude substrate<br>• Technique is not reliable as no improvement in overall conversion was obtained even after removal of impurities in CG | [70] |
| Glyceric acid | Glycerol 40.3<br>Methanol 24.7<br>Water 7.4<br>Ash 6.6<br>MONG 45.7 | Oxidation | 60 $^0$C; 175 mins; 5 bar $O_2$; 1500 rpm; NaOH/Gly. 4; 1wt% $Pt/Al_2O_3$ Catalyst | C: 47.2% | • Mineral salts have negative effect on the oxidation process.<br>• Residual organic matter present in crude glycerol strongly hinders the reaction rates. | [76] |

| | | | | | |
|---|---|---|---|---|---|
| Triacetin | Glycerol 78<br>NaCl 8<br>KCl 3<br>Methanol 1<br>Water 10 | Esterification | 120 $^0$C; 4 h;1 atm;<br>Acetic acid/Gly 10;<br>6 wt.% HSiW/$ZrO_2$ catalyst | C: 99.6%<br>S: 30.5% | • Not Available | [77] |
| Monoglycerides | Glycerol 70<br>Monoglycerides 3.7<br>Sodium hydroxide 2 | Glycerolysis | 200 $^0$C;<br>Gly/Palm stearin 2.5:1;<br>20 mins | Y: 60.8%<br>P: 62.2% | • Impurities present in CG significantly reduced the yield and purity of the product | [78] |
| Dioxolane and dioxane | Glycerol 87<br>Impurities NC | Condensation | 100 $^0$C, 20 min,<br>Furfural/Gly 5<br>1% Aluminosilicate MCM-41 catalyst | Y: ≤ 1% | • Large amount of catalyst required.<br>• Reduced catalytic activity due to absorption of water present in CG by the catalyst | [50] |
| Glycerol carbonate | Glycerol 70<br>Water 9.82<br>Salt 20.18<br>Soap 35,100 ppm | Esterification | 300 °C; 20 MPa; 15 mins | Y: ~ 40% | • Water and salts present in CG partly decomposed the formed glycerol carbonate into glycidol thus reducing the overall yield | [74] |
| Polyesters | Glycerol 15.4<br>Soap 22.4<br>FFA 2.1<br>FAME 30.9<br>Methanol 5.1<br>Ash 4.2 | Polycondensation | 180 $^0$C; 250 rpm; 6 h;<br>Glycerol/succinic acid 1.9:1 | Y: 53.9% | • Impurities (mainly soap and free fatty acids) present in CG reduced the yield of the reaction and resulted in of product with different chemical structures as that obtained with pure glycerol | [53] |
| Glycerol ethers | NC | Etherification | 80 $^0$C; Catalyst loading > 5 wt%; 1-2 h;<br>Gly. / Isobutylene ~3. | NC | • Poisoning of catalyst by the impurities present in CG | [79] |

NC: Not Communicated; Gly: Glycerol; FFA Free fatty acids; C: Conversion; Y: Yield; S: Selectivity; P: Purity

### 2.5.2. Biological conversion routes

Biological processes do not need the starting reactant to be pure and is considered environmentally safe and cost effective in many cases. Various authors have thus explored the possibility of converting crude glycerol into different commercially useful compounds. Some of such compounds include hydrogen, 1,3 propanediol, n-butane, citric acid, trehalose, propanoic acid, ethanol, single cell oil, etc. [45]. All the biological conversion processes used for crude glycerol conversion make use of either bacteria, yeast, fungi or microalgae. *Klebsiella, Clostridium, Propionibacterium, Yarrowia, Candida, Rhodosporidium and Schizochytrium* are some of the microbes that can convert crude glycerol into useful compounds [43, 45, 80–84]. But their commercial feasibility is not easy. Table 2.4 lists some of the products that can be obtained from crude glycerol using different types of microorganisms.

Table 2.4. Bioconversion of crude glycerol into various value added products using different microorganisms

| Products | Crude glycerol composition | Microorganism | Species | Yield (g/g glycerol) | References |
|---|---|---|---|---|---|
| Hydrogen | Glycerol 1% (w/v)<br>Methanol Removed<br>Other impurities NC | Bacteria | *Thermotoga neapolitana* DSM 4359 | 0.029 | [85] |
| 1,3-Propanediol | Glycerol 55 wt.%<br>Fatty acids 1 wt.%<br>Chloride 31,408 ppm | Bacteria | *Clostridium butyricum* AKR102a | 0.514 | [86] |
| n-butanol | NC | Bacteria | *Clostridium pasteurianum* MTCC 116 | 0.35 | [87] |
| Trehalose | Glycerol 13.2 wt.%<br>Soap 0.4 wt.%<br>Ash 1.4 wt.% | Bacteria | *Propionibacterium freudenreichii subsp. Shermanii* NCIM 5137 | 0.104 | [84] |
| Propanoic acid | Glycerol 86 wt.%<br>(partially refined, desalinated and devoided of methanol) | Bacteria | *Propionibacterium freudenreichii ssp. shermanii 1* | 0.56 | [88] |
| Ethanol | Glycerol 80 -85% (w/v)<br>Methanol 0.5% (w/v)<br>Salts 5–6% (w/v)<br>Water 10% (w/v)<br>Nonglycerol organics 2% (w/v) | Bacteria | *Kluyvera cryocrescens* S26 | 0.80 | [89] |
| Succinic acid | Glycerol 42% wt.<br>Impurities NC | Bacteria | *Pasteurellaceae strain* DD1 | 1.20 | [90] |
| PHB | Glycerol 80 wt.%<br>Water 10.8 wt.%<br>Methanol < 0.01 wt.%<br>NaCl 5.5 wt.% | Bacteria | *Cupriavidus necator* JMP 134 | 0.14 | [91] |

| | | | | | |
|---|---|---|---|---|---|
| DHA | Glycerol 56.45-62.35 wt.%<br>Methanol 12.79-28.27 wt.%<br>Soap 15.28-25.17 wt.% | Microalgae | *Schizochytrium limacinum* SR21 | NC | [83] |
| Citric acid | Glycerol 65 wt.%<br>Methanol 3 wt.%<br>Water 26 wt.%<br>Heavy metals & lignin 1 wt.%<br>Potassium & sodium salts 4-5 wt.%<br>Other organic matter 0.5 wt.% | Yeast | *Yarrowia lipolytica* ACA-DC 50109 | 0.56 | [92] |
| Single cell oil | Glycerol 42.2% (w/v)<br>Impurities NC | Yeast | *Candida* sp. LEB-M3 | 0.33 | [82] |
| Single cell oil | Glycerol 44.56 wt.%<br>Methanol 13.86 wt.%<br>Ash 10.47 wt.%<br>FFA 0.48 wt.%<br>Soap 32.9 wt.% | Yeast | *Rhodosporidium toruloides* ATCC 10788 | 0.44 | [43] |
| Single cell oil | Glycerol 80 wt.%<br>Methanol 1 wt.%<br>Water 13.5 wt.%<br>Potassium & sodium salts 4 wt.%<br>Heavy metals & lignin 1 wt.%<br>NGOM 0.5 wt.% | Fungi | *Cunninghamella echinulata* ATHUM 4411 | 0.10 | [93] |
| Single cell oil | Glycerol 65 wt.%<br>Methanol 3 wt.%<br>Water 26 wt.%<br>Heavy metals & lignin 1 wt.%<br>Potassium & sodium salts 4-5 wt.%<br>Other organic matter 0.5 wt.% | Fungi | *Mortierella isabellina* ANTHUM 2935 | 0.08 | [92] |

NC: Not communicated; FFA: Free fatty acid; NaCl: Sodium Chloride; NGOM: Non-glycerol organic matter; DHA: Docosahexaenoic Acid; PHB: poly(3-hydroxybutyrate)

## 2.6. Problems associated in bioconversion of crude glycerol

During the bioconversion of crude glycerol, impurities (mainly methanol, salt and soap) present in it inhibit growth and product formation. For instance, during the production of phytase enzyme by *Pichia pastoris*, reduced cell density was due to inhibition by impurities present in crude glycerol [23, 94]. Methanol present in crude glycerol has been found to reduce the growth of *Schizochytrium limacinum* SR21 [46] and *Rhodosporidium toruloides* 32489 [95] during the conversion to microbial lipids.

Presence of saponified fatty acids (soap), a component in crude glycerol, in the fermentation media was found to inhibit the growth of *Rhodopseudomonas palustris* NCIMB 11774, thus reducing the overall yield of hydrogen produced by this strain [96]. Similar effects of soap were observed when microalgae was used for the production of docosahexaenoic acid (DHA) from crude glycerol [83].

During the anaerobic digestion of crude glycerol into biogas, the salts present in the former negatively affect the metabolism of microbes used [97]. Similarly, Mothes et al. (2007) reported the reduction in poly-3-hydroxybutyrate (PHB) production by *Paracoccus denitrificans* DSMZ 413 and *Cupriavidus necator* JMP 134, when crude glycerol contaminated with sodium chloride salt was used as a carbon source [91]. Fig 2.2 summarizes the effect of some of the impurities present in crude glycerol during its bioconversion into various products.

In order to address these issues, it is important to find a robust microbial strain that can grow in a harsh chemical environment and produce higher amount of commercially useful compounds is important. In addition, developing a process that can be easily integrated to an existing biodiesel plant would make such processes commercially viable. With respect to energy balances, Zhang et al. (2016) found crude glycerol conversion to microbial lipid is a better option when compared to its conversion to hydrogen, biogas or ethanol [98]. Hence, we decided to focus on the production of microbial lipids in our study.

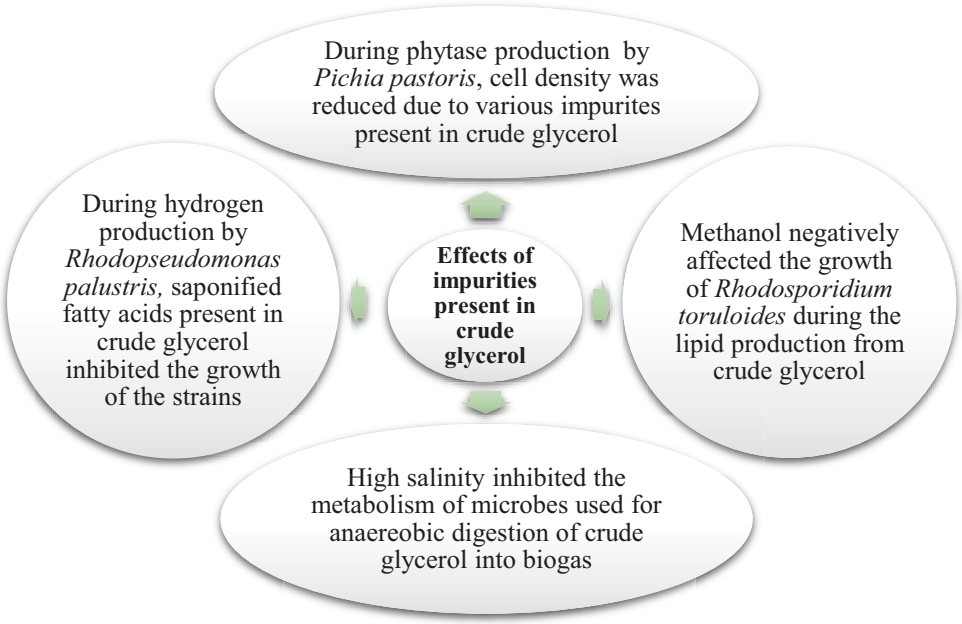


Fig 2.2. Some known effects of impurities on the bioconversion of crude glycerol

## 2.7. Microbial lipids and its production from crude glycerol

Microbial lipids (single cell oil, SCO) are obtained from the oleaginous microorganisms such as bacteria, fungi, and algae. If a microorganism accumulates lipids to levels higher than 20% of its dried cell biomass, it is known as an oleaginous microbe. The obtained lipid from oleaginous strains are chemically similar to vegetable oils and can be converted into biodiesel and biopolymers [99–101]. Hence, microbial lipids are considered as potential alternatives to the plant and animal based oil [102]. Unlike plant and animal oil, microbial lipid production does not require the use of huge arable lands and also poses less risk as they are not dependent on agricultural practices, weather and has no effect on food security [43, 103].

Most of the eukaryotic cells such as fungi, yeast, plants, microalgae and animals accumulate lipid in the form of triacylglycerol or TAG [14, 104, 105]. TAG's plays an important role in intracellular energy storage and energy balance. Microbial lipid (single cell oil or microbial oil) production from crude glycerol is an attractive concept as such processes can easily be integrated to present biodiesel production plants, thus saving transportation cost and startup costs [43]. Production of biopolymers from such oil can add to revenues of the industries involved. Some of the bacteria belonging to actinomycetes group accumulate up to 70% of

intracellular lipids inside them. However, most other bacteria store their energy in the form of complex lipoids, such as polyhydroxyalkanoic acids [14, 103]. Thus, many fungi, yeast or microalgae have been explored for their ability to grow on crude glycerol and produce lipids. Details of this will be discussed in the following sections.

### 2.7.1. Microalgae

Certain microalgae grow fast and accumulate large amount of lipid and thus explored for their ability to consume crude glycerol. The quantity of lipid accumulated by microalgae varies between species. Additionally, other factors that affects the lipid production by microalgae includes media compositions, irradiance and temperature [106]. Among the different microalgae species, *Schizochytrium limacinum* SR21 has been studied the most for its use in crude glycerol bioconversion [46, 83, 107–109]. The microbial lipid obtained from this strain was found to be rich in Docosahexaenoic acid (C22:6; DHA), a type of polyunsaturated fatty acid (PUFA), which is of considerable commercial interest. Chi et al. (2007) reported a maximum of 33.62% of DHA in the obtained microbial lipid. *Schizochytrium limacinum* SR21 is also capable of growing in harsh environmental conditions posed by crude glycerol. While growing in a crude glycerol (23 g/L) containing high concentration of methanol (22.7%) and soap (3%) and other impurities, this strain was reported to accumulate approximately 65.8 wt.% of lipid at the end of incubation. O'Gardy and Morgan (2011) and [57] explored the ability of *Chlorella protothecoides* UTEX 256 to produce microbial lipid from crude glycerol. Chen and Walker (2011) also studied the lipid production ability of this strain using batch and fed-batch fermentation with crude glycerol as carbon source. With batch fermentation 23.5 g/L of biomass containing 62% of lipid was obtained whereas 45.1 g/L of biomass with 54% of lipid was obtained when fed-batch was carried out. Even though the lipid content was less when fed-batch was carried out, the lipid productivity (g/L-day) was higher with this approach. Besides these two microalgae, Choi and Yu (2015) studied the ability of *C. vulgaris* (KMMCC-355), *B. braunii* (KMMCC-1681) and *Scenedesmus sp.* (KMMCC-1235) to grow on crude glycerol from biodiesel industry. The obtained lipid from these strains were predominantly richer in oleic acid, a monounsaturated fatty acid (MUFA). Such lipid can be fed back into the biodiesel plant as feedstocks rich in MUFA are quite suitable for biodiesel production [43].

However, microbial lipid production using microalgae have some drawbacks such as the need for phosphorus for their growth, high upfront capital costs, need for considerable large amounts of land and water and the requirement to maintain temperature conditions [14, 112–114]. All together these requirements have made the process less competitive and uneconomical. This might be one of the reasons for fewer algal strains (compared to yeast and fungus) to have been studied for microbial lipid production using crude glycerol as a sole carbon substrate. Table 2.5 summarizes the advantages and disadvantages of using oleaginous microbes including microalgae for the production of microbial lipids from crude glycerol.

Table 2.5. Advantages and disadvantages of utilizing different types of microbes to produce lipids from crude glycerol

| Microbes | Advantages | Disadvantages |
|---|---|---|
| Bacteria | • Highest growth rate<br>• Involves easy culture method<br>• Some actinomycetes spp. can accumulate up to 70% of lipid | • Very few produce oils suitable for biodiesel productions.<br>• Instead of lipid, most of the bacteria accumulate complicated lipoid (i.e. polyhydroxyalkanoates) |
| Microalgae | • Fast grower and produce huge amount of biomass and lipid.<br>• Utilize carbon-dioxide thus reducing the greenhouse gases<br>• Live in harsh conditions due to their unicellular or simple multicellular structure | • Need to be grown under controlled temperature conditions<br>• Requires phosphorus as a fertilizer which is becoming scarce<br>• Relatively high upfront capital costs<br>• Requires a considerable amount of land and water.<br>• Presently cost of production is too high. |
| Fungi (molds) | • Can withstand harsh growth conditions<br>• Accumulate significant quantity of lipids predominantly rich in triacylglycerol | • Filamentous fungi can stick to the wall of bioreactor during the fermentation.<br>• Slow growers compared to other oleaginous microbes |
| Yeast | • Grows fast and does not require especial growth requirements like microalgae<br>• Can accumulate high quantities of lipids | • More robust strains are required |

### 2.7.2. Fungi

Fungi have the capacity to produce a variety of products. Endophytic fungi are presently being explored for their ability to produce various useful products [115]. Apart from enzymes, vitamins, nutraceuticals, primary and secondary metabolites, fungi can produce single cell oil and low molecular mass hydrocarbons (i.e. mycodiesel) which could be used as alternative sources of fuels [116–119]. Filamentous fungi can withstand harsher environmental condition and accumulate high quantity of intracellular lipids. This makes them a suitable candidate to convert crude glycerol to microbial lipid. Various authors have reported the use of *Mortierella isabellina, Mortierella alpine, Cunninghamella echinulate, Thamnidium elegans, and Aspergillus niger* for the conversion of crude glycerol from biodiesel industry. The lipids accumulated by these fungal species range between 41.4 to 71.1 wt.%. In shake flask experiments carried out by Chatzifragkou et al. (2012), *Thamnidium elegans* CCF 1465 utilized crude glycerol as a carbon source and was found to accumulate 16.3 g/L of biomass with a lipid content of 71.1 wt.% [120]. Similarly, certain *Aspergillus* species have shown to be tolerant to high concentration of salts and other impurities present in crude glycerol. When *Aspergillus niger* LFMB 1 and *Aspergillus niger* NRRL 364 was grown on a crude glycerol (70 wt.% glycerol, 12 wt.% of salts, non-glycerol organic material 1 wt.%, methanol 2 wt.% and water 14 wt.%) with higher amount of salts, biomass with lipid content of 57.4 wt.% and 41.4 wt.% respectively were obtained at the end of fermentation [121]**.** The obtained lipids were mainly rich in oleic and linoleic acids which makes them suitable for their use in fuel and food industries. However, compared to yeast and microalgae, fungi are slow growers. Additionally, due to the filamentous nature of molds (a type of fungi), they can stick to the walls and probes of bioreactors and create uncalled for issues during the fermentation and separation of intracellular lipids [122]**.**

### 2.7.3. Yeast

Yeasts are a type of single cell fungus which are widely distributed in natural environment and are capable of growing in under extreme environmental conditions such as low temperature and low oxygen levels [123]. Many yeasts have been reported to accumulate significant quantities of lipids (more than 20%) and are thus considered to be oleaginous microbes. Yeasts can grow faster than other group of fungus (mainly molds) thus enhancing the lipid productivity. They do

not require special growth conditions like algae (requirement of light source and phosphorus) which can be expensive. Various oleaginous yeasts belonging to the genera *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus and Lipomyces* have been studied for their ability to use crude glycerol as a carbon source. Cells containing a lipid up to 69.5 wt.% have been reported for *Rhodosporidium toruloides* AS2.1389, while growing in a crude glycerol [124]. Liang et al. (2010) used *Cryptococcus curvatus* ATCC 20509 to consume crude glycerol containing glycerol, soap, methanol, and water in the percentages of 48.7, 3.0, 22.7, and 25.6 wt.% respectively [52]. In order to reduce the effect of impurities (present in crude glycerol) on the growth of this strain, experiments were carried out in a two-stage fed -batch operation. At the end of 12 days, 32.9 g/L of biomass containing 52 wt.% of lipids were obtained. Furthermore, *Yarrowia lipolytica* QU 21, *Trichosporondoides spathulata* JU4-57, *Lipomyces starkeyi* DSM 70296, *Candida* spp. LEB-M3, *Trichosporon fermentans* CICC 1368, *Trichosporon cutaneum* AS 2.0571 and *Kodamaea ohmeri* BY4-523 have been studied for their abilities to produce lipids from crude glycerol as a carbon source [125–129]. Recently, we reported a robust yeast strain, *Rhodosporidium toruloides* ATCC 10788, capable of growing on crude glycerol with high concentration of soap (32.97 wt.%) and methanol (13.86 wt.%) to produce 21.16 g/L of biomass containing 53.28 wt.% of lipid at the end of 7 days [43]. The lipid obtained was predominantly rich in oleic acid (47.16 %), making it suitable for its use in oleochemical and polymer industries. After a few initial experiments with other organisms, we decided to work with this species in our studies. Different oleaginous microorganisms capable of utilizing crude glycerol to produce lipid has been listed in Table 2.6.

Table 2.6. Biomass and lipid concentration of various oleaginous microbes grown on crude glycerol as a carbon source

| Strains | Microbes type | Culture mode | Biomass concentration (g/L) | Lipid content (wt.%) | References |
|---|---|---|---|---|---|
| *Rhodosporidium toruloides* NRRL Y-27012 | Yeast | Shake flask | 30.1 | 40.0 | [127] |
| *Rhodosporidium toruloides* AS2.1389 | Yeast | Batch | 26.7 | 69.5 | [124] |
| *Cryptococcus curvatus* ATCC 20509 | Yeast | Fed batch | 32.9 | 52.9 | [52] |
| *Yarrowia lipolytica* QU 21 | Yeast | Shake flask | 3.85 | 22.1 | [125] |
| *Trichosporondoides spathulata* JU4-57 | Yeast | Fed batch | 13.8 | 56.4 | [126] |
| *Lipomyces starkeyi DSM 70296* | Yeast | Shake flask | 34.4 | 35.9 | [127] |
| *Candida sp.* LEB-M3 | Yeast | Shake flask | 19.7 | 50.2 | [128] |
| *Trichosporon fermentans* CICC 1368 | Yeast | Shake flask | 16.0 | 32.4 | [129] |
| *Trichosporon cutaneum* AS 2.0571 | Yeast | Shake flask | 17.4 | 32.2 | [129] |
| *Kodamaea ohmeri* BY4-523 | Yeast | Shake flask | 10.3 | 53.3 | [126] |
| *Rhodosporidium toruloides* ATCC 10788 | Yeast | Shake flask | 21.16 | 53.28 | [43] |

| | | | | | |
|---|---|---|---|---|---|
| *Mortierella isabellina* ATHUM 2935 | Fungus | Shake flask | 8.5 | 51.7 | [92] |
| *Cunninghamella echinulate* ATHUM 4411 | Fungus | Shake flask | 7.8 | 25.6 | [93] |
| *Thamnidium elegans* CCF 1465 | Fungus | Shake flask | 16.3 | 71.1 | [120] |
| *Mortierella alpine* LPM 301 | Fungus | Shake flask | 15.6 | 33.3 | [130] |
| *Aspergillus niger* LFMB 1 | Fungus | Shake flask | 5.4 | 57.4 | [121] |
| *Aspergillus niger* NRRL 364 | Fungus | Shake flask | 8.2 | 41.4 | [121] |
| *Schizochytrium limacinum* SR21 | Microalgae | Shake flask | 13.1 | 71.1 | [46] |
| *Schizochytrium limacinum* SR21 | Microalgae | Batch | 18.0 | 51.0 | [107] |
| *Chlorella protothecoides* UTEX 256 | Microalgae | Batch | 23.5 | 62.0 | [57] |
| *Scenedesmus sp.* KMMCC-1235 | Microalgae | Batch | 60.0 | 16.24 | [111] |
| *Botryococcus braunii* KMMCC-1681 | Microalgae | Batch | 57.82 | 16.41 | [111] |

### 2.7.4. Biochemistry of lipid production

Even though there are similarities in fatty acid biosynthesis pathway among eukaryotes, there are some crucial differences between oleaginous and non-oleaginous organisms. The biochemistry of lipid accumulation within the oleaginous fungus and yeasts are quite similar. However, in microalgae, a slight difference in lipid accumulation mechanism exists. Details on pathways for algal oil production has been discussed in literature [131–133]. Here, we will discuss the mechanism of lipid production in oleaginous yeasts and fungus.

Oleaginous and non-oleaginous microbes mainly differ in their ability to direct the consumed carbon source towards the production of lipid at nutrient limited condition, mainly nitrogen [134]. When oleaginous microbes are grown in a media with excess of carbon but nitrogen limited conditions, it continues to assimilate available carbon source and directs it towards the production of lipids. However, a non-oleaginous organism under similar growth conditions would either stop further proliferation of cells or directs the excess carbon towards the production of polysaccharides such as glycogen, glucan and mannans but not towards lipids. Oleaginous microbes usually possess two main attributes [105, 134, 135] :

I. They should continuously produce Acetyl-CoA (a precursor for fatty acid synthesis) and
II. Have sufficient supply of NADPH reductant from malic acid or similar alternative NADPH generating sources.

In case of oleaginous microbes, isocitrate dehydrogenase, a component of TCA cycle, is uniquely dependent on adenosine monophosphate (AMP). The concentration of AMP is regulated by the activity of enzyme known as AMP deaminase. Under nitrogen limited condition, as there is no nitrogen source in the media, the cell tries to get the nitrogen source (in the form of ammonia) from within the cell by converting AMP into inosine monophosphate (IMP) and free ammonia. This reduces the amount of AMP in the cytosol and mitochondrion [136]. Due to reduction in the amount of AMP, the activity of isocitrate dehydrogenase (which is linked to the presence of AMP) also decreases, thus leading to a higher accumulation of isocitrate in the TCA cycle (inside mitochondrion). The higher amounts of isocitrate equilibrate with the citrate making a higher concentration of citric acid across the mitochondrion. This then enters the cytosol and is cleaved by ACL to give acetyl-CoA and oxaloacetate. In this manner,

the cell constantly receives acetyl-CoA which then enters the fatty acid biosynthesis pathway [134–136]. A simplified schematic diagram of this process is shown in Fig 2.3.

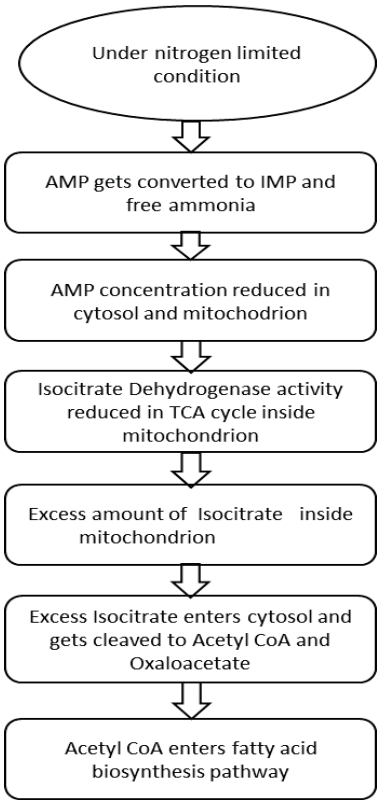


Fig 2.3. Schematic diagram to show the continuous production of Acetyl-CoA by oleaginous yeast and fungi under nitrogen limited conditions

Generally, malic enzyme present in most oleaginous microbes are responsible for continuous supply of NADPH required during fatty acid biosynthesis. NADPH is an essential reductant in fatty acid biosynthesis and is used to reduce 3-keto- fatty acyl group produced by condensation reaction between acetyl-CoA with malonyl-CoA. NADPH is produced by malic acid by converting $NADP^+$ into pyruvate, carbon dioxide and NADPH. However, malic acid is absent in some of oleaginous microorganisms (such as *Lipomyces* spp. and *Candida* spp.). Under such circumstances, it is believed that NADPH is produced by alternative NADPH generating enzymes such as cytosolic NAPDH-dependant isocitrate dehydrogenase [134]. The end-product of fatty acid biosynthesis is commonly saturated C16 or C18 which is then modified into various unsaturated and polyunsaturated fatty acids through a series of activities of elongases and

desaturases enzymes [136]. Palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic acid (C18:2) are the common fatty acids present in lipid obtained from oleaginous microbes [134, 136]. Conversion of C16:0 to C18:0, C18:0 to C18:1, C18:1 to C18:2 are catalyzed by enzyme elongase, Δ9 desaturase and Δ12 desaturase respectively [134].

### 2.7.5. Fatty acids profile of microbial lipids

The lipids produced by microbes vary widely depending on the types of species and strains used. The commercial applications of lipids obtained from oleaginous microbes depend on the fatty acid composition of the obtained lipids. Triacylglycerol (TAG) is the most common lipid obtained from most of the oleaginous microbes as it is a primary storage form of carbon and energy [137]. In oleaginous microbes two type of lipids are mainly present i.e. polar and non-polar (neutral). Polar lipids mainly make up the extracellular components of these organisms [137, 138] and store energy in the form of neutral lipids, which are found to be suitable for biodiesel production [105, 139]. The amount and types of neutral lipids accumulated by these microbes also varies with species, strain and culture conditions. For example, in oleaginous yeast, TAG and/or sterol esters (SE) are the primary neutral lipids accumulated inside the cell, whereas in specific types of bacteria and archaea, smaller amounts of TAG are accumulated, with polyhydroxyalkanoates (PHA) being the major compound produced [105]. Similarly, oleaginous microalgae contain both neutral and polar lipids. Under stressed conditions, certain microalgae can convert polar lipid to its neutral form [137]. Fatty acids present in the microbial lipid range from lauric acid (C12:0) to docosahexaenoic acids (C22:6). However, as mentioned in section 2.7.4., the major fatty acids present include palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic acid (C18:2). Generally, lipids obtained from yeast and fungus are rich in polyunsaturated fatty acids with oleic (18:1), linoleic (18:2) acids and palmitoleic acids (C16:1) being the common constituents [14]. Certain strains of microalgae can accumulate lipid richer in eicosapentaenoic acid (C20:5; EPA) and docosahexaenoic acid (C22:6; DHA) which have high nutritional value [140, 141]. Table 2.7 shows the fatty acids compositions of lipid obtained from crude glycerol using different oleaginous microbes.

Table 2.7. Fatty acid composition of lipids obtained from various oleaginous microbes using crude glycerol as carbon source and comparison with vegetable oils

| Name | Type | % C16:0 | % C16:1 | % C18:0 | % C18:1 | % C18:2 | % C18:3 | % Others | References |
|---|---|---|---|---|---|---|---|---|---|
| *Rhodosporidium toruloides* Y4 | Yeast | 20.1 | - | 12.7 | 55.3 | 11.9 | - | - | [125] |
| *Rhodosporidium toruloides* AS2.1389 | Yeast | 29.2 | - | 13.9 | 41.4 | 10.4 | 2.9 | 2.2 | [124] |
| *Rhodosporidium toruloides* NRRL Y-27012 | Yeast | 35.4 | - | 12.2 | 44 | 2.4 | - | 6 | [127] |
| *Cryptococcus curvatus* ATCC 20509 | Yeast | 23 | 0.9 | 16.7 | 39.6 | 15.2 | 0.66 | 3.94 | [52] |
| *Yarrowia lipolytica* QU 21 | Yeast | 15.52 | 5.49 | 17.49 | 52.52 | 6.49 | - | 2.49 | [142] |
| *Trichosporondoides spathulata* JU4-57 | Yeast | 35.1 | 2.1 | 7.5 | 41.1 | 11.2 | - | 3.0 | [126] |
| *Trichosporon fermentans* CICC 1368 | Yeast | 27.3 | - | 11.1 | 46.8 | 12.2 | - | 2.6 | [129] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Trichosporon cutaneum* AS 2.0571 | Yeast | 24.6 | - | 12.6 | 47.1 | 13.9 | - | 1.9 | [129] |
| *Candida sp.* LEB-M3 | Yeast | 19.2 | 7.45 | 3.73 | 56.43 | 9.15 | 0.85 | 3.2 | [143] |
| *Rhodosporidium toruloides* ATCC 10788 | Yeast | 24.39 | - | 16.38 | 47.16 | 12.05 | - | 0.02 | [43] |
| *Mortierella isabellina* ATHUM 2935 | Fungus | 22.0 | 4.2 | 4.3 | 50.0 | 16.7 | 3.7 | - | [92] |
| *Mortierella ramanniana* MUCL 9235 | Fungus | 21.6 | - | 4.1 | 48.8 | 19.3 | 5.1 | 1.1 | [144] |
| *Thamnidium elegans* CCF 1465 | Fungus | 23.1 | 4.1 | 5.1 | 55.3 | 9.9 | 2.4 | 0.1 | [120] |
| *Mortierella alpine* LPM 301 | Fungus | 15.3 | - | 21.1 | 14.4 | 41.4 | 5.3 | 3.1 | [130] |
| *Schizochytrium limacinum* SR21 | Microalgae | 54.70 | - | 1.04 | - | - | - | 44.26* | [107] |
| *Botryococcus braunii* KMMCC-1681 | Microalgae | 13.23 | 2.49 | 5.84 | 48.36 | 19.73 | 7.03 | 3.32 | [111] |
| *Scenedesmus sp.* KMMCC-1235 | Microalgae | 15.04 | 2.10 | 2.85 | 47.05 | 17.65 | 11.12 | 4.19 | [111] |
| Palm | Vegetable oil | 42.70 | - | 2.13 | 39.37 | 10.62 | 0.21 | 4.97 | [43] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Canola | Vegetable oil | 3.75 | 0.21 | 1.87 | 62.41 | 20.12 | 8.37 | 3.27 | [43] |
| Corn | Vegetable oil | 10.34 | - | 2.04 | 25.54 | 59.27 | 1.07 | 1.74 | [43] |
| Olive | Vegetable oil | 11.8 | 1.5 | 2.7 | 74.2 | 8.5 | 0.7 | 0.6 | [143] |
| Sunflower | Vegetable oil | 6.2 | 0.1 | 3.7 | 25.2 | 63.1 | 0.2 | 1.5 | [145] |

*5.16% Myristic acid (C14:0) + 5.48% Docosapentaenoic acid (C22:5; DPA) + 33.62% Docosahexaenoic acid (C22:6; DHA)

### 2.7.6. Methods of lipid extraction from cell biomass

Separation of lipids from cell biomass consist of two steps (i) cell disruption and (ii) extraction. Several researchers have explored different mechanical and non-mechanical cell disruption methods to extract lipid from biomass [146]. Use of mechanical methods such as bead milling, homogenization, ultrasonication and non-mechanical methods such as osmotic shock, microwave treatment, drying, use of acids and enzymes have been previously reported [147–153]. Similarly, extraction using classical methods, pressurized method and supercritical fluids have been studied [154–157]. Details of these methods are discussed in a recent review [146]. The most commonly used extraction techniques to obtain lipid from cell biomass will be discussed here. These include Soxhlet extraction, Folch et al. extraction and Bligh-Dyer method for lipid extraction.

The Soxhlet extraction method is a classical technique used to obtain lipid from microalgae and yeast biomass [135, 158–160]. This method requires a Soxhlet apparatus which is suitable for solid-liquid extraction. The extraction takes place by continuous washing of the sample by an extractant. Use of Soxhlet extractor is one of the oldest methods to extract lipids. However, the methods reported by Folch et al. (1957) and Bligh and Dyer (1959) to extract lipids from biological sources including oleaginous microbes are also gaining acceptance [161, 162]. In 1957, Folch et al reported a method to extract lipid from animal tissues. The method makes use of chloroform, methanol and water mixture (8:4:3) as an extraction solvent to obtain lipid from a homogenised sample. The ratio of sample to extractant was 1:20 [161]. This method was adapted and modified by various authors to extract lipid from different sources [135, 156]. Bligh and Dyer (1959) suggested another important alternative method for the extraction of lipid from biological sources [143, 162, 163]. The method is similar to Folch et al. (1957) but uses different ratios of chloroform, methanol and water. It also uses smaller amounts of sample to solvent ratio compared to the Folch et al (1957) method. In the Bligh and Dyer (1959) method, the sample needs to have 80% of water and thus water can be added to obtain the required ratio [135, 162]. The method makes use of chloroform, methanol and water in the ratio of 2:2:1.8 (after dilution of sample). The ratio of sample to solvent used during extraction is lowered to 3 as compared to 20 in case of Folch et al.'s method, thus reducing the amount of solvent used [135, 156].

### 2.7.7. Hurdles in commercial production of microbial lipid from crude glycerol

One of the major problems in commercial production of microbial lipids from crude glycerol is the variation in crude glycerol composition. As discussed above (section 2.4), the chemical composition of crude glycerol differs from batch to batch and amongst different biodiesel producers. Hence, a suitable method for crude glycerol obtained from one biodiesel producer, might have to be re-tuned for crude glycerol from another source. Secondly, fluctuating prices of vegetable oils and fossil based resources have a huge impact on the economics of production of microbial lipid from biodiesel derived crude glycerol. However, with the increase in demand of energy and food to meet the need of the growing world population, the prices of fossil fuel and vegetable oils is expected to grow immensely in the coming years [164, 165]. Under such circumstances, microbial lipids will certainly be a useful alternative to vegetable oils for biodiesel production.

Another major problem in microbial lipid production from crude glycerol is the low productivity due to lack of robust oleaginous strain that can withstand impurities present. Such problems could be solved by screening or adapting for more robust oleaginous strains or through the development of genetically engineered species, metabolic engineering and molecular biology.

### 2.7.8. Potential applications of microbial lipids

Lipids obtained from various microbial sources can be used as feedstocks in biodiesel industries, oleochemical industries, food and pharmaceutical industries. Some of the oleaginous yeast strains have been found to withstand the inhibiting effect of impurities present in crude glycerol and accumulate considerable quantity of lipids. We reported a strain of *Rhodosporidium toruloides* which can grow in crude glycerol media containing as high as 13.83% wt. of methanol and 32.97 wt.% of soap. The strain was reported to accumulate ~53.26 wt.% of lipid. In the referred publication, we have also discussed the unique ability to consume some of the impurities present in crude glycerol as a carbon source [43]. Similarly, Liang et al (2010) reported the ability of *Cryptococcus curvatus* ATCC 20509 to grow in crude glycerol and accumulate up to 52 wt.% of lipids in a two-step fed batch process [52]. Methanol present in crude glycerol did not have much inhibitory effect on this strain. The obtained lipids were found to be rich in monounsaturated fatty acids, thus making them suitable for use in biodiesel

production [48, 52]. Similarly, Duarte et al. (2013) [128], Saenge et al. (2011) [166], Chatzifragkou et al. (2011) [120] and Chen and Walker (2011) [57] have reported the use of *Candida* spp. (yeast), *Rhodotorula glutinis* TISTR 5159 (yeast), *Thamnidium elegans* CCF 1465 (fungi) and *Chlorella protothecoides* UTEX 256 (microalgae) respectively to convert crude glycerol to lipids suitable as biodiesel feedstocks.

Lipids from various microalgae and fungi are mostly rich in polyunsaturated fatty acid (PUFA) which makes them suitable for their use in food and pharmaceutical industries. Chatzifragkou et al. (2011) reported the ability of *T. elegans* CCF 1465 to produce lipid rich in linolenic acid (GLA), a type of PUFA [120]. The fungal strain was able to consume 90 g/L of crude glycerol and produce 371 mg/L of GLA. Similar abilities have been reported for certain strains of *Cunninghamella* and *Mortierella* [93]. Using crude glycerol as a substrate, *Cunninghamella echinulata* ATHUM 4411 and *Mortierella isabellina* ATHUM 2935 were able to produce 190 mg/L and 116 mg/L of GLA respectively [93]. Bioconversion of crude glycerol to docosahexanoic acid (DHA) using *Schizochytrium limacinum* SR21 and eicosahexanoic acid (EPA) using *Pythium irregular* have been reported by Chi et al. (2007) [107] and Athalye et al. (2009) [167] respectively. Both DHA and EPA are types of omega-3 fatty acids. Omega-3 fatty acids are a type of PUFA which provides health benefits to the people suffering from cancer, Alzheimer's and cardiovascular disease. Similarly, inclusion of DHA in infant's diets is very important to ensure their normal development [146]. At present, fish oil is the major source of DHA and EPA [168]. Thus, production of PUFA from algal and fungal source could be of great interest for vegetarians across the globe.

As mentioned previously, the lipids obtained from oleaginous microbes are chemically similar to vegetable oils. Thus, their potential use as an alternative to vegetable oil to produce biopolymer has also been explored. Petrović et al. (2013) [100] and Pawar et al. (2016) [99] reported the conversion of algal oil to polyols suitable for polyurethane production. Conversion of crude glycerol into microbial lipid using *R. toruloides* ATCC 10788 and its subsequent conversion to polyurethane has been successfully carried out in our lab [101]. Thus, microbial lipid produced from cheaper substrate such as crude glycerol is of commercial interest as it has number of possible applications.

## 2.8. Present and future scope of conversion of crude glycerol to microbial lipid

Very few companies are involved in the commercial production of microbial lipids from any carbon sources. Commercial production of PUFA as an alternative to expensive evening primrose oil began in 1985 using *Mucor cicinelloides* [134]. However, the process became cost-inefficient and the company had to shut down six years down the line. This might be one of the reasons why many industries are still apprehensive about commercial production of microbial oil. Additionally, the fluctuating prices of agriculture products and oleaginous chemicals are also factors which deter companies from investing on research in single cell oil production. At present, most of the companies working towards the production of microbial lipids have focused their interest on using algal strains and glucose as a carbon source. However, use of glucose as a starting material makes the overall process economically inefficient [170]. Hence, cheap alternative feedstocks should be used to make the process commercially feasible. Some of the companies working towards the production of microbial lipids are listed in Table 2.8.

Table 2.8. List of some of the algal fuel producers and technology providers across the world

| Name | Company Type | Location | References |
|---|---|---|---|
| Neste Oil | Microbial lipid producer | Finland | [171] |
| British Algoil | Technology provider | Britain | [172] |
| Buggypower | Produces food, feed, cosmetics and nutraceuticals from algal biomass | Portugal | [173] |
| Pond Biofuels | Technology provider | Canada | [174] |
| Algaeneers Inc. | Algal oil with customized properties | Canada | [175] |
| Algae Farms | Producer of algae pellets, oil and briquettes | Greece | [176] |
| Sapphire Energy | Algal oil producers | USA | [177] |

The search for alternative sources of carbon such as glycerol from biodiesel industry, cellulose from lignocellulosic biomass and waste have been explored by different researchers [178].

However, most experiments have been restricted to a lab scale. Considerable research involving the genetic manipulation at molecular level is also being done at present [179–182]. However, no work has reached the commercial scale. This can be due to insufficient understanding of microbial lipid production and control at a molecular level. However, with more researchers focusing on ways to control the biochemical pathways of these oleaginous strains [104, 183–185], the scenario is expected to change in the near future.

At present, compared to the use of microbial lipid, vegetable oils feedstocks for biodiesel production is cost effective. However, the price of lipids from plant and animal keeps fluctuating [186]. Recently, the cost of vegetable oils has increased and is expected to increase even further in the future [164]. There will be a competition between vegetable oils for food and biodiesel production. When that situation arises, the use of microbial lipid as an alternative to vegetable oils will certainly become feasible. Additionally, with the increasing demands of fuels across the world and the deterioration of climate due to use of fossil fuels [43], biodiesel production is expected to grow in the future as well. Under such circumstances, lipids from microbes will be required to meet the demand.

Furthermore, cost of microbial lipids production depends on various factors including type of species used, obtained biomass concentration and lipid content within such biomass [187]. The selection of either algae, fungus or yeasts to produce such oils with regards to economics will depend on the environmental condition in which it is proposed to be produced. For instance, in cold countries like Canada, oil production from algal biomass might be expensive as such process must be undertaken in closed photo-fermenters. Both the installation and running cost of such equipment are very expensive when compared to the growth requirements of yeast based system [187, 188]. However, if we think of tropical countries like India, Brazil and certain parts of USA, it would be better idea to produce oil from algal biomass than from other microbes as open race-track cultivation of such microbes may be cheap and feasible. There is a need for techno-economic evaluation of oil production from microbes using crude glycerol as a carbon source to be carried out taking these parameters into account. Assessment of economic feasibility of microbial oil production using glucose as a carbon substrate has been done by Koutinas et al. (2014) [170] and Ratledge and Cohen (2008) [189]. The result showed

commercial production from glucose to be economically non-feasible. However, the authors also support the idea of its production from cheaper feedstocks such as crude glycerol, waste effluents, lignocellulosic biomass, etc. as they are likely to make the process commercially viable. It is worthwhile mentioning that all such work takes into account phase out of fossil fuels due to its effect on the environment in the short term and its depletion in the long term.

### 2.9. Summary

Due to increased biodiesel production, crude glycerol has started to accumulate in large quantities. Conversion of crude glycerol has become important both from an economic and environmental point of view. Bioconversion of crude glycerol into microbial lipids is one of the options to valorize crude glycerol. Various fungus, yeast and microalgae have been explored for their ability to convert crude glycerol to lipids. Crude glycerol from different biodiesel industries varies in their chemical compositions. Besides, this by-product also contains methanol, soap and salts as major impurities. The impurities present in crude glycerol inhibit the growth of microorganisms used for lipid production. This subsequently reduce the overall lipid produced from the crude glycerol used. Thus, a robust strain capable of growing in crude glycerol with varying concentrations of impurities is important for biodiesel producers. Microbial lipids obtained from crude glycerol have various applications in food, pharma and oleochemical industries. Most of the studies carried out in the past focus on the conversion of microbial lipid into biodiesel. Recently, use of such lipid for the production of polyols and subsequent conversion to bioplastics have also been explored. Currently, microbial lipid from crude glycerol cannot compete with vegetable based oils as a feedstock for biodiesel. However, with the increasing cost of vegetable oil and rising demands for biodiesel production, there is a possibility of spiraling increase in vegetable oil prices. The use of alternative resources for vegetable oil will then be needed to bridge the gap.

**References**


1. Zabeti M, Daud W, Aroua M (2009) Activity of solid catalysts for biodiesel production: A review. Fuel Process Technol 90:770–777. doi: 10.1016/j.fuproc.2009.03.010
2. Guldhe A, Singh B, Mutanda T, et al (2015) Advances in synthesis of biodiesel via enzyme catalysis: Novel and sustainable approaches. Renew Sustain Energy Rev 41:1447–1464. doi: 10.1016/j.rser.2014.09.035
3. Bharathiraja B, Chakravarthy M, Kumar RR, et al (2014) Biodiesel production using chemical and biological methods – A review of process, catalyst, acyl acceptor, source and process variables. Renew Sustain Energy Rev 38:368–382. doi: 10.1016/j.rser.2014.05.084
4. Bryan R. Moser (2009) Biodiesel production, properties and feedstocks. Vitr CellDevBiol—Plant 45:229–266. doi: 10.1007/s11627-009-9204-z
5. Chakrabarti PP, Prasad RBN (2012) Biodiesel Production from Jatropha curcas Oil. In: Carels N, Sujatha M, Bahadur B (eds) Jatropha, Challenges a New Energy Crop. 1. Springer Science & Business Media, New York, NY, p 482
6. Akoh CC, Chang S-W, Lee G-C, Shaw J-F (2007) Enzymatic Approach to Biodiesel Production. J Agric Food Chem 55:8995–9005. doi: 10.1021/jf071724y
7. Joshi H, Toler J, Moser BR, Walker T (2009) Biodiesel from canola oil using a 1 : 1 molar mixture of methanol and ethanol *. Eur J Lipid Sci Technol 111:464–473. doi: 10.1002/ejlt.200800071
8. Zhang Y, You H (2015) Study on Biodiesel Production from Rapeseed Oil through the Orthogonal Method. Energy Sources, Part A Recover Util Environ Eff 37:422–427. doi: 10.1080/15567036.2011.576417
9. Alkabbash AN, Alam MZ, Mirghani MES, Al-Fusaiel AMA (2009) Biodiesel Production from Crude Palm Oil by Transesterification Process. J Appl Sci 9:3166–3170. doi: 10.3923/jas.2009.3166.3170
10. Mata TM, Sousa IRBG, Vieira SS, Caetano NS (2012) Biodiesel Production from Corn Oil via Enzymatic Catalysis with Ethanol. Energy & Fuels 26:3034–3041. doi: 10.1021/ef300319f
11. Sanchez F, Vasudevan PT (2006) Enzyme catalyzed production of biodiesel from olive oil. Appl Biochem Biotechnol 135:1–14.

12. Indexmundi 2017 (2017) Commodity Prices - Price Charts, Data, and News - IndexMundi. In: Indexmundi. http://www.indexmundi.com/commodities/. Accessed 24 Apr 2017
13. Canola 2017 (2017) Current Canola Oil, Meal, and Seed Prices - Canola Council of Canada. In: Canola Counc. Canada. http://www.canolacouncil.org/markets-stats/statistics/current-canola-oil,-meal,-and-seed-prices/. Accessed 24 Apr 2017
14. Meng X, Yang J, Xu X, et al (2009) Biodiesel production from oleaginous microorganisms. Renew Energy 34:1–5. doi: 10.1016/j.renene.2008.04.014
15. Sorda G, Banse M, Kemfert C (2010) An overview of biofuel policies across the world. Energy Policy 38:6977–6988. doi: 10.1016/j.enpol.2010.06.066
16. OECD-FAO (2016) OECD-FAO Agricultural Outlook (Edition 2016). OECD Agric Stat. doi: http://dx.doi.org/10.1787/60b7ee42-en
17. Fox 2016 (2017) World biodiesel production/consumption to rise 14% by 2020: OECD/FAO. In: S&P Glob. Platts. http://www.platts.com/latest-news/agriculture/london/world-biodiesel-productionconsumption-to-rise-26485632. Accessed 18 Feb 2017
18. REN21 (2015) Renewables 2015-Global Status Report. REN21 Renewables. doi: 10.1016/0267-3649(88)90030-1
19. Statista 2015 The world's biggest biodiesel producers in 2015, by country (in billion liters). In: Stat. Stat. Portal. https://www.statista.com/statistics/271472/biodiesel-production-in-selected-countries/. Accessed 18 Feb 2017
20. OECD C (2016) OECD-FAO Agricultural Outlook 2016-2025. In: Stat Technol. http://stats.oecd.org/viewhtml.aspx?datasetcode=HIGH_AGLINK_2016&lang=en#. Accessed 18 Feb 2017
21. Dessureault D (2016) Canada Biofuels Annual 2016.
22. Tan HW, Abdul Aziz AR, Aroua MK (2013) Glycerol production and its applications as a raw material: A review. Renew Sustain Energy Rev 27:118–127. doi: 10.1016/j.rser.2013.06.035
23. Ardi MS, Aroua MK, Hashim NA (2015) Progress, prospect and challenges in glycerol purification process: A review. Renew Sustain Energy Rev 42:1164–1173. doi: 10.1016/j.rser.2014.10.091

24. Sunita G, Devassy BM, Vinu A, et al (2008) Synthesis of biodiesel over zirconia-supported isopoly and heteropoly tungstate catalysts. Catal Commun. doi: 10.1016/j.catcom.2007.08.007
25. Freedman B, Pryde EH, Mounts TL (1984) Variables affecting the yields of fatty esters from transesterified vegetable oils. J Am Oil Chem Soc 61:1638–1643. doi: 10.1007/BF02541649
26. Ejikeme PM, Anyaogu ID, Ejikeme CL, et al (2010) Catalysis in Biodiesel Production by Transesterification Process-An Insight. E-Journal Chem 7:1120–1132. doi: 10.1155/2010/689051
27. Mosali R, Bobbili S (2011) Homogenous Catalyst and Effects on Multifeedstock Processing. In: Biodiesel Mag. http://www.biodieselmagazine.com/articles/7793/homogenous-catalyst-and-effects-on-multifeedstock-processing. Accessed 1 Mar 2017
28. Lee J-S, Saka S (2010) Biodiesel production by heterogeneous catalysts and supercritical technologies. Bioresour Technol 101:7191–7200. doi: 10.1016/j.biortech.2010.04.071
29. Vyas AP, Verma JL, Subrahmanyam N (2010) A review on FAME production processes. Fuel 89:1–9. doi: 10.1016/j.fuel.2009.08.014
30. Liu X, He H, Wang Y, et al (2008) Transesterification of soybean oil to biodiesel using CaO as a solid base catalyst. Fuel 87:216–221. doi: 10.1016/j.fuel.2007.04.013
31. Dossin TF, Reyniers M-F, Marin GB (2006) Kinetics of heterogeneously MgO-catalyzed transesterification. Appl Catal B Environ 62:35–45. doi: 10.1016/j.apcatb.2005.04.005
32. Uprety BK, Chaiwong W, Ewelike C, Rakshit SK (2016) Biodiesel production using heterogeneous catalysts including wood ash and the importance of enhancing byproduct glycerol purity. Energy Convers Manag 115:191–199. doi: 10.1016/j.enconman.2016.02.032
33. Alba-Rubio AC, Santamaría-González J, Mérida-Robles JM, et al (2010) Heterogeneous transesterification processes by using CaO supported on zinc oxide as basic catalysts. Catal Today 149:281–287. doi: 10.1016/j.cattod.2009.06.024
34. Zabeti M, Daud WMAW, Aroua MK (2010) Biodiesel production using alumina-

supported calcium oxide: An optimization study. Fuel Process Technol 91:243–248. doi: 10.1016/j.fuproc.2009.10.004

35. Mbaraka IK, Shanks BH (2006) Conversion of oils and fats using advanced mesoporous heterogeneous catalysts. J Am Oil Chem Soc 83:79–91. doi: 10.1007/s11746-006-1179-x
36. Ilgen O, Akin AN (2009) Development of Alumina Supported Alkaline Catalysts Used for Biodiesel Production. Turkish J Chem 33:281–287. doi: 10.3906/kim-0809-29
37. Luo X, Ge X, Cui S, Li Y (2016) Value-added processing of crude glycerol into chemicals and polymers. Bioresour Technol 215:144–154. doi: 10.1016/j.biortech.2016.03.042
38. Bajaj A, Lohan P, Jha PN, Mehrotra R (2010) Biodiesel production through lipase catalyzed transesterification: An overview. J Mol Catal B Enzym 62:9–14. doi: 10.1016/j.molcatb.2009.09.018
39. Dizge N, Aydiner C, Imer DY, et al (2009) Biodiesel production from sunflower, soybean, and waste cooking oils by transesterification using lipase immobilized onto a novel microporous polymer. Bioresour Technol 100:1983–1991. doi: 10.1016/j.biortech.2008.10.008
40. Gog A, Roman M, Toşa M, et al (2012) Biodiesel production using enzymatic transesterification – Current state and perspectives. Renew Energy 39:10–16. doi: 10.1016/j.renene.2011.08.007
41. Helwani Z, Othman MR, Aziz N, et al (2009) Technologies for production of biodiesel focusing on green catalytic techniques: A review. Fuel Process Technol 90:1502–1514. doi: 10.1016/j.fuproc.2009.07.016
42. Fukuda H, Kondo A, Noda H (2001) Biodiesel fuel production by transesterification of oils. J Biosci Bioeng 92:405–416. doi: 10.1016/S1389-1723(01)80288-7
43. Uprety BK, Dalli SS, Rakshit SK (2017) Bioconversion of crude glycerol to microbial lipid using a robust oleaginous yeast Rhodosporidium toruloides ATCC 10788 capable of growing in the presence of impurities. Energy Convers Manag 135:117–128. doi: 10.1016/j.enconman.2016.12.071
44. Nanda M, Yuan Z, Qin W (2014) Purification of crude glycerol using acidification: Effects of acid types and product characterization. Austin Chem Eng 1:1–7.

45. Garlapati VK, Shankar U, Budhiraja A (2016) Bioconversion technologies of crude glycerol to value added industrial products. Biotechnol Reports 9:9–14. doi: 10.1016/j.btre.2015.11.002
46. Liang Y, Sarkany N, Cui Y, Blackburn JW (2010) Batch stage study of lipid production from crude glycerol derived from yellow grease or animal fats through microalgal fermentation. Bioresour Technol 101:6745–6750. doi: 10.1016/j.biortech.2010.03.087
47. Gholami Z, Abdullah AZ, Lee KT (2014) Dealing with the surplus of glycerol production from biodiesel industry through catalytic upgrading to polyglycerols and other value-added products. Renew Sustain Energy Rev 39:327–341. doi: 10.1016/j.rser.2014.07.092
48. Yang F, Hanna M a, Sun R (2012) Value-added uses for crude glycerol-a byproduct of biodiesel production. Biotechnol Biofuels 5:13. doi: 10.1186/1754-6834-5-13
49. Ciriminna R, Pina C Della, Rossi M, Pagliaro M (2014) Understanding the glycerol market. Eur J Lipid Sci Technol 1432–1439. doi: 10.1002/ejlt.201400229
50. Kong PS, Aroua MK, Daud WMAW (2016) Conversion of crude and pure glycerol into derivatives: A feasibility evaluation. Renew Sustain Energy Rev 63:533–555. doi: 10.1016/j.rser.2016.05.054
51. Almeida JRM, Fávaro LCL, Quirino BF (2012) Biodiesel biorefinery: opportunities and challenges for microbial production of fuels and chemicals from glycerol waste. Biotechnol Biofuels 5:48. doi: 10.1186/1754-6834-5-48
52. Liang Y, Cui Y, Trushenski J, Blackburn JW (2010) Converting crude glycerol derived from yellow grease to lipids through yeast fermentation. Bioresour Technol 101:7581–7586. doi: 10.1016/j.biortech.2010.04.061
53. Valerio O, Horvath T, Pond C, et al (2015) Improved utilization of crude glycerol from biodiesel industries: Synthesis and characterization of sustainable biobased polyesters. Ind Crops Prod 78:141–147. doi: 10.1016/j.indcrop.2015.10.019
54. Hansen CFA, Hernandez AA, Mullan BPB, et al (2009) A chemical analysis of samples of crude glycerol from the production of biodiesel in Australia , and the effects of feeding crude glycerol to growing- fi nishing pigs on performance , plasma metabolites and meat quality at slaughter. 154–161.
55. Hu S, Luo X, Wan C, Li Y (2012) Characterization of crude glycerol from biodiesel

plants. J Agric Food Chem 60:5915–5921. doi: 10.1021/jf3008629

56. Xiao Y, Xiao G, Varma A (2013) A Universal Procedure for Crude Glycerol Purification from Different Feedstocks in Biodiesel Production : Experimental and Simulation Study. Ind Eng Chem Res 52:14291.
57. Chen Y-H, Walker TH (2011) Biomass and lipid production of heterotrophic microalgae Chlorella protothecoides by using biodiesel-derived crude glycerol. Biotechnol Lett 33:1973–1983. doi: 10.1007/s10529-011-0672-y
58. Ayoub M, Abdullah AZ (2012) Critical review on the current scenario and significance of crude glycerol resulting from biodiesel industry towards more sustainable renewable energy industry. Renew Sustain Energy Rev 16:2671–2686. doi: 10.1016/j.rser.2012.01.054
59. Quispe CAG, Coronado CJR, Carvalho JA (2013) Glycerol: Production, consumption, prices, characterization and new trends in combustion. Renew Sustain Energy Rev 27:475–493. doi: 10.1016/j.rser.2013.06.017
60. Sarma SJ, Brar SK, Sydney EB, et al (2012) Microbial hydrogen production by bioconversion of crude glycerol: A review. Int J Hydrogen Energy 37:6473–6490. doi: 10.1016/j.ijhydene.2012.01.050
61. Thompson JC, He BB (2006) Characterization of Crude Glycerol from Biodiesel Production From Multiple Feedstocks. Appl Eng Agric 22:261–265.
62. Dasari M (2007) Crude glycerol potential described. Feedstuffs
63. Mclea L, Ball MEE, Kilpatrick D, Elliott C (2011) The effect of glycerol inclusion on broiler performance and nutrient digestibility. Br Poult Sci 52:368–375. doi: 10.1080/00071668.2011.584520
64. Cerrate S, Yan F, Wang Z, et al (2006) Evaluation of Glycerine from Biodiesel Production as a Feed Ingredient for Broilers. Int J Poult Sci 5:1001–1007. doi: 10.3923/ijps.2006.1001.1007
65. Hassannia J (2011) Crude Glycerol Combustion System- Introduction and Commercialization Plan.
66. Fan X, Burton R, Zhou Y (2010) Glycerol (Byproduct of Biodiesel Production) as a Source for Fuels and Chemicals - Mini Review~!2009-08-26~!2010-02-02~!2010-04-09~! Open Fuels Energy Sci J 3:17–22. doi: 10.2174/1876973X01003010017

67. Fermoso J, He L, Chen D (2012) Production of high purity hydrogen by sorption enhanced steam reforming of crude glycerol. Int J Hydrogen Energy 37:14047–14054. doi: 10.1016/j.ijhydene.2012.07.084
68. Sharma R V., Kumar P, Dalai AK (2014) Selective hydrogenolysis of glycerol to propylene glycol by using Cu:Zn:Cr:Zr mixed metal oxides catalyst. Appl Catal A Gen 477:147–156. doi: 10.1016/j.apcata.2014.03.007
69. Cheng L, Liu L, Ye XP (2013) Acrolein production from crude glycerol in sub- and super-critical water. JAOCS, J Am Oil Chem Soc 90:601–610. doi: 10.1007/s11746-012-2189-5
70. Sullivan JA;, Burnham S (2014) Title The selective oxidation of glycerol over model Au/TiO2 catalysts -the influence of glycerol purity on conversion and product selectivity. Catal Commun. doi: 10.1016/j.catcom.2014.06.026
71. Gao Z, Ma Y, Wang Q, et al (2016) Effect of crude glycerol impurities on lipid preparation by Rhodosporidium toruloides yeast 32489. Bioresour Technol 218:373–379. doi: 10.1016/j.biortech.2016.06.088
72. Behera S, Singh R, Arora R, et al (2015) Scope of Algae as Third Generation Biofuels. Front Bioeng Biotechnol 2:1–13. doi: 10.3389/fbioe.2014.00090
73. Wegenhart BL, Liu S, Thom M, et al (2012) Solvent-Free Methods for Making Acetals Derived from Glycerol and Furfural and Their Use as a Biodiesel Fuel Component. ACS Catal 2:2524–2530. doi: 10.1021/cs300562e
74. Ilham Z, Saka S (2016) Esterification of glycerol from biodiesel production to glycerol carbonate in non-catalytic supercritical dimethyl carbonate. Springerplus 923. doi: 10.1186/s40064-016-2643-1
75. Chiu CW, Dasari MA, Sutterlin WR, Suppes GJ (2006) Removal of residual catalyst from simulated biodiesel's crude glycerol for glycerol hydrogenolysis to propylene glycol. Ind Eng Chem Res 45:791–795. doi: 10.1021/ie050915s
76. Skrzyńska E, Wondołowska-Grabowska A, Capron M, Dumeignil F (2014) Crude glycerol as a raw material for the liquid phase oxidation reaction. Appl Catal A Gen 482:245–257. doi: 10.1016/j.apcata.2014.06.005
77. Zhu S, Zhu Y, Gao X, et al (2013) Production of bioadditives from glycerol esterification over zirconia supported heteropolyacids. Bioresour Technol 130:45–51.

doi: 10.1016/j.biortech.2012.12.011

78. Chetpattananondh P, Tongurai C (2008) Synthesis of high purity monoglycerides from crude glycerol and palm stearin. Songklanakarin J Sci Technol 30:515–521.

79. Noureddini H, Dailey WR, Hunt BA (1998) Production of Ethers of Glycerol From Crude Glycerol - the By- Product of Biodlesel Production Production. Pap. Biomater. 18:

80. Samul D, Leja K, Grajek W (2014) Impurities of crude glycerol and their effect on metabolite production. Ann Microbiol 64:891–898. doi: 10.1007/s13213-013-0767-x

81. Papanikolaou S, Gortzi O, Margeli E, et al (2008) Effect of Citrus essential oil addition upon growth and cellular lipids of Yarrowia lipolytica yeast. Eur J Lipid Sci Technol 110:997–1006. doi: 10.1002/ejlt.200800085

82. Duarte SH, de Andrade CCP, Ghiselli G, Maugeri F (2013) Exploration of Brazilian biodiversity and selection of a new oleaginous yeast strain cultivated in raw glycerol. Bioresour Technol. doi: 10.1016/j.biortech.2013.04.004

83. Pyle DJ, Garcia RA, Wen Z (2008) Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: Effects of impurities on DHA production and algal biomass composition. J Agric Food Chem 56:3933–3939. doi: 10.1021/jf800602s

84. Ruhal R, Aggarwal S, Choudhury B (2011) Suitability of crude glycerol obtained from biodiesel waste for the production of trehalose and propionic acid. Green Chem 13:3492. doi: 10.1039/c1gc15847c

85. Ngo TA, Kim MS, Sim SJ (2011) High-yield biohydrogen production from biodiesel manufacturing waste by Thermotoga neapolitana. Int J Hydrogen Energy 36:5836–5842. doi: 10.1016/j.ijhydene.2010.11.057

86. Wilkens E, Ringel AK, Hortig D, et al (2012) High-level production of 1,3-propanediol from crude glycerol by Clostridium butyricum AKR102a. Appl Microbiol Biotechnol 93:1057–1063. doi: 10.1007/s00253-011-3595-6

87. Khanna S, Goyal A, Moholkar VS (2013) Production of n-butanol from biodiesel derived crude glycerol using Clostridium pasteurianum immobilized on Amberlite. Fuel 112:557–561. doi: 10.1016/j.fuel.2011.10.071

88. Kośmider A, Drozdzyńska A, Blaszka K, et al (2010) Propionic acid production by propionibacterium freudenreichii ssp. shermanii using crude glycerol and whey lactose

industrial wastes. Polish J Environ Stud 19:1249–1253.

89. Choi WJ, Hartono MR, Chan WH, Yeo SS (2011) Ethanol production from biodiesel-derived crude glycerol by newly isolated Kluyvera cryocrescens. Appl Microbiol Biotechnol 89:1255–1264. doi: 10.1007/s00253-010-3076-3
90. Scholten E, Dagele D (2008) Succinic acid production by a newly isolated bacterium. Biotechnol Lett 30:2143–2146. doi: 10.1007/s10529-008-9806-2
91. Mothes G, Schnorpfeil C, Ackermann JU (2007) Production of PHB from crude glycerol. Eng Life Sci 7:475–479. doi: 10.1002/elsc.200620210
92. Papanikolaou S, Fakas S, Fick M, et al (2008) Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. Biomass and Bioenergy 32:60–71. doi: 10.1016/j.biombioe.2007.06.007
93. Fakas S, Papanikolaou S, Batsos A, et al (2009) Evaluating renewable carbon sources as substrates for single cell oil production by Cunninghamella echinulata and Mortierella isabellina. Biomass and Bioenergy 33:573–580. doi: 10.1016/j.biombioe.2008.09.006
94. Tang S, Boehme L, Lam H, Zhang Z (2009) Pichia pastoris fermentation for phytase production using crude glycerol from biodiesel production as the sole carbon source. Biochem Eng J 43:157–162. doi: 10.1016/j.bej.2008.09.020
95. Gao Z, Ma Y, Wang Q, et al (2016) Effect of crude glycerol impurities on lipid preparation by Rhodosporidium toruloides yeast 32489. Bioresour Technol 218:373–9. doi: 10.1016/j.biortech.2016.06.088
96. Pott RWM, Howe CJ, Dennis JS (2014) The purification of crude glycerol derived from biodiesel manufacture and its use as a substrate by Rhodopseudomonas palustris to produce hydrogen. Bioresour Technol 152:464–470. doi: 10.1016/j.biortech.2013.10.094
97. Santibáñez C, Varnero MT, Bustamante M (2011) Residual Glycerol from Biodiesel Manufacturing, Waste or Potential Source of Bioenergy: A Review. Chil J Agric Res 71:469–475. doi: 10.4067/S0718-58392011000300019
98. Zhang X, Yan S, Tyagi RD, et al (2016) Energy balance of biofuel production from biological conversion of crude glycerol. J Environ Manage 170:169–176. doi:

10.1016/j.jenvman.2015.09.031

99. Pawar MS, Kadam AS, Dawane BS, Yemul OS (2016) Synthesis and characterization of rigid polyurethane foams from algae oil using biobased chain extenders. Polym Bull 73:727–741. doi: 10.1007/s00289-015-1514-1
100. Petrović ZS, Wan X, Bilić O, et al (2013) Polyols and polyurethanes from crude algal oil. JAOCS, J Am Oil Chem Soc 90:1073–1078. doi: 10.1007/s11746-013-2245-9
101. Uprety BK, Reddy JV, Dalli SS, Rakshit SK (2017) Utilization of microbial oil obtained from crude glycerol for the production of polyol and its subsequent conversion to polyurethane foams. Bioresour Technol. doi: 10.1016/j.biortech.2017.03.126
102. Azócar L, Ciudad G, Heipieper HJ, Navia R (2010) Biotechnological processes for biodiesel production using alternative oils. Appl Microbiol Biotechnol 88:621–636. doi: 10.1007/s00253-010-2804-z
103. Martínez EJ, Raghavan V, González-Andrés F, Gómez X (2015) New biofuel alternatives: Integrating waste management and single cell oil production. Int J Mol Sci 16:9385–9405. doi: 10.3390/ijms16059385
104. Liang MH, Jiang JG (2013) Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. Prog Lipid Res 52:395–408. doi: 10.1016/j.plipres.2013.05.002
105. Garay LA, Boundy-Mills KL, German JB (2014) Accumulation of high-value lipids in single-cell microorganisms: A mechanistic approach and future perspectives. J Agric Food Chem 62:2709–2727. doi: 10.1021/jf4042134
106. Huerlimann R, de Nys R, Heimann K (2010) Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production. Biotechnol Bioeng 107:245–257. doi: 10.1002/bit.22809
107. Chi Z, Pyle D, Wen Z, et al (2007) A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. Process Biochem 42:1537–1545. doi: 10.1016/j.procbio.2007.08.008
108. Ethier S, Woisard K, Vaughan D, Wen Z (2011) Continuous culture of the microalgae Schizochytrium limacinum on biodiesel-derived crude glycerol for producing docosahexaenoic acid. Bioresour Technol 102:88–93. doi: 10.1016/j.biortech.2010.05.021

109. Abad S, Turon X (2015) Biotechnological production of docosahexaenoic acid using aurantiochytrium limacinum: Carbon sources comparison and growth characterization. Mar Drugs 13:7275–7284. doi: 10.3390/md13127064
110. O'Gardy J, Morgan JA (2011) Heterotrophic growth and lipid production of Chlorella protothecoides on glycerol. Bioprocess Biosyst Eng 34:121–125. doi: 10.1007/s00449-010-0474-y
111. Choi HJ, Yu SW (2015) Influence of crude glycerol on the biomass and lipid content of microalgae. Biotechnol Biotechnol Equip 29:506–513. doi: 10.1080/13102818.2015.1013988
112. Li Q, Du W, Liu D (2008) Perspectives of microbial oils for biodiesel production. Appl Microbiol Biotechnol 80:749–756. doi: 10.1007/s00253-008-1625-9
113. Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: A review. Renew Sustain Energy Rev 14:217–232. doi: 10.1016/j.rser.2009.07.020
114. Hannon M, Gimpel J, Tran M, et al (2010) Biofuels from algae: challenges and potential. Biofuels 1:763–784. doi: 10.4155/bfs.10.44
115. Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67:491–502. doi: 10.1128/MMBR.67.4.491-502.2003
116. Strobel GA, Knighton B, Kluck K, et al (2008) The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus Gliocladium roseum (NRRL 50072). Microbiology 154:3319–3328. doi: 10.1099/mic.0.2008/022186-0
117. Peng X-W, Chen H-Z (2007) Microbial oil accumulation and cellulase secretion of the endophytic fungi from oleaginous plants. Ann Microbiol 57:239–242. doi: 10.1007/BF03175213
118. McAfee BJ, Taylor A (1999) A review of the volatile metabolites of fungi found on wood substrates. Nat Toxins 7:283–303. doi: 10.1002/1522-7189(199911/12)7:6<283::AID-NT70>3.0.CO;2-N
119. Fakas S, Papanikolaou S, Batsos A, et al (2009) Evaluating renewable carbon sources as substrates for single cell oil production by Cunninghamella echinulata and Mortierella isabellina. Biomass and Bioenergy 33:573–580. doi:

10.1016/j.biombioe.2008.09.006

120. Chatzifragkou A, Makri A, Belka A, et al (2011) Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species. Energy 36:1097–1108. doi: 10.1016/j.energy.2010.11.040
121. André A, Diamantopoulou P, Philippoussis A, et al (2010) Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid. Ind Crops Prod 31:407–416. doi: 10.1016/j.indcrop.2009.12.011
122. Sisniega H, Rio J-L Del, Amaya M-J, Faus I (2005) Strategies for Large-Scale Production of Recombinant Proteins in Filamentous Fungi. Microb. Process. Prod.
123. Butinar L, Spencer-Martins I, Gunde-Cimerman N (2007) Yeasts in high Arctic glaciers: the discovery of a new habitat for eukaryotic microorganisms. Antonie Van Leeuwenhoek 91:277–289. doi: 10.1007/s10482-006-9117-3
124. Xu J, Zhao X, Wang W, et al (2012) Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast Rhodosporidium toruloides and the individual effect of some impurities on lipid production. Biochem Eng J 65:30–36. doi: 10.1016/j.bej.2012.04.003
125. Uçkun Kiran E, Trzcinski A, Webb C (2013) Microbial oil produced from biodiesel by-products could enhance overall production. Bioresour Technol 129:650–654. doi: 10.1016/j.biortech.2012.11.152
126. 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 Res 6:300–310. doi: 10.1007/s12155-012-9257-4
127. Tchakouteu SS, Kalantzi O, Gardeli C, et al (2015) Lipid production by yeasts growing on biodiesel-derived crude glycerol: Strain selection and impact of substrate concentration on the fermentation efficiency. J Appl Microbiol 118:911–927. doi: 10.1111/jam.12736
128. Duarte SH, Ghiselli G, Maugeri F (2013) Influence of culture conditions on lipid production by Candida sp. LEB-M3 using glycerol from biodiesel synthesis. Biocatal Agric Biotechnol 2:339–343. doi: 10.1016/j.bcab.2013.07.001
129. Liu L, Hu Y, Lou W, et al (2016) Use of Crude Glycerol as Sole Carbon Source for

Microbial Lipid Production by Oleaginous Yeasts. Appl Biochem Biotechnol. doi: 10.1007/s12010-016-2340-0

130. Dedyukhina EG, Chistyakova TI, Mironov AA, et al (2014) Arachidonic acid synthesis from biodiesel- derived waste by Mortierella alpina. Eur J Lipid Sci Technol 116:429–437. doi: Doi 10.1002/Ejlt.201300358
131. Sakthivel R, Elumalai S, Mohommad Arif M (2011) Microalgae lipid research, past, present: A critical review for biodiesel production, in the future. J Exp Sci 2:29–49.
132. Zhu LD, Li ZH, Hiltunen E (2016) Strategies for Lipid Production Improvement in Microalgae as a Biodiesel Feedstock. Biomed Res Int 2016:1–8. doi: 10.1155/2016/8792548
133. Hu Q, Sommerfeld M, Jarvis E, et al (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54:621–639. doi: 10.1111/j.1365-313X.2008.03492.x
134. Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. Biochimie 86:807–815. doi: 10.1016/j.biochi.2004.09.017
135. Christophe G, Kumar V, Nouaille R, et al (2012) Recent developments in microbial oils production: A possible alternative to vegetable oils for biodiesel without competition with human food? Brazilian Arch Biol Technol 55:29–46. doi: 10.1590/S1516-89132012000100004
136. Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 51:1–51. doi: 10.1016/S0065-2164(02)51000-5
137. Sharma KK, Schuhmann H, Schenk PM (2012) High Lipid Induction in Microalgae for Biodiesel Production. Energies 5:1532–1553. doi: 10.3390/en5051532
138. Dong T, Knoshaug EP, Pienkos PT, Laurens LML (2016) Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review. Appl Energy 177:879–895. doi: 10.1016/j.apenergy.2016.06.002
139. Suutari M, Leskinen E, Fagerstedt K, et al (2015) Macroalgae in biofuel production. Phycol Res 63:1–18. doi: 10.1111/pre.12078
140. Hamilton ML, Warwick J, Terry A, et al (2015) Towards the Industrial Production of Omega-3 Long Chain Polyunsaturated Fatty Acids from a Genetically Modified

Diatom Phaeodactylum tricornutum. PLoS One 10:e0144054. doi: 10.1371/journal.pone.0144054

141. Adarme-Vega T, Lim DKY, Timmins M, et al (2012) Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. Microb Cell Fact 11:96. doi: 10.1186/1475-2859-11-96
142. Poli JS, da Silva MAN, Siqueira EP, et al (2014) Microbial lipid produced by Yarrowia lipolytica QU21 using industrial waste: A potential feedstock for biodiesel production. Bioresour Technol 161:320–326. doi: 10.1016/j.biortech.2014.03.083
143. Duarte SH, Hernandez GL del P, Canet A, et al (2015) Enzymatic biodiesel synthesis from yeast oil using immobilized recombinant Rhizopus oryzae lipase. Bioresour Technol 183:175–180. doi: 10.1016/j.biortech.2015.01.133
144. Papanikolaou S, Rontou M, Belka A, et al (2016) Conversion of biodiesel-derived glycerol into biotechnological products of industrial significance by yeast and fungal strains. Eng Life Sci 0:1–20. doi: 10.1002/elsc.201500191.
145. Schlagermann P, Göttlicher G, Dillschneider R, et al (2012) Composition of algal oil and its potential as biofuel. J Combust. doi: 10.1155/2012/285185
146. Ochsenreither K, Glück C, Stressler T, et al (2016) Production Strategies and Applications of Microbial Single Cell Oils. Front Microbiol 7:1539. doi: 10.3389/fmicb.2016.01539
147. Heim A, Kamionowska U, Solecki M (2007) The effect of microorganism concentration on yeast cell disruption in a bead mill. J Food Eng 83:121–128. doi: 10.1016/j.jfoodeng.2007.02.047
148. Spiden EM, Scales PJ, Kentish SE, Martin GJO (2013) Critical analysis of quantitative indicators of cell disruption applied to Saccharomyces cerevisiae processed with an industrial high pressure homogenizer. Biochem Eng J 70:120–126. doi: 10.1016/j.bej.2012.10.008
149. Wang M, Yuan W (2015) Microalgal cell disruption in a high-power ultrasonic flow system. Bioresour Technol 193:171–177. doi: 10.1016/j.biortech.2015.06.040
150. Rakesh S, Dhar DW, Prasanna R, et al (2015) Cell disruption methods for improving lipid extraction efficiency in unicellular microalgae. Eng Life Sci 15:443–447. doi: 10.1002/elsc.201400222

151. Guldhe A, Singh B, Rawat I, et al (2014) Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production. Fuel 128:46–52. doi: 10.1016/j.fuel.2014.02.059
152. 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 Res 8:1788–1799. doi: 10.1007/s12155-015-9626-x
153. Jin G, Yang F, Hu C, et al (2012) Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast Rhodosporidium toruloides. Bioresour Technol 111:378–382. doi: 10.1016/j.biortech.2012.01.152
154. White PM, Potter TL, Strickland TC (2009) Pressurized Liquid Extraction of Soil Microbial Phospholipid and Neutral Lipid Fatty Acids. J Agric Food Chem 57:7171–7177. doi: 10.1021/jf901257n
155. Walker TH, Cochran HD, Hulbert GJ (1999) Supercritical carbon dioxide extraction of lipids from Pythium irregulare. J Am Oil Chem Soc 76:595–602. doi: 10.1007/s11746-999-0009-3
156. Iverson SJ, Lang SL, Cooper MH (2001) Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 36:1283–1287. doi: 10.1007/s11745-001-0843-0
157. Manirakiza P, Covaci A, Schepens P (2001) Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer Extraction Methods. J Food Compos Anal 14:93–100. doi: 10.1006/jfca.2000.0972
158. Dai C, Tao J, Xie F, et al (2007) Biodiesel generation from oleaginous yeast Rhodotorula glutinis with xylose assimilating capacity. 6:2130–2134.
159. Li Y, Naghdi FG, Garg S, et al (2014) A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. Microb Cell Fact 13:1–9. doi: 10.1186/1475-2859-13-14
160. Prommuak C, Pavasant P, Quitain AT, et al (2012) Microalgal Lipid Extraction and Evaluation of Single-Step Biodiesel Production. Eng J 16:26–27. doi: 10.4186/ej.2012.16.5.157

161. Folch J, Lees M, Sloan Stanley GH (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J Biol Chem 226:497–509.
162. Bligh EG, Dyer WJ (1959) A Rapid Method of Total Lipid Extraction and Purification. Can. J. Biochem. Physiol. 37:
163. Axelsson M, Gentili F (2014) A single-step method for rapid extraction of total lipids from green microalgae. PLoS One 9:17–20. doi: 10.1371/journal.pone.0089643
164. Kojima Y, Parcell J, Cain J (2016) A Global Demand Analysis of Vegetable Oils for Food and Industrial Use: A Cross-Country Panel Data Analysis with Spatial Econometrics. In: Agric. Appl. Econ. Assoc. Boston, pp 0–27
165. Lane J (2016) Biofuels Mandates Around the World : 2016. BiofuelsDigest 1–10.
166. Saenge C, Cheirsilp B, Suksaroge TT, Bourtoom T (2011) Potential use of oleaginous red yeast Rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. Process Biochem 46:210–218. doi: 10.1016/j.procbio.2010.08.009
167. Athalye SK, Garcia RA, Wen Z (2009) Use of Biodiesel-Derived Crude Glycerol for Producing Eicosapentaenoic Acid (EPA) by the Fungus Pythium irregulare. J Agric Food Chem 57:2739–2744. doi: 10.1021/jf803922w
168. Swanson D, Block R, Mousa SA (2012) Omega-3 Fatty Acids EPA and DHA: Health Benefits Throughout Life. Adv Nutr An Int Rev J 3:1–7. doi: 10.3945/an.111.000893
169. Petrović ZS, Wan X, Bilić O, et al (2013) Polyols and Polyurethanes from Crude Algal Oil. J Am Oil Chem Soc 90:1073–1078. doi: 10.1007/s11746-013-2245-9
170. Koutinas AA, Chatzifragkou A, Kopsahelis N, et al (2014) Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. Fuel 116:566–577. doi: 10.1016/j.fuel.2013.08.045
171. Neste oil 2012 Neste Oil inaugurates Europe's first pilot plant for producing microbial oil from waste and residues | Neste. https://www.neste.com/en/neste-oil-inaugurates-europes-first-pilot-plant-producing-microbial-oil-waste-and-residues. Accessed 22 Feb 2017
172. Algoil 2017 British Algoil - Our Technology. http://www.britishalgoil.com/our-technology. Accessed 22 Feb 2017
173. Buggypower 2017 Buggypower-Microalgae life creators.

http://www.buggypower.eu/index.php?lang=en. Accessed 22 Feb 2017

174. Pondtech 2017 Pond Technologies Inc. http://pondtechnologiesinc.com/. Accessed 22 Feb 2017
175. Algaeneers 2015 Products and Benefits. http://algae.io/products-and-benefits/. Accessed 22 Feb 2017
176. Algaefarms 2017 Algae Farms/Greece: Company Profile. https://www.bloomberg.com/profiles/companies/1040821D:GA-algae-farms/greece. Accessed 22 Feb 2017
177. Sapphire 2016 Sapphire Energy, Inc. http://www.sapphireenergy.com/. Accessed 22 Feb 2017
178. Xu J, Du W, Zhao X, et al (2013) Microbial oil production from various carbon sources and its use for biodiesel preparation. Biofuels, Bioprod Biorefining 7:65–77. doi: 10.1002/bbb.1372
179. Cao Y, Liu W, Xu X, et al (2014) Production of free monounsaturated fatty acids by metabolically engineered Escherichia coli. Biotechnol Biofuels 7:59. doi: 10.1186/1754-6834-7-59
180. Tai M, Stephanopoulos G (2013) Engineering the push and pull of lipid biosynthesis in oleaginous yeast Yarrowia lipolytica for biofuel production. Metab Eng 15:1–9. doi: 10.1016/j.ymben.2012.08.007
181. Shimada H, Kondo K, Fraser PD, et al (1998) Increased carotenoid production by the food yeast Candida utilis through metabolic engineering of the isoprenoid pathway. Appl Environ Microbiol 64:2676–2680.
182. Abghari A, Chen S (2014) Yarrowia lipolytica as an Oleaginous Cell Factory Platform for Production of Fatty Acid-Based Biofuel and Bioproducts. Front Energy Res 2:1–21. doi: 10.3389/fenrg.2014.00021
183. Zhu Z, Zhang S, Liu H, et al (2012) A multi-omic map of the lipid-producing yeast Rhodosporidium toruloides. Nat Commun 3:1112. doi: 10.1038/ncomms2112
184. Pfleger BF, Gossing M, Nielsen J (2015) Metabolic engineering strategies for microbial synthesis of oleochemicals. Metab Eng 29:1–11. doi: 10.1016/j.ymben.2015.01.009
185. Lee JJL, Chen L, Shi J, et al (2014) Metabolomic Profiling of Rhodosporidium toruloides Grown on Glycerol for Carotenoid Production during Different Growth

Phases. J Agric Food Chem 62:10203–10209. doi: 10.1021/jf502987q

186. Papanikolaou S, Aggelis G (2011) Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. Eur J Lipid Sci Technol 113:1031–1051. doi: 10.1002/ejlt.201100014
187. Subramaniam R, Dufreche S, Zappi M, Bajpai R (2010) Microbial lipids from renewable resources: Production and characterization. J Ind Microbiol Biotechnol 37:1271–1287. doi: 10.1007/s10295-010-0884-5
188. Bruton T, Lyons H, Lerat Y, et al (2009) A Review of the Potential of Marine Algae as a Source of Biofuel in Ireland.
189. Ratledge C, Cohen Z (2008) Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? Lipid Technol 20:155–160. doi: 10.1002/lite.200800044

# Chapter 3

## Biodiesel production using heterogeneous catalysts including wood ash and the importance of enhancing byproduct glycerol purity

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**Abstract**

Transesterification of vegetable oils or animal fats with methanol in the presence of catalysts produces fatty acid methyl esters (FAME) and glycerol as a co-product. This study was focused on a comparative study of the transesterification of refined, bleached and deodorized palm oil (RBD palm oil) using a heterogeneous catalysts CaO with and without γ -alumina ($\gamma$-$Al_2O_3$) as a support. The results were also compared to that using sodium hydroxide (NaOH), which is a homogenous catalyst. Parameters like the amount of catalyst, the molar ratio of methanol to oil, reaction time and reaction temperature that affect methyl ester and glycerol formation were analyzed and the optimum conditions were determined. The FAME and glycerol content (96.75% and 92.73% respectively) obtained using CaO were lower in purity compared to that using $CaO/Al_2O_3$ (97.66% and 96.36% respectively). In the second phase of our work, wood ash from two different sources (birch bark & flyash from a biomass based power plant), which were calcined at 800 $^0$C were studied for their potential use as a cheap renewable alternative heterogeneous catalyst. Both the wood ash samples were found to have good potential for use in such production process, but needs to be optimized further to obtain biodiesel which meets fuel biodiesel specifications. Both CaO and CaO supported on alumina produces FAME to levels that meet the fuel specifications required for blending with diesel. However, the latter produces a purer form of byproduct glycerol that can be easily be converted to value added products, without the need for purification. On this basis, the supported catalyst is recommended for use in industry as it can add to profits in integrated plants which produces biodiesel and simultaneously uses the byproduct glycerol for value added products.

### 3.1. Introduction

Biofuels and renewable forms of energy are being developed to substitute conventional fossil fuels due to the finite nature, fluctuating costs and climate effects of the latter. Biodiesel has attracted considerable attention as it offers several advantages. It is a mixture of fatty acid alkyl esters derived from renewable feedstock such as vegetable oils or animal fats. Although there are several methods to produce biodiesel, fatty acid methyl esters (FAME) are commonly produced by the transesterification of triglycerides with alcohol. The resultant product has physical and chemical characteristics similar to petroleum based diesel. Therefore, biodiesel can be used in diesel engines without further modification [1]. The advantages of biodiesel over conventional diesel fuel include a high flash point, high cetane number, low viscosity, high lubricity, biodegradability, and environmentally friendly nature as it emits lower levels of carbon monoxide and sulfur dioxide [2].

Transesterification reactions for fatty acid alkyl ester formation can be catalyzed by acids, bases and enzymatic catalysts. Glycerol is produced as a co-product to a level of 10% (w/w) of the biodiesel produced [3]. With the increase in production of biodiesel over the years, there is a simultaneous accumulation of huge amounts of glycerol (also known as crude glycerol) which contains considerable amounts of impurities. Purification of crude glycerol into pharmaceutical and technical grade glycerol is an expensive process and is not commercially feasible for small and medium sized biodiesel industries [3–5]. Additionally, direct conversion of crude glycerol into value added products is hindered by the presence of toxic impurities. An alternative biodiesel production process which produces purer glycerol together with biodiesel which meets fuel specifications is very important for the biodiesel industry [3, 4, 6]. This can bring larger profits to the overall biodiesel production process by cutting the cost involved in the downstream processing and make possible the use of glycerol to produce various value added products.

Conventionally, sodium or potassium hydroxide and alkoxides are the common homogenous base catalysts used in this reaction under mild conditions and results in high yields of methyl esters in short reaction times [7]. However, its solubility in the reaction medium leads to formation of soap via saponification which is a serious problem because it reduces biodiesel

yield, consumes catalysts and causes significant difficulties in product separation and purification [8]. The use of acids such as sulfonic acid, sulfuric acid and hydrochloric acid as catalysts in biodiesel production can eliminate soap formation from free fatty acid. But the rate of reaction is slower than the use of basic catalysts and also needs high reaction temperatures [9–11]. Enzymatic reaction is highly specific and chemically clean, however the production of a lipase catalysts are considerably costlier compared to a chemical catalyst [10]. Therefore, heterogeneous alkali catalysts are being studied to overcome these problems. The latter can be easily separated, reused and regenerated due to their low solubility and consumption in the reaction. However, heterogeneous catalysts also have some drawbacks such as mass transfer limitation, low active sites availability for catalyst reaction and in some cases, they leach into the reaction media. In order to address these issues, various types of catalyst supports (mainly alumina, silica, zinc oxide and zirconium oxide) have been used for biodiesel production [7, 11–13]. Alumina, is the most commonly used catalyst support for biodiesel production because of its enhanced thermal and mechanical stability, high specific surface area (as high as 300 $m^2/g$), large pore size (5-15 nm), large specific pore volume (0.6 $cm^3/g$) and ability to be shaped into pellets and extruded. In general, attaching catalysts to a support, like alumina, increases surface area and number of pores for active species. This minimizes the diffusion limitations, a major problem with the use of heterogeneous catalyst, thus improving the rate of reaction [14]. In comparison to the unsupported CaO catalyst, the composite catalyst ($CaO/Al_2O_3$) has a larger specific surface areas and more active sites and pores. This facilitates the attachment of large triglyceride molecules with the active catalyst species [14].

This study involves biodiesel production starting from refined, bleached and deodorized palm oil (RBD palm oil) by transesterification using heterogeneous catalyst with and without support. The optimum conditions were determined taking into account the conversion to methyl esters, glycerol yield and purity. The quality of biodiesel produced was then evaluated to see if it meets the specifications of commercial biodiesel.

Calcium oxide (a solid heterogeneous catalyst) has a high basicity, low solubility, low price and is easy to handle. However, the transesterification reaction it catalyzes is inadequate for practical applications due to mass transfer limitations as mentioned above [13, 15]. Aluminum

oxide has been widely used as a support in catalysis processes and many researchers have tried to attach various compounds, including calcium oxide, for the efficient production of biodiesel [16]. However, little work involving a comparative study of calcium oxide and calcium oxide attached to γ- alumina under similar reaction conditions has been reported, in spite of the known advantages of attached catalyst. In our study, the results from these two catalysts have also been compared to sodium hydroxide, a homogeneous catalyst.

Additionally, we also explored the possibility of utilizing wood ash from two different sources (birch bark and flyash from a power station) as a cheaper alternative heterogeneous catalyst to carry out the transesterification reaction. Ash from birch bark is considered an alternative source of calcium oxide and magnesium oxide as they are present in higher levels compared to other woody plants [17, 18] . Utilization of ash from birch bark can serve as a reusable catalyst that is widely available in Canada and various other parts of the world. Flyash acquired from Ontario Power Generation Inc. (Canada) was utilized to convert triglyceride into biodiesel in our experiments. This is one of world's first power plants that has been retrofitted from a coal based unit to biomass-fueled power generation plant. Every year it produces large amount of flyash, which can serve as a cheap catalyst. Flyash has limited large scale applications besides being used as a soil stabilizer [19, 20]. Hence, utilization of this flyash would be of economic importance for the biodiesel industry. Finally, this paper also tries to make a case, that if a higher purity byproduct glycerol stream can be produced, then it can be used on-site to produce higher value products and increase profits of an integrated plant.

## 3.2. Materials and methods

### 3.2.1. Materials

The vegetable oil used in this research study was refined, bleached and deodorized (RBD) palm oil which was obtained from Oleen Company Limited, Bangkok, Thailand. The properties and fatty acid composition of the palm oil as provided by the supplier is tabulated in Table 3.1 and 3.2 below. All the chemicals used in this work were of analytical reagent grade and obtained from Sigma Aldrich, Fisher Sci & Merck. Sample analyses were performed in duplicates and percentage errors were expressed as mean ± standard deviation.

Table 3.1. Properties of Refined, Bleached and Deodorized (RBD) palm oil used in this study

| Properties | Unit | Limits | Method | RBD palm oil |
|---|---|---|---|---|
| Density at 15 $^0C$[a] | $kg/m^3$ | 860 min. and 900 max. | ASTM D 1298 | 910 |
| Kinematic Viscosity[a] | cSt | 3.5 min. and 5.0 max. | ASTM D 445 | 44.5155 |
| Free fatty acid (as palmitic acid)[b] | % | 0.1% max. | A.O.C.S Ca 5a-40 | 0.06 |
| Peroxide value[b] | meq/kg | 2.0 max. | A.O.C.S Cd 8-53 | 0.56 |
| Moisture and impurities[b] | % | 0.10% max. | A.O.C.S Ca 2c-25 | 0.03 |
| Iodine value[b] | - | 50-55 | A.O.C.S Cd 1-25 | 51.57 |

[a] European standard specification for biodiesel fuel in EN 14214-2003; [b] Thai industrial standards for palm oil in TIS 288-2521

Table 3.2. Fatty acid composition of Refined, Bleached and Deodorized (RBD) palm oil (Oleen, Co., Ltd)

| Fatty acid | Chemical Structure | Molecular weight of fatty acid | Wt.% |
|---|---|---|---|
| Caprylic acid | C 8:0 | 144.21 | 0.0143 |
| Capric acid | C 10:0 | 172.26 | 0.0158 |
| Lauric acid | C 12:0 | 200.31 | 0.2068 |
| Myristic acid | C 14:0 | 228.36 | 0.8312 |
| Palmitic acid | C 16:0 | 256.42 | 44.0902 |
| Stearic acid | C 18:0 | 284.47 | 4.7035 |
| Oleic acid | C 18:1 | 282.47 | 30.0319 |
| Linoleic acid | C 18:2 | 280.77 | 10.6056 |
| Linolenic acid | C 18:3 | 278.43 | 0.1991 |
| Arachidic acid | C 20:0 | 312.53 | 0.3023 |
| **Total fatty acid** | | | 100.0007 |

### 3.2.2. Preparation of catalyst

Calcium oxide (CaO) supported on alumina ($\gamma$-$Al_2O_3$) was prepared using an impregnation method of an aqueous solution of calcium acetate on alumina support as reported by Zabeti et al. (2009) [1] . In this method [1], $\gamma$-alumina in powder form was preheated to 600 $^0$C for 1 h to remove absorbed water. It was then added to 100.54% (w/v) solution of calcium acetate precursor in distilled water. The mixture was stirred for 4 h at 60 $^0$C. The obtained slurry was heated overnight in an oven. Prior to its use in the transesterification reaction, CaO/$Al_2O_3$ was calcined in furnace at 718 $^0$C for 5 h. Similarly, pure CaO was prepared by calcination at 600 $^0$C for 4 h.

Birch bark obtained from Trowbridge Falls Park (Canada) was cleaned with water and dried at 80 $^0$C for 24 hours in an oven. After 24 hours, dried bark was burnt to obtain ash. Similarly, flyash from burnt wood pellets was obtained from Ontario Power Generation Inc., Atikokan, Ontario (Canada). Both these ash powder were calcined at 800 $^0$C for 4 hours before they were utilized to catalyze the transesterification of palm oil [21].

### 3.2.3. Catalyst characterization

X-ray Diffraction (XRD) and Scanning Electron Microscopy (SEM) analysis techniques were used to characterize prepared CaO/$\gamma$-$Al_2O_3$ catalysts.

### 3.2.4. X-ray diffraction (XRD)

The X-ray diffraction technique was utilized to characterize and identify the internal structure, bulk phase and composition in crystalline phase of CaO/$\gamma$-$Al_2O_3$ catalysts. The prepared catalyst was analyzed at XRD/XRF X-ray Techniques Laboratory, Analysis and Testing Research Unit, National Metal and Materials Technology Center (MTEC), Thailand. A JEOL JDX-3530 theta-2theta X-ray diffractometer equipped with $CuK_\alpha$ radiation ($\lambda$ = 1.5406) was used as an X-ray source with an acceleration voltage of 30 kV and beam current of 40 mA. A detector scanned the X-ray reflection from the sample as a function of 2$\theta$ angle between $10^0$ to $80^0$ using $0.02^0$ step angle and 1.0 s. counting time at each angle step with D-, R- and S-slits at $1^0$, 0.2 mm and $1^0$, respectively.

### 3.2.5. Scanning electron microscopy

A scanning electron microscopy (SEM) was utilized to identify the microstructure and capture images of catalyst morphology. The prepared catalysts and catalyst support were placed on a stub and coated with gold in a sputtering device before being observed by the SEM, PHILIPS XL 30 CP at the Metallurgy and Materials Science Research Institute (MMRI), Chulalongkorn University, Thailand.

### 3.2.6. Transesterification of oil using heterogeneous catalyst

The transesterification of RBD palm oil using CaO or $CaO/Al_2O_3$ was carried out in a 250 ml two-necked round bottom flask equipped with reflux condenser and thermometer. 50 grams of RBD palm oil was weighed in the flask, which then was immersed in a constant temperature water bath and heated to the desired temperature. Known amounts of methanol and catalyst were mixed in a beaker for 5 minutes and then added into the reactor which was stirred using a magnetic stirrer for the desired reaction time. Subsequently, the reaction mixture was immersed in a room temperature (25 $^0$C) water bath to stop the reaction and solid catalysts were removed from the mixture by centrifugation and filtration. The reaction mixture was then evaporated to remove excess methanol under vacuum in a rotary vacuum evaporator. The obtained transesterified product was transferred to a separating funnel and allowed to stand overnight to allow complete separation of methyl ester and glycerol phases. The glycerol phase (bottom phase) was drawn off first. The methyl esters (top phase) was washed with warm distilled water at 50 $^0$C several times, until the wash-water was clear. Finally, the remaining water in the product was removed by adding 20 wt.% anhydrous $Na_2SO_4$. A similar procedure was used with birch wood ash and flyash from burnt wood pellets.

### 3.2.7. Analysis of biodiesel and glycerol

The methyl esters were analyzed by using a Gas chromatograph (Thermo Scientific, Trace 1300 model) equipped with a Flame Ionization Detector (FID) and a polar column (TG WaxMS A; 30 m x 0.25 μm x 0.25 μm). Analysis parameters were adapted from a work reported by Khan (2013), with slight modification [22]. Helium which was used as the carrier gas had a flow rate of 1 ml/min, linear velocity of 30 cm/s and a sample injection split ratio of 50:1. The temperature gradient maintained in the oven was 100 $^0$C (0.25min), 30 $^0$C/min, 220 $^0$C (0 mins) and 10

$^0$C/min, 250 $^0$C (3 mins). Air flow of 350 ml/min, $H_2$ at 35ml/min, and $N_2$ at 40ml/min were adopted throughout the runs. Each components of methyl esters were identified by known peaks of standards Supelco FAME mix C4-C24 and using methyl nonadecanoate as the internal standard. The %FAME or purity of biodiesel (mg/mg of sample) was calculated using equation (3.1):

$$\%\text{FAME} = \frac{(\sum \text{A} - \text{A}_{\text{EI}})}{\text{A}_{\text{EI}}} \text{x} \frac{\text{C}_{\text{EI}} \text{ x } \text{V}_{\text{EI}}}{\text{m}} \text{x} 100 \qquad \text{Equation 3.1}$$

Where, $\sum A$ is the total peak area from methyl esters in the range $C_{14}$ to $C_{24:1}$, $A_{EI}$ is the peak area of methyl nonadecanoate, $C_{EI}$ is the concentration of the methyl nonadecanoate solution, (mg/ml), $V_{EI}$ is the volume of the methyl nonadecanoate solution being used (ml) and *m* is the mass of the sample, mg. Similarly, biodiesel conversion (%) was calculated using equation (3.2):

$$\text{Biodiesel conversion, \%} = \frac{\text{Weight of biodiesel produced} \times \%\text{FAME}}{\text{Weight of oil}} \qquad \text{Equation 3.2}$$

The analyzed FAME mixtures consisted of mixtures of Myristic Acid Methyl Ester, Palmitic Acid Methyl Ester, Stearic Acid Methyl Ester, Oleic Acid Methyl Ester, Linoleic Acid Methyl Ester and Nonadecanoic Acid Methyl Ester.

Glycerol content was determined by using Agilent 1260 infinity HPLC using Aminex ion-exchange Biorad column (HPX-87H, 300 x 7.8mm) and Refractive Index (RI) detector. The operating condition for analysis included 5mM Sulfuric Acid as mobile phase, 0.60 ml/min flow rate, 50 $^0$C column and detector temperature.

### 3.2.8. Specification requirements for biodiesel

The physical and chemical properties of biodiesel obtained from CaO and CaO/γ-$Al_2O_3$ in this work were also tested according to the specification of the Department of Energy Business B.E. 2548 (2005) [23].

### 3.3. Results and discussion

#### 3.3.1. Catalyst characterization

##### 3.3.1.1. X-ray diffraction (XRD) data analysis

The XRD pattern of CaO/ γ-$Al_2O_3$ with 100.54% loading of calcium acetate is shown in Figure 1. The XRD pattern of CaO crystallite phase showed clear diffraction peaks at 2θ= ~32.2, ~37.4, ~53.9, ~64.2 and ~67.4 and also that of gamma-alumina crystalline phase at 2θ= ~45.7 and ~67.0. The XRD pattern exhibited high peak intensity for CaO, since residual amounts of calcium oxide remained on the composite after calcination, as a result of loading at more than the spontaneous dispersion capacity on the surface of alumina. In contrast, the peak could not be detected when calcium oxide was dispersed as a monolayer on alumina (Fig 3.1).

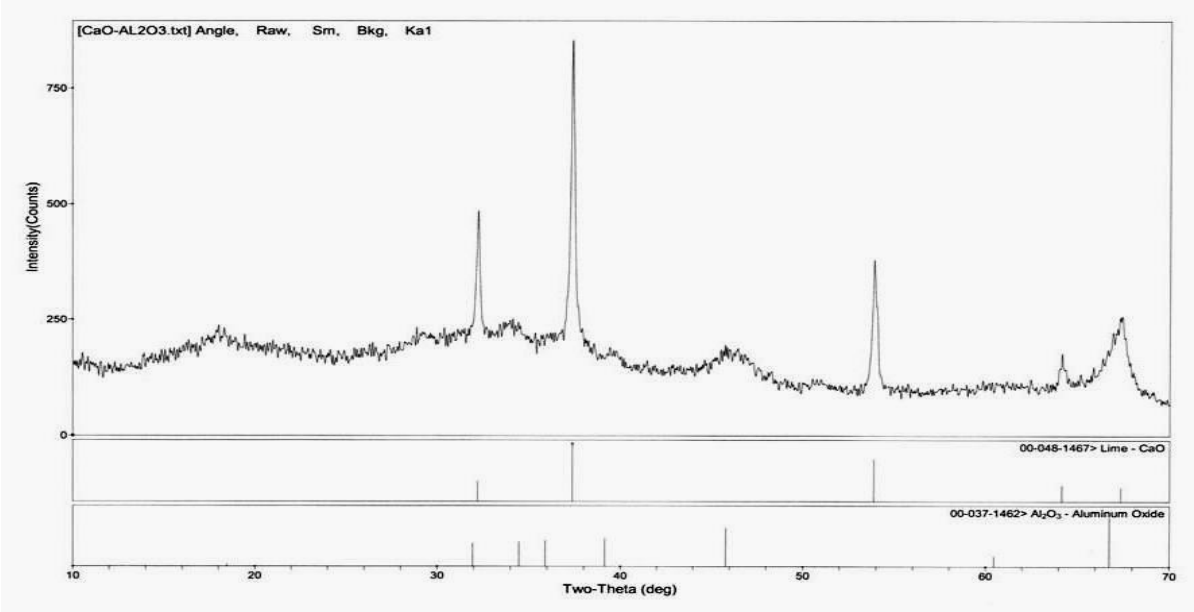


Fig 3.1. XRD patterns of 100.54% loading of calcium oxide precursor on γ-$Al_2O_3$ calcined at 718 $^0$C for 5 h

Earlier, it had been reported that the X-ray diffraction patterns were observed mainly as calcium oxide with some calcium carbonate after supporting calcium acetate on mesoporous silica by impregnation [24]. Moreover, CaO/$Al_2O_3$ prepared by modified single sol-gel method did not exhibit the diffraction peak of CaO in X-ray diffraction. This is explained on the basis that the samples absorbed water and carbon dioxide present in air [25]. In addition, the diffraction pattern result did not present peaks related to the acetate salt. Therefore, calcium oxide precursor

(calcium acetate) was completely converted to calcium oxide after calcinations at 718 $^0$C for 5 hours.

### 3.3.1.2. Scanning electron microscopy (SEM) data analysis

SEM micrographs in Fig 3.2, 3.3 and 3.4 show morphologies of unloaded γ-$Al_2O_3$, calcium oxide and CaO/ γ-$Al_2O_3$ catalyst prepared by loading CaO onto γ-$Al_2O_3$ respectively.

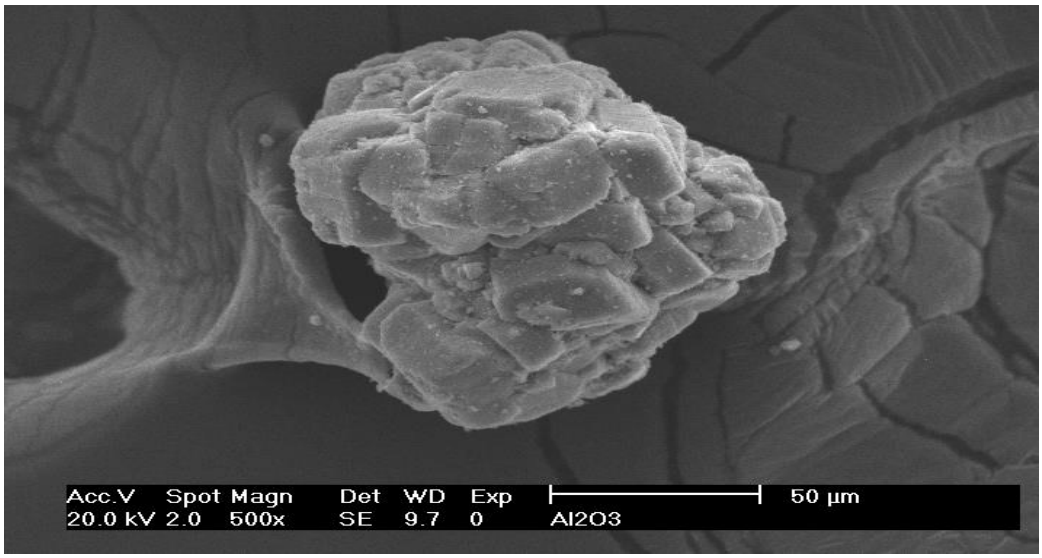


Fig 3.2. SEM micrograph of unloaded γ-$Al_2O_3$ calcined at 600 $^0$C

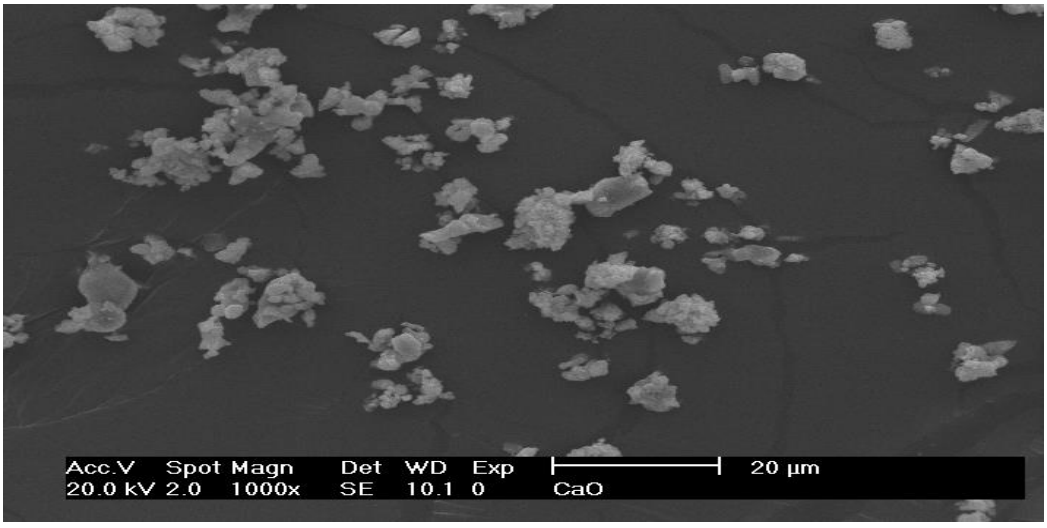


Fig 3.3. SEM micrograph of calcium oxide calcined at 600 $^0$C

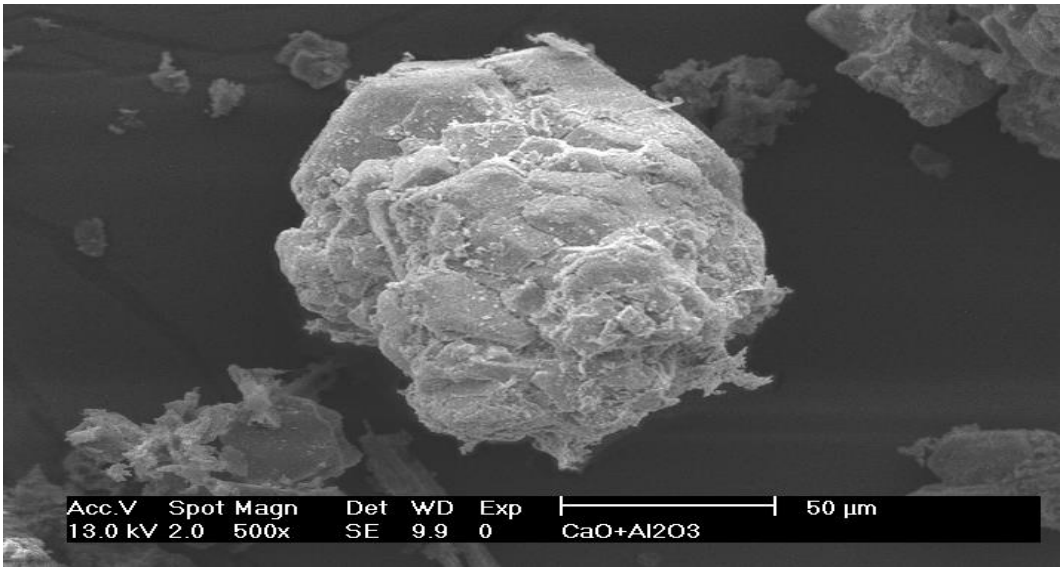


Fig 3.4. SEM micrograph of 100.54% calcium oxide precursor loading on γ-$Al_2O_3$ calcined at 718 $^0$C

The structure of γ-$Al_2O_3$ (Fig 3.2) was rough and contained pores which results in high specific surface area where active calcium oxide could be anchored. CaO was supported on alumina with 100.54% loading of calcium precursor and calcination at 718 $^0$C for 5 h (Fig 3.4). The calcium oxide converted from calcium acetate filled in the pore and also dispersed on all the surface of mesoporous alumina leading to reduction of the specific surface area. The particle size of CaO were smaller and provided the lowest specific surface areas (Fig 3.3). This confirms our expectation that the addition of γ-$Al_2O_3$ would help in increasing the loading of the catalyst.

### 3.3.2. Transesterification of biodiesel using sodium hydroxide as homogeneous catalyst

In conventional processes, sodium hydroxide is commonly used to catalyze the transesterification of oil. It has high catalytic activity, due to its high basicity and solubility in media. The operating condition of transesterification of RBD palm oil in this research were 0.5 % NaOH with molar ratio of methanol to oil of 6:1, reaction time of 0.5 h and reaction temperature of 60 $^0$C. When sodium hydroxide was employed, soap was formed due to a reaction between free fatty acid and sodium hydroxide in the presence of water that may be present in the feedstock. The purification of the methyl ester layer was done by washing with warm water (~50 $^0$C) repeatedly, until pH of washed-water was neutral. Because sodium hydroxide is very reactive, as described above, biodiesel conversion was the highest (94.21%)

among the catalysts studied (Table 3.3).

Table 3.3. Biodiesel and glycerol produced by transesterification of RBD palm oil using homogeneous and variety of various heterogeneous catalyst

| Catalyst Used | Reaction Conditions | FAME (%) | Biodiesel conversion (%) | Glycerol purity (%) | Glycerol yield (%) |
|---|---|---|---|---|---|
| CaO* | Catalyst 9 wt.%<br>MeOH/Oil 12:1<br>Time 2 hrs.<br>Temp 60 $^0$C | 96.75 ± 0.37 | 90.11 ± 1.34 | 92.73 ± 2.05 | 81.84 ± 4.44 |
| $CaO/Al_2O_3$* | Catalyst 9 wt.%<br>MeOH/Oil 15:1<br>Time 3 hrs.<br>Temp 60 $^0$C | 97.66 ± 1.37 | 86.38 ± 0.44 | 96.36 ± 0.26 | 79.32 ± 4.09 |
| NaOH | Catalyst 0.5 wt.%<br>MeOH/Oil 6:1<br>Time 0.5 hr.<br>Temp 60 $^0$C | 97.32 ± 0.93 | 94.21 ± 1.57 | 64.47 ± 1.43 | 65.61 ± 1.60 |
| Birch bark ash | Catalyst 3 wt.%<br>MeOH/Oil 12:1<br>Time 3 hrs.<br>Temp 60 $^0$C | 88.06 ± 0.72 | 69.70 ± 0.83 | 78.18 ± 1.41 | 75.01 ± 1.19 |
| Flyash from wood pellet | Catalyst 9 wt.%<br>MeOH/Oil 12:1<br>Time 3 hrs.<br>Temp 60 $^0$C | 99.92 ± 0.01 | 87.76 ± 1.14 | 88.23 ± 0.24 | 94.23 ± 0.84 |

Data are given in mean ± standard error; *Under optimized reaction conditions; MeOH: Methanol

The glycerol obtained during transesterification with sodium hydroxide was semi-solid and had a low yield and purity of 65.16% and 64.47% respectively. The formation of sodium compounds such as sodium glycerate, sodium methylate, and sodium soaps presented in the glycerol phase account for this. Additionally, other chemical substances such as water, a small amount of esters and alcohol, traces of glycerides, and vegetable colors are also present [26]. The crude glycerol obtained by using such a homogenous catalyst has to go through considerable purification before it can be used in any way. This makes the glycerol utilization process too costly for any practical purpose [27, 28]. Hence, the possibility of increasing revenue by utilizing the glycerol stream becomes difficult when a homogenous catalyst is used for biodiesel production.

### 3.3.3. Optimization of biodiesel synthesis using heterogeneous catalysts, CaO & CaO/ γ-$Al_2O_3$

Triglycerides in palm oil can be converted into methyl esters by reacting with methanol in the presence of a solid based catalyst. During the transesterification reaction, initially the hydroxyl bond in a molecule of methanol is broken by the basic site of metal oxide catalyst to form methoxide anion and hydrogen cation. The methoxide anion formed then acts as a nucleophile and attacks the carbonyl carbon of triglyceride to form an unstable tetrahedral intermediate which then breaks down into FAME and diglyceride anion [29]**.** Four process variables in transesterification reaction using CaO and $CaO/Al_2O_3$ as heterogeneous catalysts were studied. These include the amount of catalysts, methanol to oil ratio, reaction time and reaction temperature.

### 3.3.4. Effect of amount of catalyst

The catalyst concentration is one of the most important parameters that affects the rate of transesterification reaction. The effect of four different catalysts concentrations were evaluated. In preliminary tests, CaO and $CaO/Al_2O_3$ provided different rates of reaction. Free calcium oxide reacted faster than the one with support. Significant amount of calcium oxide transesterification occurred under conditions of 600 rpm mixing rate, 9:1 methanol to oil ratio, 1 h reaction time and 60 $^0$C reaction temperature. The results shown in Fig 3.5 indicate that when the amount of catalyst was not sufficient (at 3% catalyst), the biodiesel conversion was relatively low (21.67%) and glycerol was not produced. Biodiesel yields increased from 21.67% to 73.27% as the amount of calcium oxide increased from 3% to 9%. However, there was no significant change at a higher level of catalyst (12 wt.%) due to mixing problems involving reactants, products and solid catalysts. It has been reported that the presence of excess catalysts could cause phase separation of methanol, oil and catalyst and increase viscosity which in turn effects mixing and transesterification [1, 24]. At the same time, the glycerol yield increased as catalyst concentration increased to 9 wt.% based on oil and remained constant at higher catalyst levels.

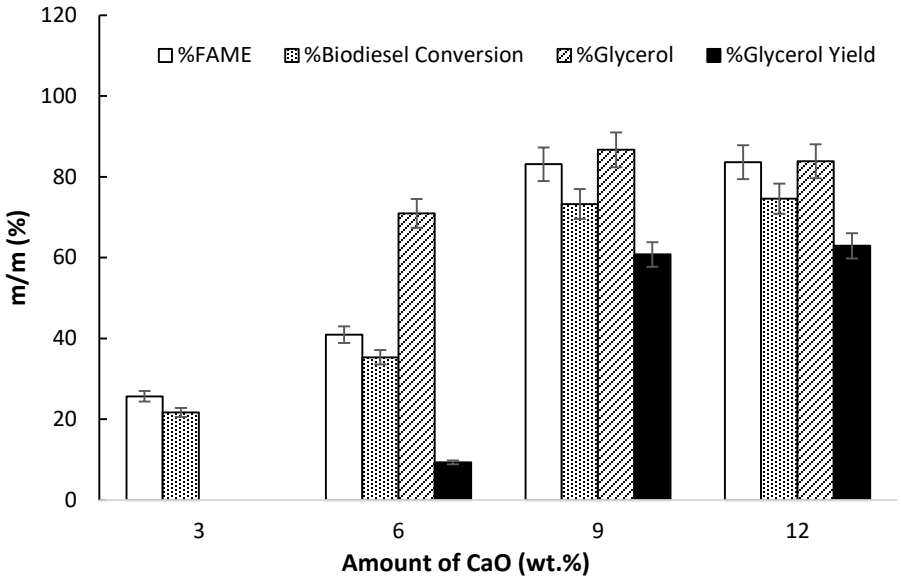


Fig 3.5. Effect of amount of calcium oxide on methanolysis (with RBD palm oil, molar ratio of methanol to oil of 9:1, reaction time of 1 h and reaction temperature of 60 $^0$C)

When alumina supported calcium oxide was applied, the results (Fig 3.6) show that significant transesterification reaction occurred at conditions of molar ratio of methanol to oil of 12:1, the reaction time of 2 h and the reaction temperature of 60 $^0$C. The FAME content and biodiesel conversion increased with the increase in catalyst concentration (starting from 3 wt. % catalyst) and finally remained constant at 9 wt.% catalyst. But glycerol concentration was not measurable with 3 wt.% catalyst concentration. Glycerol concentration increased with catalyst concentration but remained insignificantly different when 6 wt.% and 9 wt.% catalysts were employed. At both catalyst levels, the glycerol content was high and had approximately 95% purity. However, based on the amount of biodiesel produced and its purity, it can be concluded that 9 wt.% of $CaO/Al_2O_3$ catalyst was optimum for the conversion of biodiesel. At the higher concentrations of the catalyst, the biodiesel yields were almost constant because of mixing limitation.

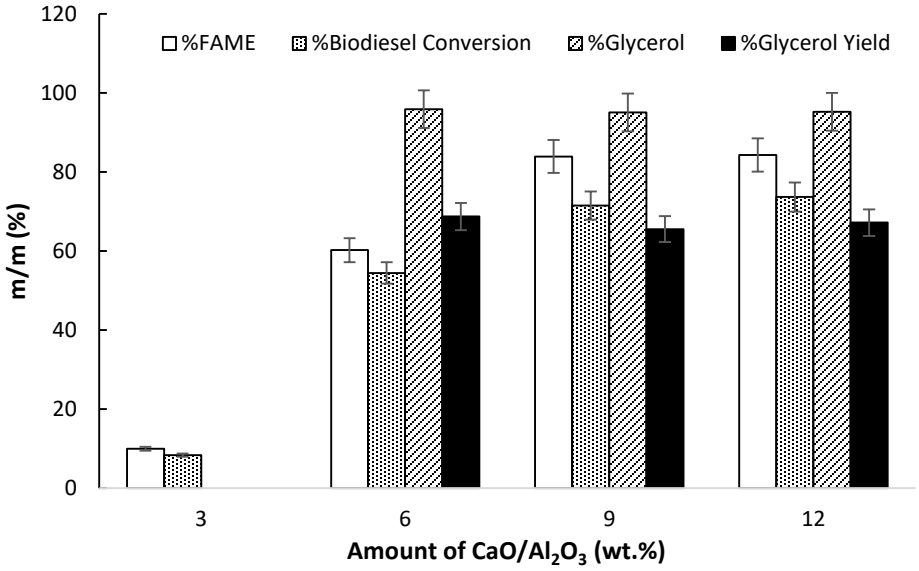


Fig 3.6. Effect of amount of alumina supported calcium oxide on methanolysis (with RBD palm oil, molar ratio of methanol to oil of 12:1, reaction time of 2 h and reaction temperature of 60 $^0$C

### 3.3.5. Effect of molar ratio of methanol to oil

According to reaction stoichiometry, the transesterification of vegetable oil including palm oil with methanol requires that the molar ratio of methanol to oil should be 3:1. Since the transesterification process is reversible reaction, the excess of the methanol is required in order to shift the equilibrium forward. As shown in Fig 3.7, biodiesel conversion was too low (30.67%) at molar ratio of methanol to oil of 3:1 and the byproduct glycerol could not be measured. An increase of methanol to oil ratio resulted in an increase of ester yield. Above 12:1 methanol to oil ratio, the ester yield increased insignificantly as the reaction may have reached its equilibrium point. Additionally, there was a reduction in the glycerol yield at 15:1 mole ratio of methanol to oil because of the difficulty in the separation of glycerol from the biodiesel layer at conditions over optimum level (12%) of amount of methanol.

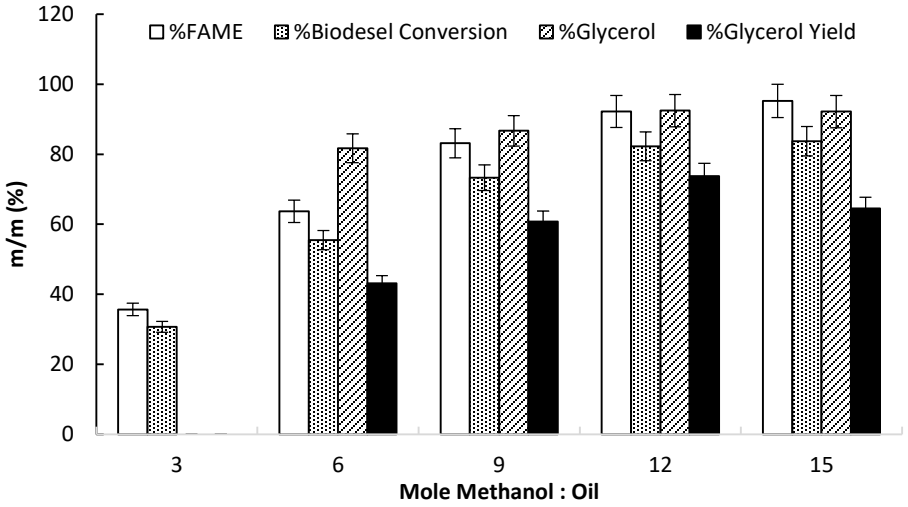


Fig 3.7. Effect of molar ratio of methanol to oil on methanolysis using calcium oxide (with RBD palm oil, 9 wt. % catalyst, the reaction time of 1 h and the reaction temperature of 60 $^0$C)

The transesterification reaction using $CaO/Al_2O_3$, the biodiesel conversion and glycerol yield was similar to that using CaO (Fig 3.8). However, the optimum methanol to oil molar ratio was 15:1. At levels higher than 15:1 of methanol to oil ratio, biodiesel conversion and glycerol yield decreased due to slow separation of methyl ester and glycerol as described above. Many studies have reported that the optimum molar ratio of methanol to oil was 15:1 using heterogeneous catalysts with supports [30–34]. This is similar to the results obtained in our experiment even though they used other supports.

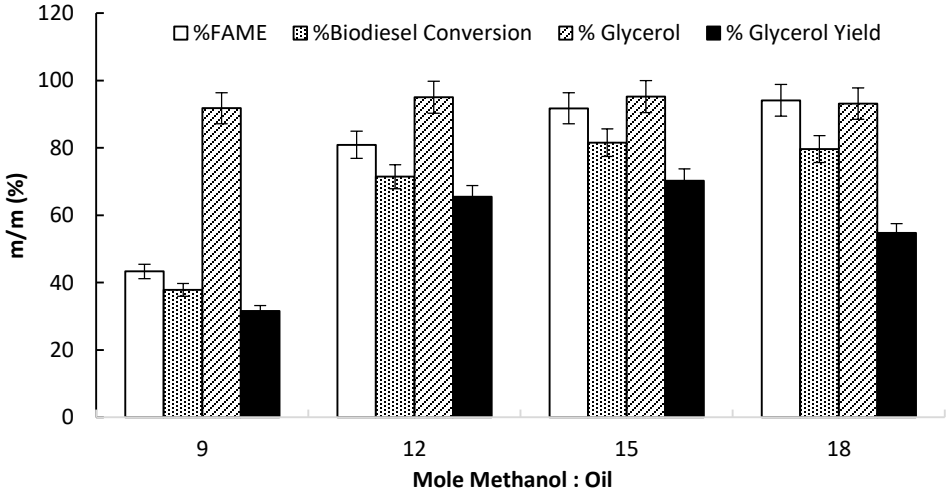


Fig 3.8. Effect of molar ratio of methanol to oil on methanolysis using alumina supported calcium oxide (with RBD palm oil, 9 wt.% catalyst, the reaction time of 2 h and the reaction temperature of 60 $^0$C)

### 3.3.6. Effect of reaction time

The effect of reaction time on biodiesel conversion and glycerol yield was studied by varying it but keeping all the other reaction parameters constant in a series of experiments. The reaction conditions used for transesterification of palm oil using calcium oxide was methanol/oil molar ratio of 12:1, 9 wt.% catalyst, 600 rpm mixing speed and 60 $^0$C reaction temperature. Similarly, for the calcium oxide supported on alumina the reaction conditions of 15:1 methanol/oil molar ratio, 9 wt.% catalyst, 600 rpm of stirrer speed and 60 $^0$C reaction temperature was used. The result shown in Fig 3.9 for calcium oxide catalyst shows biodiesel conversion increased with time from 24.48% at 0.5 h to 89.18% at 2 h and subsequently remained nearly constant. The purity of FAME produced after one hour of reaction time was 92.21%. Even though the subsequent change was not significant, the required purity ( i.e. 96.65%) to meet the biodiesel specification was obtained only after 2 hours [23]. Glycerol production yield was similar to biodiesel conversion as per stoichiometry, but not much difference was observed after 2 hours. Thus, it was concluded that the transesterification of RBD palm oil reached the equilibrium at 2 h using calcium oxide as heterogeneous catalyst.

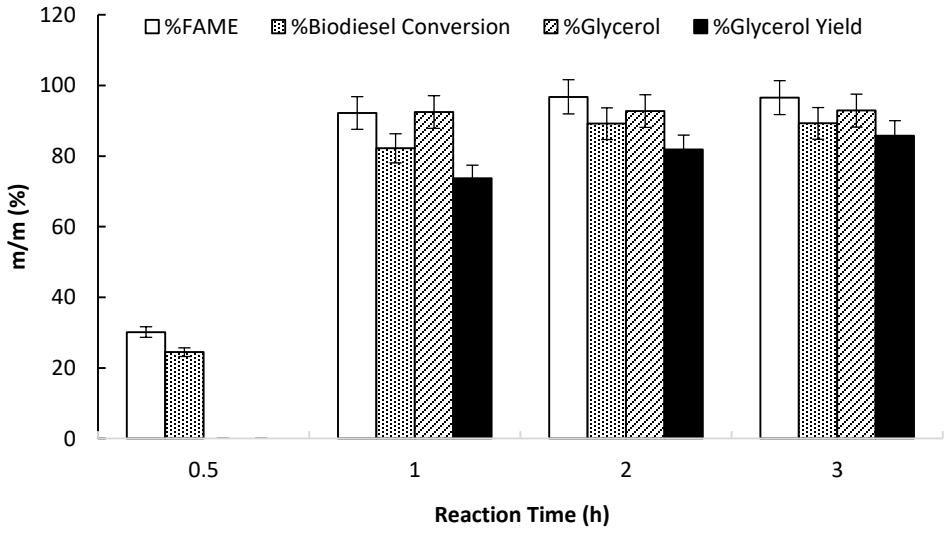


Fig 3.9. Effect of reaction time on methanolysis using calcium oxide (with RBD palm oil, 9 wt.% catalyst, the molar ratio of methanol to oil of 12:1 and the reaction temperature of 60 $^0$C)

Similarly, with the $CaO/Al_2O_3$ catalyst, biodiesel conversion and glycerol yield significantly increased in the first three hours of reaction and afterwards remained nearly constant (Fig 3.10). The optimum reaction time was found to be 3 h at which point biodiesel conversion and glycerol yields were 86.38% and 79.32%, respectively. In addition, %FAME and %glycerol, indicating purity of the products, were 97.66% and 96.36% respectively.

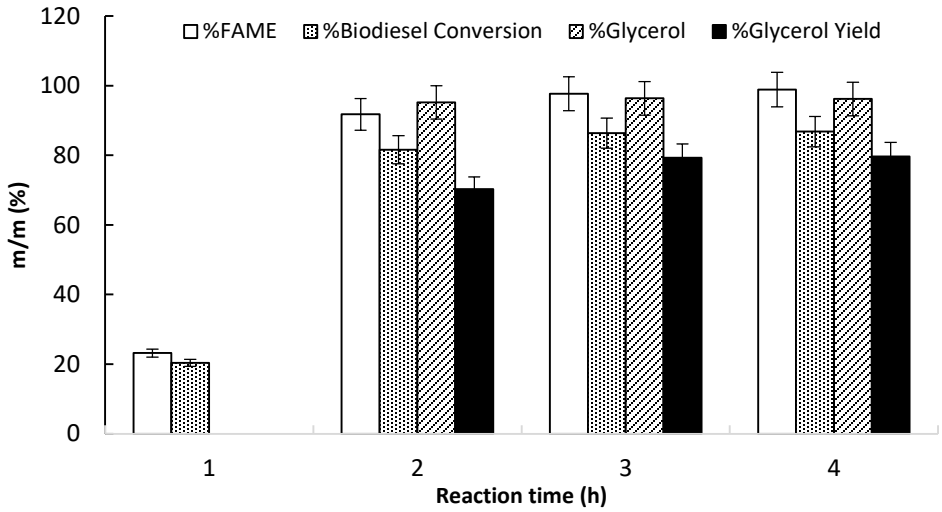


Fig 3.10. Effect of reaction time on methanolysis using alumina supported calcium oxide (with RBD palm oil, 9 wt.% catalyst, the molar ratio of methanol to oil of 15:1 and the reaction temperature of 60 $^0$C)

### 3.3.7. Effect of reaction temperature

As reaction temperature can affect biodiesel conversion and glycerol yield, experiments were carried out at 50 $^0$C, 60 $^0$C and 70 $^0$C. The transesterification reactions of RBD palm oil were conducted under identical conditions using the optimum conditions which were obtained from the previous experimental section (600 rpm stirrer speed, 9 wt.% calcium oxide, 12:1 molar ratio methanol to oil, and 2 h reaction time). As can be seen from Figure 11, at 50 $^0$C reaction temperature, the biodiesel conversion was low (37.59%). At the same time, glycerol yield was also low. However, percentage glycerol purity was quite high. The highest biodiesel conversion and glycerol yield was obtained when reaction temperature was maintained 60 $^0$C. At this temperature, these parameters were higher than 50 $^0$C and 70 $^0$C. This could be due to the fact that increasing the reaction temperature above the boiling point of methanol (64.5 $^0$C) causes its evaporation and reduces the extent of methanol and catalyst interaction. It has also been

reported, that if the reaction temperature was higher than boiling point, a large number of bubbles are formed and this inhibit the reaction on the three-phase interface of oil, methanol, and catalyst [35].

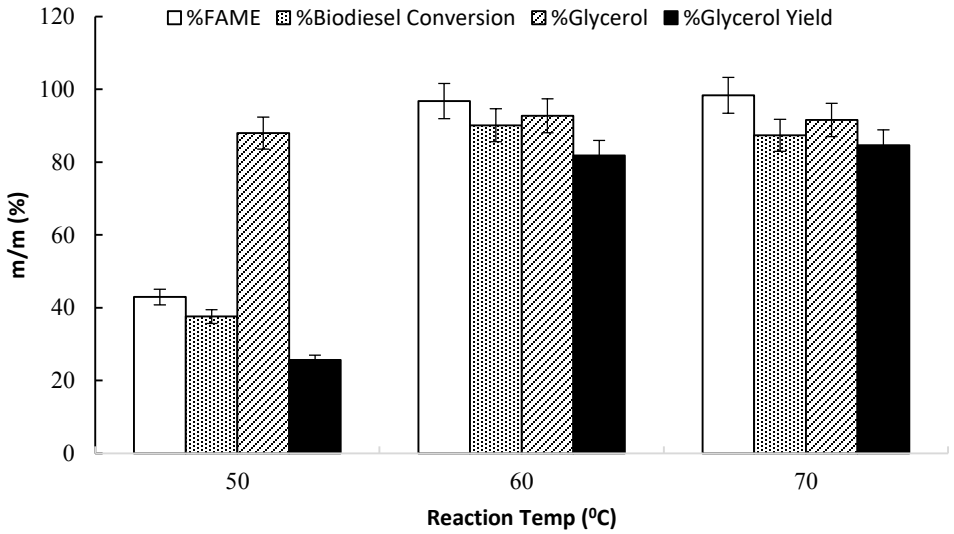


Fig 3.11. Effect of reaction temperature on methanolysis using calcium oxide (with RBD palm oil, 9 wt.% catalyst, molar ratio of methanol to oil of 12:1 and reaction time of 2 h)

The transesterification reaction using $CaO/Al_2O_3$ at 60 $^0$C was found to be similar to that using calcium oxide (Fig 3.12). The biodiesel conversion was 14.51% at 50 $^0$C reaction temperature and increased up to a temperature of 60 $^0$C and remained nearly constant beyond this temperature. The highest biodiesel yield and glycerol conversion were 86.38% and 78.49%, respectively. The effect of temperature above the boiling point of methanol is described in the previous paragraphs. The optimum conditions of CaO and $CaO/Al_2O_3$ are summarized in Table 3.3 above.

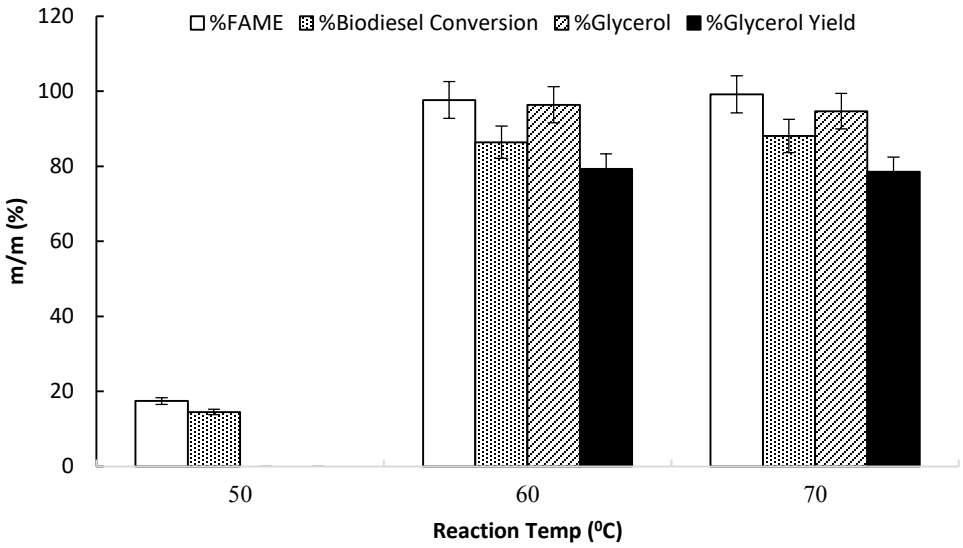


Fig 3.12. Effect of reaction temperature on methanolysis using alumina supported calcium oxide (with RBD palm oil, 9 wt.% catalyst, molar ratio of methanol to oil of 15:1 and reaction time of 3 h)

### 3.3.8. Properties of biodiesel

The physical and chemical properties of biodiesel obtained from CaO and CaO supported on $Al_2O_3$ were tested according to the specification of the Department of Energy Business B.E. 2548 (2005) [23]. These properties of biodiesels obtained on transesterification of RBD palm oil using homogeneous and heterogeneous catalysts are shown in Table 3.4. It can be seen from the table that the properties of biodiesel produced on transesterification by using heterogeneous catalysts, calcium oxide and alumina supported calcium oxide, are within the required limits specified by Department of Energy Business B.E. 2548 (2005) [23, 36, 37]. They are similar to homogeneous catalyzed transesterification reaction by conventional process using sodium hydroxide as catalyst.

Table 3.4. Properties of RBD palm oil biodiesel (obtained by using CaO, $CaO/Al_2O_3$ & NaOH catalyst) compared to the specifications of Department of Energy Business B.E. 2548 (2005) [23]

| Properties | Unit | Limits* | Method | Heterogeneous catalyzed methanolysis | | Homogeneous catalyzed methanolysis |
|---|---|---|---|---|---|---|
| | | | | CaO | $CaO/Al_2O_3$ | NaOH |
| Methyl Ester | wt.% | >96.5 | EN 14103 | 96.75 | 97.66 | 97.32 |
| Density at 15°C | $kg/m^3$ | 860-900 | ASTM D 1298 | 885 | 887 | 880 |
| Viscosity at 40°C | cSt | 3.5-5.0 | ASTM D 445 | 4.82 | 4.75 | 4.70 |
| Acid Number | mg KOH/g | 0.50 max | ASTM D 664 | 0.34 | 0.38 | 0.21 |

*Department of Energy Business B.E. 2548 (2005)

### 3.3.9. Wood ash as a cheaper alternative heterogeneous catalyst

Wood ash is rich in a variety of alkaline earth oxides including calcium oxides. The amount of calcium oxide present in a wood ash depends on the type of wood. Wood ash from birch bark contains approximately 69% of calcium oxide. Similarly, flyash from wood pellet industries has also shown to have significant amount of calcium oxides in it [17, 18]. It has been reported that the calcium oxide amount in wood flyash from various sources across Canada and USA ranges between 2.2 to 36.7% with an average of 16% [38, 39]. As these two sources of wood ashes are rich in calcium oxide, we made an initial attempt to study their potential to be used as a cheap alternative heterogeneous catalyst for the transesterification of palm oil into biodiesel. Flyash used in this experiment was obtained from the OPG Power plant in Atikokan, Ontario. These substances were initially calcined at 800 $^0$C for 4 hours in a muffle furnace and then stored in a desiccator, after cooling it to room temperature (25 $^0$C). Calcination at this temperature was carried out to remove water and carbon dioxide from the ash and convert calcium carbonate (calcite) into calcium oxide [21, 40]. Initially, for the transesterification of oil using birch bark ash and flyash, methanol/oil ratio of 12:1, reaction time of 3 hours, temperature of 60 $^0$C and catalyst amount of 3 wt.% were maintained. With birch bark ash, a biodiesel conversion of 69.70% and 88.06% of %FAME content was obtained. At the same time, glycerol yield and

purity were 75.01% and 78.18% respectively. In contrast, when flyash from the power plant was utilized at a level of 3 wt.%, no biodiesel or glycerol production was observed. But when the catalyst amount was increased to 9 wt.%, 87.76% of biodiesel conversion with a FAME purity (%) of 88.06% was obtained. Additionally, the glycerol yield in this case was 75.01% and glycerol purity was 78.18%. Both the natural catalysts, which are generally considered as a waste, have thus been shown to possess a promising ability to catalyze the transesterification reaction for biodiesel production. Further optimization of the process taking into account all process parameters is being carried out to obtain biodiesel which would meet the required specifications required for use as a fuel.

### 3.3.10. Importance of purity of biodiesel byproduct glycerol

The glut in glycerol available in the market has reduced its cost. However, it also can result in environmental problems if it is not suitable utilized. At present, some of the crude glycerol obtained from the biodiesel industry is purified to technical and pharmaceutical grade glycerol which has a higher market value. However, the profit margins are low. The crude glycerol is also used as an additive to animal feed. The use of glycerol as a substrate to potential value added products through bioconversion is limited, as certain toxic components in it limit microbial growth. Production of purer forms of byproduct glycerol, may allow its use directly and add to the revenues of integrated biodiesel and value added chemical production plants.

Table 3.3 summarizes the production of biodiesel and glycerol using homogeneous and heterogeneous catalysts. It can be seen from the table that when $CaO/Al_2O_3$ was used as catalyst, lower biodiesel conversion was produced, but high quality glycerol was obtained. In contrast , when CaO was used without support, comparatively higher biodiesel conversion (with almost the same quality as with support) was obtained, but glycerol obtained contained higher levels of impurities. The percentage FAME obtained with both catalysts are to levels (>96.5 wt.%) that would meet the biodiesel standards in Europe (EN 14214:2003) and Thailand (B.E. 2548 :2005) which is minimum of 96.5 wt.% [23, 41, 42]. One interesting fact observed in this work was that biodiesel conversion decreased when catalyst support was used. This can be due to a decrease in basic strength of catalyst due to its attachment to catalytic support. Even though attachment of metal oxide to alumina has shown to enhance the catalytic activity of a catalyst

in many of the cases [14, 16, 43], this is not always true. Apart from specific area and pore volume, surface basicity is the major determinant of catalyst activity [44, 45]. Thus, attaching of metal oxides to such support does not guarantee enhanced biodiesel conversion. Dall'Oglio et al. (2014) [46] have reported a comparative study between variety of metal oxide with and without support during the transesterification of sunflower oil. They found that when CaO was used alone it gave higher biodiesel conversion (100%) but produced a large amount of soap. When this was then attached to aluminum oxide and titanium oxide support, the amount of soap formation decreased significantly but the biodiesel conversion also declines to 46.2% and 36.6% respectively. They had also attributed this decreases to the lower basic strength of the supported calcium oxide.

Thus, for a biodiesel industry, making a choice of catalyst which produces biodiesel which meets fuel specification and at the same time a byproduct (glycerol) that can be used directly for bioconversion would be recommended. This would allow them to develop an integrated bioconversion process on-site and enhance profit margins.

### 3.4. Summary

The following conclusions and recommendations can be drawn from the results of all the experiments carried out in this study. Firstly, the optimum conditions of methanolysis of RBD palm oil using calcium oxide were 9 wt.% catalyst, with 12:1 molar ratio of methanol to oil, reaction time of 2 h and reaction temperature of 60 $^{0}$C. This resulted in 90.11% biodiesel conversion and 81.84% glycerol yield. Similarly, the optimum conditions with supported catalyst $CaO/Al_2O_3$ were 9% catalyst, with 15:1 molar ratio of methanol to oil, 3 h reaction time and 60 $^{0}$C. This produced the highest biodiesel conversion and glycerol yield at 86.38% and 79.32%, respectively. Secondly, use of wood ash from two different sources showed a potential ability to be utilized as an alternative to prevailing chemical based heterogeneous catalysts. Thirdly, the use of byproduct glycerol is often limited by the presence of large amounts of inhibitors. Although the methanolysis by using calcium oxide showed higher biodiesel conversion and glycerol yield than that using calcium oxide precursor supported on alumina, the catalyst with support exhibited higher purity of both biodiesel and glycerol produced. The purity of biodiesel meets the specifications required for blending with diesel directly. The

presence of lower impurities in glycerol has its advantages as it can be used directly for fermentation production to a number of value added products. Hence, CaO attached to alumina, which simultaneously produces a purer form of glycerol that can be used for production of value added compounds in the same premises (integrated system) is recommended. This will add to overall profits of such processes.

**References**


1. Zabeti M, Daud W, Aroua M (2009) Optimization of the activity of CaO/Al2O3 catalyst for biodiesel production using response surface methodology. Appl Catal A Gen 366:154–159. doi: 10.1016/j.apcata.2009.06.047
2. Dorado MP, Ballesteros E, Arnal JM, et al (2003) Exhaust emissions from a Diesel engine fueled with transesterified waste olive oil. Fuel 82:1311–1315. doi: 10.1016/S0016-2361(03)00034-6
3. Johnson DT, Taconi KA (2007) The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ Prog 26:338–348. doi: 10.1002/ep.10225
4. Ciriminna R, Pina C Della, Rossi M, Pagliaro M (2014) Understanding the glycerol market. Eur J Lipid Sci Technol 116:1432–1439. doi: 10.1002/ejlt.201400229
5. Santibáñez C, Varnero MT, Bustamante M (2011) Residual Glycerol from Biodiesel Manufacturing, Waste or Potential Source of Bioenergy: A Review. Chil J Agric Res 71:469–475. doi: 10.4067/S0718-58392011000300019
6. Ardi MS, Aroua MK, Hashim NA (2015) Progress, prospect and challenges in glycerol purification process: A review. Renew Sustain Energy Rev 42:1164–1173. doi: 10.1016/j.rser.2014.10.091
7. Zabeti M, Daud W, Aroua M (2009) Activity of solid catalysts for biodiesel production: A review. Fuel Process Technol 90:770–777. doi: 10.1016/j.fuproc.2009.03.010
8. Longloilert R (2008) Factors affection transesterification of palm oil into biodiesel in the presence of NaOH and ZrO2. Chulalongkorn University
9. Lotero E, Liu Y, Lopez DE, et al (2005) Synthesis of biodiesel via acid catalysis. Ind Eng Chem Res 44:5353–5363. doi: 10.1021/ie049157g
10. Sivasamy A, Cheah KY, Fornasiero P, et al (2009) Catalytic applications in the production of biodiesel from vegetable oils. ChemSusChem 2:278–300. doi: 10.1002/cssc.200800253
11. Bharathiraja B, Chakravarthy M, Kumar RR, et al (2014) Biodiesel production using chemical and biological methods – A review of process, catalyst, acyl acceptor, source and process variables. Renew Sustain Energy Rev 38:368–382. doi: 10.1016/j.rser.2014.05.084

12. Mbaraka IK, Shanks BH (2006) Conversion of oils and fats using advanced mesoporous heterogeneous catalysts. J Am Oil Chem Soc 83:79–91. doi: 10.1007/s11746-006-1179-x
13. Zabeti M, Daud WMAW, Aroua MK (2010) Biodiesel production using alumina-supported calcium oxide: An optimization study. Fuel Process Technol 91:243–248. doi: 10.1016/j.fuproc.2009.10.004
14. Ilgen O, Akin AN (2009) Development of Alumina Supported Alkaline Catalysts Used for Biodiesel Production. Turkish J Chem 33:281–287. doi: 10.3906/kim-0809-29
15. Peterson GR, Scarrah WP (1984) Rapeseed oil transesterification by heterogeneous catalysis. J Am Oil Chem Soc 61:1593–1597. doi: 10.1007/BF02541639
16. Sharma YC, Singh B, Korstad J (2011) Latest developments on application of heterogenous basic catalysts for an efficient and eco friendly synthesis of biodiesel: A review. Fuel 90:1309–1324. doi: 10.1016/j.fuel.2010.10.015
17. Chowdhury S, Mishra M, Suganya O (2015) The incorporation of wood waste ash as a partial cement replacement material for making structural grade concrete: An overview. Ain Shams Eng J 6:429–437. doi: 10.1016/j.asej.2014.11.005
18. Vassilev S V., Baxter D, Andersen LK, Vassileva CG (2010) An overview of the chemical composition of biomass. Fuel 89:913–933. doi: 10.1016/j.fuel.2009.10.022
19. OPG Ontario Power Generation- Atikokan Generating Station. http://www.opg.com/generating-power/thermal/stations/atikokan-station/Pages/atikokan-station.aspx. Accessed 24 Nov 2015
20. Mackiewicz SM, Ferguson EG (2005) Stabilization of Soil with Self-Cementing Coal Ashes. 2005 World Coal Ash (WOCA), April 11-15, 2005, Lexington, Kentucky, USA 1–7.
21. Sharma M, Khan AA, Puri SK, Tuli DK (2012) Wood ash as a potential heterogeneous catalyst for biodiesel synthesis. Biomass and Bioenergy 41:94–106. doi: 10.1016/j.biombioe.2012.02.017
22. Khan AI (2013) Fast Analysis of FAMEs Using Conventional GC Instrumentation. Thermo Fish. Sci.
23. DOEB Department of Energy Business B.E. 2548 (2005). http://www.poweringprogress.org/new/. Accessed 1 Dec 2015

24. Samart C, Chaiya C, Reubroycharoen P (2010) Biodiesel production by methanolysis of soybean oil using calcium supported on mesoporous silica catalyst. Energy Convers Manag 51:1428–1431. doi: 10.1016/j.enconman.2010.01.017
25. Umdu ES, Tuncer M, Seker E (2009) Transesterification of Nannochloropsis oculata microalga's lipid to biodiesel on Al2O3 supported CaO and MgO catalysts. Bioresour Technol 100:2828–2831. doi: 10.1016/j.biortech.2008.12.027
26. Bournay L, Casanave D, Delfort B, et al (2005) New heterogeneous process for biodiesel production: A way to improve the quality and the value of the crude glycerin produced by biodiesel plants. Catal Today 106:190–192. doi: 10.1016/j.cattod.2005.07.181
27. Potthast R, Chung CP, Mathur I (2009) Purification of glycerin obtained as a bioproduct from the transesterification of triglycerides in the synthesis of biofuel.
28. Hájek M, Skopal F (2010) Treatment of glycerol phase formed by biodiesel production. Bioresour Technol 101:3242–3245. doi: 10.1016/j.biortech.2009.12.094
29. Kouzu M, Yamanaka SY, Hidaka JS, Tsunomori M (2009) Heterogeneous catalysis of calcium oxide used for transesterification of soybean oil with refluxing methanol. Appl Catal A Gen 355:94–99. doi: 10.1016/j.apcata.2008.12.003
30. Boz N, Kara M, Sunal O, et al (2009) Investigation of the fuel properties of biodiesel produced over an alumina-based solid catalyst. Turkish J Chem 33:433–442. doi: 10.3906/kim-0809-28
31. Boey PL, Maniam GP, Hamid SA (2009) Biodiesel production via transesterification of palm olein using waste mud crab (Scylla serrata) shell as a heterogeneous catalyst. Bioresour Technol 100:6362–6368. doi: 10.1016/j.biortech.2009.07.036
32. Nantiyakul N (2007) Biodiesel production from rice bran oil using immobilized lipase biocatalyst. Asian Institute of Technology
33. Xie W, Peng H, Chen L (2006) Calcined Mg-Al hydrotalcites as solid base catalysts for methanolysis of soybean oil. J Mol Catal A Chem 246:24–32. doi: 10.1016/j.molcata.2005.10.008
34. Xie W, Peng H, Chen L (2006) Transesterification of soybean oil catalyzed by potassium loaded on alumina as a solid-base catalyst. Appl Catal A Gen 300:67–74. doi: 10.1016/j.apcata.2005.10.048

35. Liu X, He H, Wang Y, Zhu S (2007) Transesterification of soybean oil to biodiesel using SrO as a solid base catalyst. Catal Commun 8:1107–1111. doi: 10.1016/j.catcom.2006.10.026
36. BS EN 14103:2003 (2003) Fat and oil derivatives- Fatty acid methyl esters (FAME)- Determination of ester and linolenic acid methyl ester contents. Eur. Stand.
37. ASTM international (2006) Petroleum Products, Lubricants, and Fossil Fuels. Annu. B. ASTM Stand.
38. Naik TR, Kraus RN (2003) A New Source of Pozzolanic Material. Concr Int 25:55–62.
39. Reimann C, Ottesen RT, Andersson M, et al (2008) Element levels in birch and spruce wood ashes: green energy? Sci Total Environ 393:191–7. doi: 10.1016/j.scitotenv.2008.01.015
40. Regev L, Eckmeier E, Mintz E, et al (2011) Radiocarbon Concentrations of Wood Ash Calcite: Potential for Dating. Radiocarbon 53:117–127.
41. Biodiesel Standards. https://www.biofuelsystems.com/specification.htm. Accessed 12 Jan 2016
42. Knothe G (2006) Analyzing Biodiesel : Standards and Other Methods. JAOCS 83:823–833.
43. Lukić I, Krstić J, Jovanović D, Skala D (2009) Alumina/silica supported K2CO3 as a catalyst for biodiesel synthesis from sunflower oil. Bioresour Technol 100:4690–6. doi: 10.1016/j.biortech.2009.04.057
44. Sun H, Hu K, Lou H, Zheng X (2008) Biodiesel Production from Transesterification of Rapeseed Oil Using KF/Eu2O3 as a Catalyst. Energy & Fuels 22:2756–2760.
45. Guo F, Fang Z (2011) Biodiesel Production with Solid Catalysts. In: Stoytcheva M (ed) Biodiesel - Feed. Process. Technol. pp 339–358
46. Dall'Oglio EL, T. de Sousa Jr. P, Tiago de Jesus Oliveira P, et al (2014) Use of Heterogeneous Catalysts in Methylic Biodiesel Production Induced by Microwave Irradiation. Quim Nova 37:411–417. doi: 10.5935/0100-4042.20140081

# Chapter 4

## Bioconversion of crude glycerol to microbial lipid using a robust oleaginous yeast and effects of major impurities on lipid production by this strain

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[*]**Uprety BK**, Dalli SS, Rakshit SK (2017) Bioconversion of crude glycerol to microbial lipid using a robust oleaginous yeast *Rhodosporidium toruloides* ATCC 10788 capable of growing in the presence of impurities. Energy Convers Manag 135:117–128

**Abstract**

In this work, crude glycerol, a by-product from a biodiesel industry was characterized and converted to microbial lipid. The sample of crude glycerol was found to contain 44.56 wt.% glycerol, 13.86 wt.% methanol, 10.74 wt.% of ash and 32.97 wt.% of soap. *Rhodosporidium toruloides* ATCC 10788 was studied for the first time to determine its ability to grow on crude glycerol as a carbon source. The use of crude glycerol doubled the biomass (21.16 g/L) and tripled the lipid concentration (11.27 g/L) obtained using *Rhodosporidium toruloides* ATCC 10788, compared to pure glycerol. Methanol, as low as 0.5% (w/v), in crude glycerol severely affected the biomass and lipid obtained from this strain. Unlike many other oleaginous microorganisms, the ability of this yeast to consume other impurities present in crude glycerol enhanced the growth and lipid production in such a way that the effect of methanol was masked or became insignificant. The effect of major impurities in crude glycerol on lipid production ability of this strain was also studied. This strain was found to be tolerant to a salt levels of 1.5% (w/v) in the media. Soap and FAME present in crude glycerol had a positive influence on biomass and lipid production. *R. toruloides* ATCC 10788 also had the unique capacity to consume soap as a carbon source. While growing on 3.5% (w/v) of soap as the sole carbon substrate, 12.87 g/L of biomass and 5.40 g/L of lipid were obtained at the end of 168h. This study also helped us ascertain the reason for such a large improvement in biomass and lipid when crude glycerol containing large quantities of soap (32.97 wt.%) was used. The lipid obtained while growing this strain on crude glycerol contained 47.16% of oleic acid (a monounsaturated fatty acid), making it a very good feedstock for some oleochemical industries and the production of biodiesel. This bioconversion process which involves the production of lipid from crude glycerol waste stream will be of great interest to the biodiesel industry as it can be easily integrated into the existing biodiesel production plant. The unique ability of this strain to consume soap can be exploited to treat waste effluents rich in soap such as obtained from car wash centers, soap manufacturing and textile industries.

## 4.1. Introduction

Fluctuating prices of fossil based diesel, depletion of its sources, environmental issues and increasing energy demand make biodiesel an important alternative renewable source of energy [1–3]. Most developed countries have encouraged its use by implementing policies which mandate its blending (generally between 2- 20% v/v) with fossil based diesel [4, 5]. As a result, production of biodiesel has increased substantially across the world in the last decade [6]. However, biodiesel is still costlier than its fossil based counterpart and this constraint has to be addressed in order to make it more economically acceptable [1, 7]. Another issue related to the use of biofuels like biodiesel is that arable land is used to grow the required oil crops. This has been perceived as a threat to food security leading to "food vs fuel" debates [8, 9]. At present, the majority of biodiesel industries utilize vegetable based oils such as canola, rapeseed, and corn. These feedstocks are costly and contribute to approximately 70-85% of the overall biodiesel production process [10, 11]. Cheaper feedstocks for biodiesel production can bring down the overall cost of production and make it competitive to fossil based diesel. Ideally, the alternate feedstock should not compete with food crops used at present [7, 11, 12].

Utilization of single cell oil (SCO) or microbial lipid as a non-food feedstock for biodiesel industries has gained importance in recent times as its chemical component is similar to vegetable based oils used for biodiesel production [13–17]. Various microorganisms (algae, yeast and fungi) are capable of accumulating lipids to levels more than 20 wt.% of their biomass. Such microbes are called oleaginous microorganisms and the lipids they produce are known as single cell oils or microbial lipids [11, 18, 19]. Use of microbial lipids for biodiesel production has many advantages over plant based oils because their production life-cycles are short, they do not require the utilization of large land areas for their production, are unaffected by climatic condition and require comparatively less manpower [6, 14, 20]. However, techno-economic evaluations of such processes (i.e. biodiesel from single cell oil) show that this production route also depends on the cost of feedstock. The use of cheap feedstocks to produce such oil is thus very important to make the processes economically feasible [18, 19].

Numerous alternative resources including wastewater from breweries and municipality waste, whey from diary industries, and crude glycerol from biodiesel industries have been investigated

for microbial oil production [15, 21–25]. Crude glycerol from biodiesel industries is the focus of considerable attention as it is easily available and cheap. The biodiesel industry generates approximately 10% (w/w) of glycerol with every batch of biodiesel produced [26, 27]. With increased production of biodiesel over the past decade, there has been glut of glycerol in the market. Even though crude glycerol can be purified to different grades for its use in pharmaceutical, food and cosmetic industries, the purification processes are always costly and thus not suitable for small and medium sized plants [20, 26–29]. The availability of crude glycerol has also resulted in a slump in pure glycerol prices [28]. Chemical or biological conversion of such impure crude glycerol into various products have been reported [30]. However, such processes are inhibited by the presence of various impurities in crude glycerol, mainly methanol, soap, free fatty acids and salt [20, 31–37]. At present, crude glycerol holds a very low value and is even treated as a waste by biodiesel industries [38]. The proposed use of crude glycerol for the production of microbial lipid is a highly beneficial and attractive option. This is because utilization of crude glycerol to produce lipid and its subsequent conversion into biodiesel can be easily integrated into existing biodiesel plants. This can save transportation and disposal costs of this by-product as well as contribute to an increase in the profits of the industry. However, the design of an integrated plant for the conversion of crude glycerol to biodiesel (via. the production of microbial lipid) has to address an important issue. Due to the variation in the feedstocks used and the methods applied to produce biodiesel in industry, the type and concentration of impurities present in crude glycerol differ. Hence, selecting a robust strain that can grow in high concentrations of impurities is extremely important.

In this study, crude glycerol obtained from a biodiesel producer in Ontario (Canada) has been characterized using various techniques. The crude glycerol was found to contain very high concentrations of impurities (mainly methanol, salt and soap). These values are higher than many other crude glycerol samples that have been used by various researchers in similar type of work [39–42]. Utilization of such a sample of crude glycerol in our experiments allows us to confirm the robustness of the microbial strain used. In this study, *Rhodosporidium toruloides* ATCC 10788 (also known as *Rhodosporidium toruloides* CBS 14) was grown on crude glycerol (without purification) to produce microbial lipid which is suitable for biodiesel production. To the best of our knowledge, it is the first time this strain has been grown on crude glycerol. Even

though many researchers in the past reported the ability of this strain to grow in various carbon substrates such as pure glycerol, glucose, xylose and hydrolysate obtained from pre-treatment of straw, there are no reports of this strain being utilized to grow in crude glycerol from biodiesel industry [43–47]. Its ability to grow and accumulate high concentrations of lipids in the presence of high levels of impurities is reported in this paper. Furthermore, we have reported detailed experiments on the effects of some of the major components present in crude glycerol on its biomass and lipid production ability. The ability of this strain to utilize some of the impurities in crude glycerol and significantly improve the growth and lipid production was not expected. To the best of our knowledge, no such studies have been previously carried out on this specific strain of *R. toruloides*. Similar studies have been previously carried out on different strains of *R. toruloides* [20, 48]. However, MUFA content of lipids obtained from both these strains are quite low. More importantly, the impurities levels at which *R. toruloides* ATCC 10788 was able to grow and produce lipids is high making it a very good strain for potential application in industry.

## 4.2. Materials and methods

### 4.2.1. Microorganism and chemicals

The oleaginous microorganism *R. toruloides* ATCC 10788 was obtained from the American Type Culture Collection (ATCC). It was grown in YPG media (10 g/L yeast extract, 20 g/L peptones and 20 g/L glycerol; 30 $^0$C, 24 hours, 200 rpm) and maintained at -80 $^0$C in the presence of glycerol (50% v/v) before subsequent use. All the chemicals and reagents used in these experiments were analytical grade and were obtained from Fisher Scientific, Canada. Crude glycerol samples were provided by a biodiesel producer located in Ontario, Canada.

### 4.2.2. Culture conditions

The inocula for the fermentation were prepared by growing the organism in YPG media (10 g/L yeast extract, 20 g/L peptones and 20 g/L glycerol) for 48 hours in a rotary shaker maintained at 200 rpm, 30 $^0$C until the viable cell count reached about 1 x $10^8$ cells/mL.

The shake flask experiments were carried out in minimal medium prepared in distilled water and contained (g/L): $KH_2PO_4$ 1.0, $Na_2HPO_4.12H_2O$ 1.0, $MgCl_2.6H_2O$ 1.5, pH 6.0 [49]. Pure

glycerol was used as a carbon source at a concentration of 40 g/L, unless otherwise specified. The media was also supplemented with 10 mL/L of trace elements. The supplemented trace elements solution consisted of $CaCl_2.2H_2O$ 4.0 g, $FeSO_4.7H_2O$ 0.55 g, Citric acid. $H_2O$ 0.52 g, $ZnSO_4.7H_2O$ 0.10 g, $MnSO_4.H_2O$ 0.076 g, and 18M $H_2SO_4$ 100 µl (in 1L of distilled water) [50]. Ammonium chloride ($NH_4Cl$) was used as a nitrogen source maintaining an overall carbon to nitrogen (C: N) molar ratio of 100. Most experiments were carried out using crude glycerol as the carbon source instead of pure glycerol. The amount of glycerol in the media was 48.2 g/L, unless otherwise specified. The designation of the different media used in this study is given in Table 4.1.

Table 4.1. Glycerol, methanol and soap concentration of various types of media used in this study

| **Media (Designation)** | **Glycerol percentage in the media (g/L)** | **Methanol in the media (% w/v)** | **Soap in the media (% w/v)** |
|---|---|---|---|
| Pure glycerol (PGA) | 16.1 | - | - |
| Pure glycerol (PGB) | 32.2 | - | - |
| Pure glycerol (PGC) | 48.2 | - | - |
| Pure glycerol with methanol (PGM) | 40 | 0.25 to 2.5 | - |
| Diluted crude glycerol (CGA) | 16.1 | 0.5 | 1.1 |
| Diluted crude glycerol (CGB) | 32.2 | 1 | 2.3 |
| Diluted crude glycerol (CGC) | 48.2 | 1.5 | 3.5 |
| Dilute crude glycerol topped up with pure glycerol (CGAT) | 48.2* | 0.5 | 1.1 |
| Dilute crude glycerol topped up with pure glycerol (CGBT) | 48.2** | 1 | 2.3 |
| Pure glycerol with 3.5% (w/v) soap and pure glycerol (PGS) | 48.2 | - | 3.5 |
| Pure glycerol with only 3.5% (w/v) (PSNG) | - | - | 3.5 |

*16.1 g/L crude glycerol + 32.1 g/L pure glycerol; ** 32.2 g/L crude glycerol + 16.0 g/L pure glycerol

All other condition remained the same as that for the pure glycerol experiment. Crude glycerol, however, contained other impurities like soap, ash, salt, fatty acid methyl esters, etc. at levels reported in Table 4.4. Analyses were done in duplicates and percentage errors were expressed as mean ± standard deviation.

### 4.2.3. Analytical methods

#### 4.2.3.1. Determination of physical characteristics of crude glycerol

The density of crude glycerol was determined by using pycnometer at room temperature (25 ± 0.5 $^0$C). A 2% (w/v) solution of crude glycerol was used for measuring the pH (Sartorius PB-11 pH meter) at room temperature (23 ± 0.5 $^0$C). The viscosity of crude glycerol was measured at 25 ± 0.5 $^0$C according to the ASTM D 4878-08 method using a Brookfield viscometer (model: LVDVE).

#### 4.2.3.2. Determination of chemical composition of crude glycerol

##### 4.2.3.2.1. Soap content determination

For the determination of the soap content, 500 mg of crude glycerol sample was dissolved in 100 mL of acetone containing 2% (v/v) distilled water. In order to neutralize the free catalyst in this solution, it was first titrated against 0.1M HCl using 1% (w/v) phenolphthalein indicator (in isopropyl alcohol) until the red color of the solution disappeared. The volume of titrant consumed in this step was designated as "A". Once the solution became colorless, 1 mL of bromophenol blue indicator (0.4% w/v in water) was added to the neutralized solution and titration was continued using 0.1 M HCl until the second indicator color changed from blue into yellow. The volume of titrant consumed in the second titration (designated as "B") to change the color of bromophenol indicator from blue to yellow was used to calculate the soap content by using the following formula [51]:

$$\% \text{ Soap (as sodium oleate)} = \frac{Volume\ of\ titrant\ "B"\ (mL)\ x\ 0.001\ x\ 0.1\ x\ 304.04}{Amount\ of\ sample\ taken\ (g)} \text{ x } 100$$

#### 4.2.3.2.2. Water and ash content determination

For the determination of water content in crude glycerol sample, 0.5 g of sample was taken and volumetric Karl Fischer titration was carried out using a T70 automatic titration system (Mettler Toledo Karl Fisher titrator, Columbus). The ash content of crude glycerol was determined by incinerating approximately 1 g of sample in a furnace at 750 °C for 3 h.

#### 4.2.3.2.3. Elemental analysis

The C, H, N and S content of crude glycerol were determined by using an Elementar Vario EL Cube analyzer. Measurement of Na, Mg, Al, P, K, Ca, Mn, Fe, Co, Cu, and Zn was done using the ICP-AES Varian located at the Lakehead University Instrumentation Lab (LUIL). For the latter analysis, the ash from a crude glycerol sample was digested in a CEM MARSXPRESS microwave oven in I-CHEM vials using nitric and hydrochloric acid [52]. After digestion, the samples were diluted to 40 mL with double distilled water and analysed using an ICP-AES.

#### 4.2.3.2.4. Quantification of glycerol and methanol

Glycerol and methanol were determined quantitatively by using an Agilent 1260 infinity HPLC with an Aminex ion-exchange Biorad column (HPX-87H, 300 x 7.8mm) and a Refractive Index (RI) detector. Operating conditions as described in chapter 3 (section 3.2.7.) were adopted for the analysis of both these components.

#### 4.2.3.2.5. Determination of FAME

Sample preparation for the determination of fatty acid methyl esters (FAME) content of crude glycerol was carried out by adopting the method reported by Hu et al. (2012) with slight modification [53].

FAME analysis was carried out using a Gas Chromatograph (Thermo Scientific, Trace 1300 model, polar column TG WaxMS A; 30 m x 0.25 µm x 0.25 µm) equipped with a FID detector. Approximately 100 mg of prepared sample was taken. To this sample, 2 mL of 5 mg/mL of methyl nonadecanoate (in hexane) was added as an internal standard. The parameters we reported in chapter 3 were adopted for the GC analysis. Helium was used as the carrier gas at a flow rate of 1 mL/min and a sample injection split ratio of 50:1 was adopted. The temperature

gradient maintained in the oven was 100 $^0$C (0.25 min), 30 $^0$C/min, 220 $^0$C (0 mins) and 10 $^0$C/min, 250 $^0$C (3 mins). Air flow of 350 mL/min, $H_2$ at 35 mL/min, and $N_2$ at 40 mL/min were adopted throughout the runs. Each fatty acid methyl ester component was identified using standard mixtures including methyl nonadecanoate which served as the internal standard.

#### 4.2.3.2.6. Determination of Free Fatty Acid (FFA) content

The FFA contents of crude glycerol were determined using a modified AOCS Official Method Ca 5a-40 [54]. 0.5 g of crude glycerol was dissolved in 100 mL of ethyl alcohol and titrated against 0.1 N KOH in the presence of a phenolphthalein indicator (1% w/v in ethanol) until a pink color appears. FFA was then calculated using the formula below,

$$\text{FFA} = \frac{Volume\ of\ alkali\ consumed\ (mL)\ x\ Alkali\ normality\ x\ 28.2}{weight\ of\ sample\ taken\ (g)} \text{ x } 100$$

#### 4.2.3.3. Determination of biomass, lipid and characterization of lipid

In order to estimate the yeast dry biomass weight, 5 mL of fermentation broth was centrifuged for 10 minutes at 4400 rpm. The supernatant was discarded and the wet cells were washed using deionized water, dried overnight at 80 $^0$C and weighed. Lipid accumulated inside the biomass was estimated by using the Bligh-Dyer method with some modifications [55, 56]. 25 mg of dried biomass was taken in a 2 mL vial and 0.33 mL of 4M HCl was added. This was kept at 80 $^0$C in a water bath for 1 hour. After an hour, the sample was centrifuged at 6000 rpm for ten minutes and the supernatant was disposed of. To the biomass, 0.2 mL of methanol and 0.1 mL of chloroform were added and vortexed for 2 minutes. After this, 0.1 mL of chloroform was added to the above mixture and rigorously shaken for another 2 minutes. Subsequently, 0.18 mL of distilled water was added to this mixture of chloroform and methanol to make the final chloroform: methanol: water ratio of 2:2:1.8. The solution mixture was then centrifuged at 6000 rpm for 10 minutes and the chloroform layer was withdrawn using a Pasteur pipette and collected in a pre-weighed vial. The extraction was again repeated by adding 0.2 mL of 10% (v/v) methanol solution (in chloroform) into the remaining layer of methanol and water. Finally, the collected chloroform layers from both the extraction stages were combined and dried at 105 $^0$C for 4 hours and the final weight of the vial was taken. The difference between the initial

weight and final weight of the tube was used to obtain the lipid extracted from the sample.

Extracted lipid was trans-esterified using the method reported by Ichihara & Fukubayashi (2010) [57]. Approximately 1 mg of the extracted lipid was dissolved in 0.20 mL of toluene. To this solution, 0.20 mL of methyl nonadecanoate (5 mg/mL) was added as internal standard. This was followed by the addition of 1.5 mL of methanol and 0.30 mL of 8% (w/v) HCl solution in methanol. This mixture was then maintained at 100 $^0$C for 1 hour. At the end of 1 hour, the solution was allowed to cool to room temperature and was extracted using 1 mL of hexane and 1 mL of water. The hexane layer was used for fatty acid characterization using GC-FID. The GC parameters mentioned in chapter 3 (section 3.2.7) was adopted. Biomass concentration ($C_B$), Lipid concentration ($C_L$), and Lipid content ($C_Y$) were defined as follows;

$$\text{Biomass concentration; } C_B\,(\text{g/L}) = \frac{weight\ of\ dried\ biomass\ (g)}{volume\ of\ fermentation\ broth\ (L)}$$

$$\text{Lipid concentration; } C_L\,(\text{g/L}) = \frac{weight\ of\ lipid\ (g)}{volume\ of\ fermentation\ broth\ (L)}$$

$$\text{Lipid content; } C_Y\,(\%\text{ wt.}) = \frac{weight\ of\ lipid\ (g)}{Weight\ of\ dried\ cell\ biomass\ (g)} \text{ x } 100\%$$

### 4.2.4. Production of soap and biodiesel used in this study

For the preparation of soap, 13.2 g of sodium hydroxide was dissolved in 35 ml of distilled water in a 250 ml beaker and the solution allowed to cool down to approximately 40 $^0$C. 100 ml of preheated canola oil (~ 40 $^0$C) was then added into it. Mixing of oil into the basic solution was facilitated with gentle stirring and this was continued until the mixture began to thicken. Once the mixture turned thick (also known as trace stage), the slurry was left at room temperature for 7 days. Biodiesel (FAME) was prepared from canola oil using sodium hydroxide as the catalyst as per method described in our previous work [26].

## 4.3. Result and discussion

### 4.3.1. Characterization of crude glycerol

The physical and chemical composition of crude glycerol from biodiesel industries vary due to variation in the type of feedstock, type of catalyst used, transesterification efficiency and the

type of downstream processing used during the biodiesel production [1, 58, 59]. For example, characterization of crude glycerol from various biodiesel industries across Australia by Hansen et al. (2009) showed the glycerol, methanol and ash content to be in the range of 38-96 wt.%, < 0.01-13.94 wt.%, and 0-29.4 wt.% respectively [60]. In addition to methanol and ash, the crude glycerol may also contain soap, catalysts, salts, water, free fatty acids, fatty acid methyl esters (FAME), mono-glycerides and diglycerides [1, 53].

In this work, initial physical characterization of crude glycerol (result shown in Table 4.2) showed that it was more viscous and had lower density than pure glycerol. The low density of crude glycerol is due to the presence of some lighter impurities such as fatty acids methyl esters (FAMEs), fatty acids, methanol, and water [53]. The higher pH value (i.e. 10.41± 0.09) of crude glycerol was due to the presence of residual alkali (NaOH or KOH) used during the production of biodiesel.

Table 4.2. Comparison of physical characteristics of crude and pure glycerol

| **Physical properties** | **Crude glycerol (wt.%)** | **Pure glycerol (wt.%)** |
|---|---|---|
| Color | Dark brown | Colorless |
| Density at 25 $^0$C (g/cm$^3$) | 1.06 ± 0.01 | 1.26* |
| pH | 10.41 ± 0.09 | 5.71±0.04 |
| Viscosity at 25 $^0$C (centipoise) | 1600 ± 14.14 | 885±7.07 |

*Value obtained from [61]

Elemental analysis of crude glycerol sample (Table 4.3) shows that 15.63 wt.% of carbon is present in the samples used in this study. This value is slightly lower than that reported by Hu et al. (2012) based on analysis of crude derived from five different biodiesel industries [53]. They reported the carbon content to be in the range of 24.3 to 54.2%. Similarly, Thompson & He (2006) [59] reported the percentage carbon in crude glycerol (obtained by transesterification of seven different types of oil with methanol) to be in the range of 24 to 37.7 wt.%. The low value of carbon obtained in our study (i.e. 15.63 wt.%) compared to previous studies imply that

the total carbon contributing factors such as glycerol, FAME, methanol, diglycerides, monoglyceride, FFA and residual triglycerides in crude glycerol were higher in those studies. The nitrogen value obtained in this study (i.e. 0.03 wt.%) is similar to that reported by Thompson and He (2006), which ranged between 0.04 - 0.12 wt.% [59]. Additionally, from Table 4.3 we can also see that the crude glycerol sample used in this study contains very high concentration of sodium metal (i.e. 376062.00 ppm). The high residual amount of such metals can be due to the fact that most of the biodiesel industries use basic homogeneous catalysts (like sodium or potassium hydroxide) [1, 62–65].

Table 4.3. Elemental analysis of crude glycerol obtained from a local biodiesel industry

| Description | Minimum Detection Level (MDL) | Measured value |
|---|---|---|
| Ca (ppm) | 0.06 | 2592.82± 266.86 |
| Co (ppm) | 0.20 | < Detection limit |
| Cu (ppm) | 0.05 | 4.96±0.94 |
| Fe (ppm) | 0.05 | 192.93±3.92 |
| Mg (ppm) | 0.20 | 31.29±18.29 |
| Mn (ppm) | 0.05 | 3.22±0.36 |
| Na (ppm) | 0.20 | 376062.00± 9073.59 |
| P (ppm) | 0.00 | 622.96±27.68 |
| Zn (ppm) | 0.03 | 25.79±1.73 |
| C (wt. %) | 0.01 | 15.63±1.31 |
| N (wt. %) | 0.01 | 0.03±0.01 |

HPLC analysis of crude glycerol showed that it contains 44.56 wt.% of glycerol and 13.86 wt.% of methanol. Details of the chemical components present in the crude glycerol sample are shown in Table 4.4. Methanol, salt and soap are considered inhibiting components in crude glycerol for its bioconversion to value added products [28, 66]. The amount of methanol (13.86 wt.%) and soap (32.97 wt.%) present in our sample was much higher than many of the crude glycerol samples reported in previous studies [36, 38, 40]. The reason for higher amount of such impurities in our sample can be due to a variety of factors including type of feedstock used, type

of catalyst used, size of biodiesel production plant, lesser downstream processing, etc. However, this made the crude glycerol used in these experiments a good sample to test the robustness of the oleaginous yeast used in this study.

Table 4.4. Comparison of chemical composition of crude and pure glycerol

| **Composition** | **Crude glycerol (wt.%)** | ***Pure glycerol (wt.%)** |
|---|---|---|
| Glycerol | 44.56±0.45 | 100.3 ± 2.6 |
| Methanol | 13.86±0.83 | BDL |
| Ash Content | 10.74±0.34 | BDL |
| % Free fatty Acid | 0.48±0.05 | BDL |
| Soap (as Sodium oleate) | 32.97±0.06 | BDL |
| Water | 0.82±0.01 | BDL |
| FAME | 4.38±0.05 | BDL |
| **Total** | **107.81** | **100.3** |

*Values adapted from [67]; BDL: Below Detection level.

It should be noted that in Table 4.4 the sum of all components present in crude glycerol makes a total of 107.81 wt.%. In the determination of soap content via titration method, the molecular weight of sodium oleate is used to convert acid number into soap content, assuming that all the soaps present in crude glycerol is in the form of sodium oleate. As the fatty acid composition of soap present crude glycerol depends on type of feedstock used for biodiesel production this assumption is not very accurate, though it is often used in order to simplify cumbersome calculations. The use of higher molecular weight of sodium oleate for the determination of soap calculation results in the overestimation of soap present and the total composition of crude glycerol [67]. Another reason for this is that compounds such as sodium or potassium present in crude glycerol are accounted for more than once, for example, during the determination of soap and ash content [53]. This causes the sum of all the components present in crude glycerol to be over 100 wt.%.

### 4.3.2. Effect of methanol on the growth of *R. toruloides* ATCC 10788 in pure glycerol

After characterization of crude glycerol, a well-studied oleaginous microbial yeast strain *R. toruloides* ATCC 10788 was selected to study its growth on crude glycerol. To understand the inhibiting effect of methanol, this strain was initially grown at various concentration of methanol (up to level of 2.5% w/v; PGM) (Fig. 4.1). Minimal media was used with 40 g/L of pure glycerol as a carbon source and 0.697 g ammonium chloride as a nitrogen source thus maintaining a C: N molar ratio of 100. Fermentation was carried out in shake flask culture for 192 hours at 30 $^0$C and shaker speed of 200 rpm. As the stationary phase of biomass production was reached after 168 h of fermentation (Fig 4.1), all the experimental results were calculated on the basis of this amount of time.

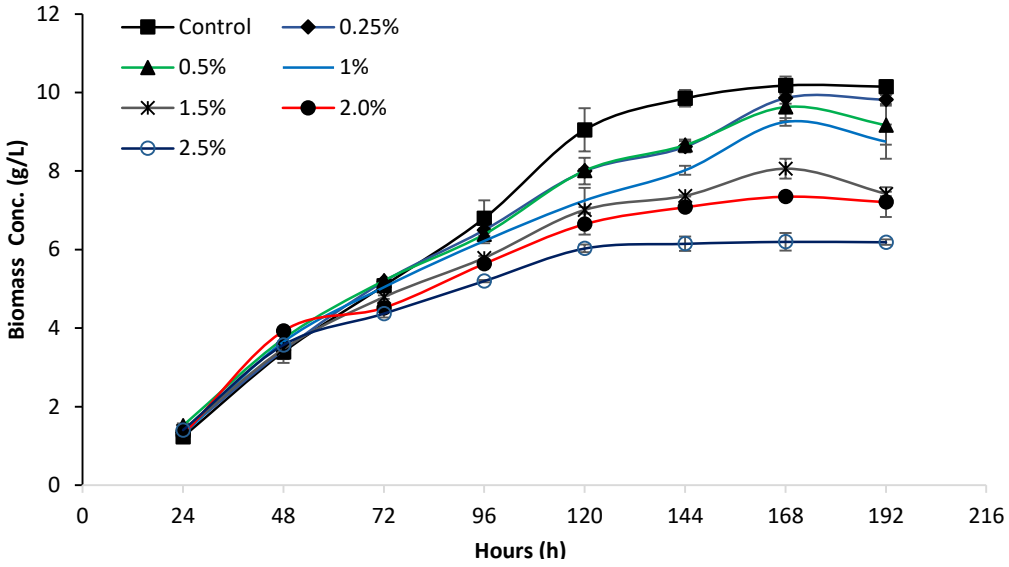



Fig 4.1. Biomass growth curves of *R. toruloides* ATCC 10788 on 40 g/L pure glycerol with pure methanol (PGM) concentrations in the range of 0.25 to 2.5% (w/v). The control media contained 40 g/L pure glycerol but no methanol

From Fig 4.1 and 4.2, it can be seen that with the increase in methanol concentration there was decrease in biomass and lipid production. In the absence of methanol in the media (i.e. control), at the end of 168 h of fermentation time, *R. toruloides* ATCC 10788 produced 10.18 g/L, 3.73 g/L and 34.08 wt.% of biomass, lipid concentration and lipid content respectively. With the increasing levels of methanol, the biomass produced, lipid concentrations and lipid content

decreased by about 8–41%, 19–53%, and 7–23%, respectively (Fig 4.2).

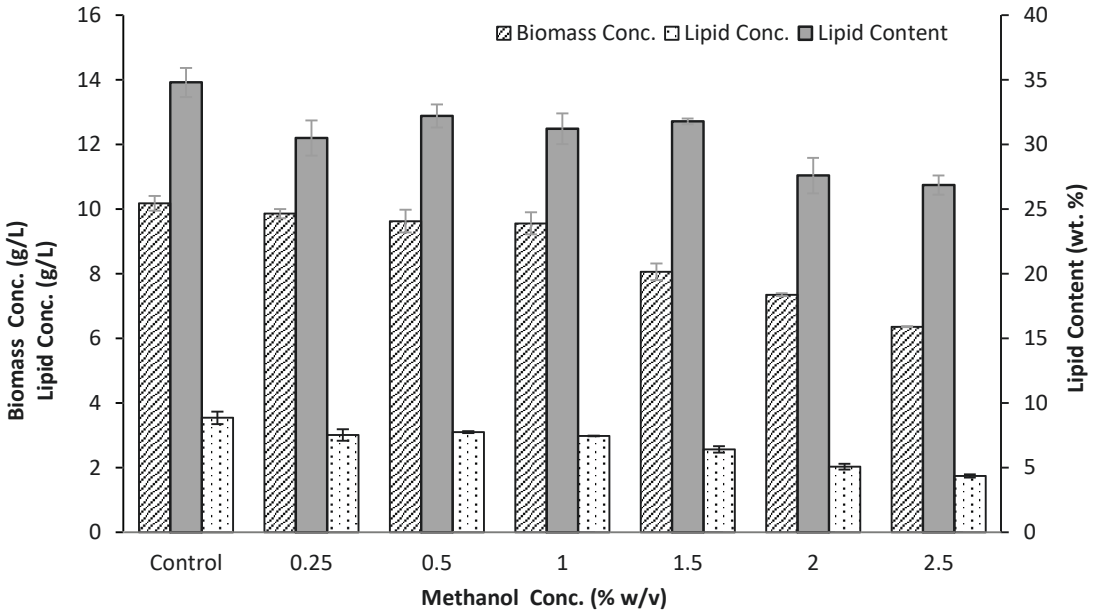



Fig 4.2. Final biomass concentration (g/L), lipid concentration (g/L) and lipid content (wt.%) produced by *R. toruloides* ATCC 10788 on 40 g/L pure glycerol after 168 hours and methanol in the range of 0.25 to 2.5% (w/v). The control media contained 40 g/L pure glycerol but no methanol.

Similar effects of methanol on biomass growth of other strains of this species have been reported previously [20, 36, 68] . For instance, Xu et al. (2012) reported a decrease in overall biomass, lipid concentrations and lipid yield (content) of *Rhodosporidium toruloides* AS2.1389, a type of oleaginous yeast, by about 0–5%, 6–24%, and 6–23% respectively, when three different concentration of methanol (4 g/L, 8 g/L and 16 g/L) were added [20]. Similarly, Pyle et al (2008) [36] reported a decrease in biomass and docosahexaenoic acid (DHA) yield, in case of oleaginous algae *Schizochytrium limacinum* SR-21, at high concentration of methanol (> 1% w/v) in the media**.** Although the exact mechanism of the action of methanol on oleaginous microbes is not well understood, it is hypothesized that methanol is absorbed into the cell and metabolized. Metabolism of methanol produces certain products which influences biomass production and lipid accumulation ability of microbes [20, 38]. With this background, the effect of methanol on the biomass production and lipid accumulation ability of this organism at different phases of its growth was further studied. Since significant amount of biomass concentration changed only when the methanol level reached 1.5% (w/v) in the media (Fig 4.1

and 4.2), the change in biomass concentration, lipid concentration and lipid content was measured on a regular interval for 192 hours in media containing 1.5% (w/v) methanol (Fig 4.3). It was found that the biomass concentration increased with time and remained constant beyond 120h. Whereas a significant amount of lipid was found to be produced (measured as lipid concentration) after 72 hours, it increased with time and remained constant after 168 hours.

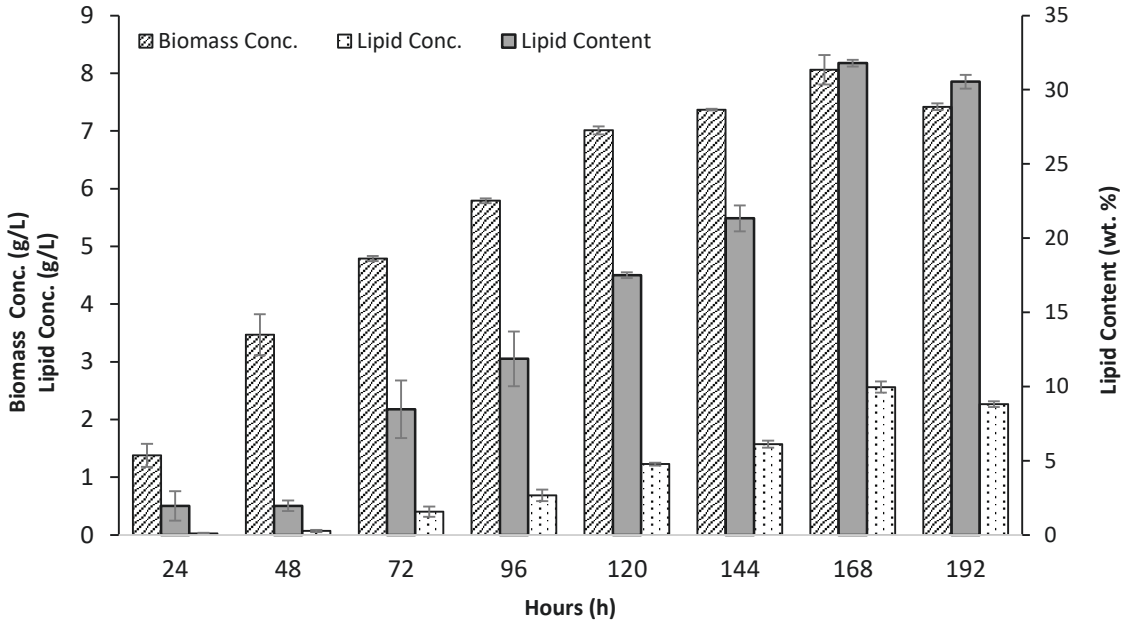
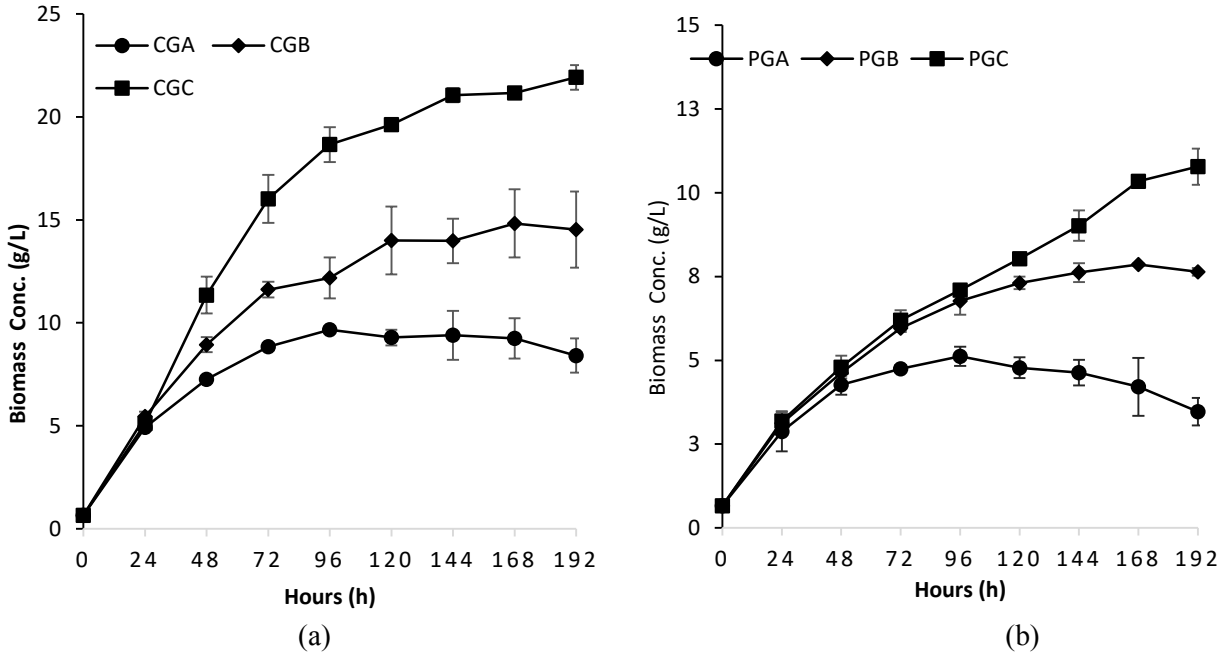



Fig 4.3. Biomass and lipid produced by *R. toruloides* ATCC 10788 in minimal media containing 40 g/L pure glycerol and 1.5% (w/v) pure methanol at various time interval.

### 4.3.3. Growth of *R. toruloides* ATCC 10788 in crude glycerol and the effect of impurities present

After understanding the effect of methanol on the biomass and lipid accumulation by *R. toruloides* ATCC 10788 (section 4.3.2) on pure glycerol, this organism was grown in minimal salt media with crude glycerol as a carbon source. As found in section 4.3.2, the presence of very small amounts of methanol in the media (i.e. 0.5% w/v) could reduce the biomass production and this growth inhibition becomes significant when the methanol concentration reaches 1.5% (w/v). Keeping this in mind, for the study involving crude glycerol, three different types of media with increasing concentration of methanol from (0 to 1.5% w/v) were prepared by diluting the crude glycerol (originally containing 44.56 wt.% glycerol and 13.86 wt.%

methanol) to different extents. The crude glycerol media diluted to 0.5% (w/v), 1% (w/v), and 1.5% (w/v) methanol were designated as CGA, CGB and CGC respectively. The amount of glycerol present in CGA, CGB and CGC media were found to be 16.1 g/L, 32.2 g/L and 48.2 g/L respectively. The control media for CGA, CGB and CGC were prepared using pure glycerol and were designated as PGA, PGB and PGC respectively and contained pure glycerol at a concentration of 16.1 g/L, 32.2 g/L and 48.2 g/L respectively. No methanol was added to these media samples. The pure glycerol control experiments without methanol would allow us to understand the overall effect of impurities (including methanol) upon the biomass production at three different levels of methanol concentration and other impurities. The biomass concentration, lipid concentration and lipid content of *R. toruloides* ATCC 10788 grown on crude glycerol media and their controls are shown in Fig 4.4(a) and (b). From Fig 4.4(a) and (b), it is seen that irrespective of the type of glycerol used (i.e. pure or crude glycerol), there was an increase in biomass production with the increasing glycerol concentration. At 168 h, the biomass obtained with PGA (16.1 g/L of pure glycerol) was 4.21 g/L whereas it increased to 10.34 g/L when pure glycerol concentration increased to 48.2 g/L (Fig 4.4b). Similar trends were obtained when crude glycerol was used instead of its purer form (Fig 4.4a). At 168 h, with CGA media, biomass obtained was 9.25 g/L which increased to 21.16 g/L when CGC was used. More interestingly, however, was the huge improvement in biomass observed at equal levels of glycerol concentration in crude glycerol compared to pure glycerol. For instance, when one compares PGC to CGC media, biomass produced nearly doubled from 10.34 g/L to 21.16 g/L. Lipid content of the obtained biomass also increased by 56.9% when CGC media was used instead of PGC. Lipid content of biomass obtained with CGC and PGC media were found to be 53.28 wt.% and 33.95 wt.% respectively. Similar increments were observed in CGA and CGB media compared to PGA and PGB respectively. Thus, instead of inhibiting growth, the impurities present in the crude glycerol media the microorganism seem to enhance the amount of biomass and lipids produced.




(a) (b)

Fig 4.4. Biomass produced by *R. toruloides* ATCC 10788 on minimal media containing crude glycerol diluted to different levels and pure glycerol. Figure 4 (a), growth on crude glycerol CGA, CGB, and CGC in which crude glycerol was diluted to bring the methanol level to 0.5%, 1% and 1.5% (w/v) respectively and the resultant glycerol content was 16.1 g/L, 32.2 g/L and 48.2 g/L respectively. Figure 4 (b), growth in pure glycerol media (control) PGA, PGB and PGC containing 16.1 g/L, 32.2 g/L and 48.2 g/L of pure glycerol respectively and containing no methanol.

This is further confirmed when one compares the biomass concentration obtained with CGC to that in pure glycerol with 1.5% (w/v) methanol (Fig 4.2). Biomass concentration in the former was much higher than the pure glycerol media with similar methanol level (i.e. 1.5% w/v). In section 4.3.2 (Fig 4.2), we found that addition of 1.5% (w/v) of methanol into the pure glycerol media decreased the biomass from 10.18 g/L (i.e. in control experiment with no methanol) to 8.06 g/L. However, in case of CGC, the biomass obtained was 21.16 g/L, approximately 105% more than that obtained with pure glycerol media (i.e. PGC control media). From this we can infer that that presence of various impurities in crude glycerol supported the growth in such a way that the effect of methanol was either masked or became insignificant. This allows the microorganism to grow even at higher concentration of methanol (i.e. 1.5% w/v). Thus, it is apparent that in the presence of various impurities present in crude glycerol, *R. toruloides* ATCC10788 can grow well even in the presence of 1.5% (w/v) methanol.

In order to understand this better, another experiment was carried out. In this experiment, two of the crude glycerol media, CGA and CGB, were topped up with pure glycerol to bring the glycerol levels equal to that present in CGC (i.e. 48.2 g/L glycerol). The topped up CGA and CGB media were designated as CGAT and CGBT and contained 48.2 g/L of glycerol. CGAT and CGBT thus had similar glycerol concentration but differing amounts of methanol (0.5% and 1% w/v) and other impurities. Growth of *R. toruloides* in CGAT and CGBT were found to be less than that obtained with CGC media (Fig 4.5). At the end of 168 h, the biomass obtained with CGC (crude glycerol media) was 21.16 g/L whereas that obtained with CGAT and CGBT were found to be 10.59 g/L and 12.75 g/L respectively. From this, we can infer that other than glycerol and nitrogen, the other impurities (e.g. soap, ash, FAME, salt, etc.) found in crude glycerol served as a carbon and nitrogen sources and enhanced the growth of this microorganism.

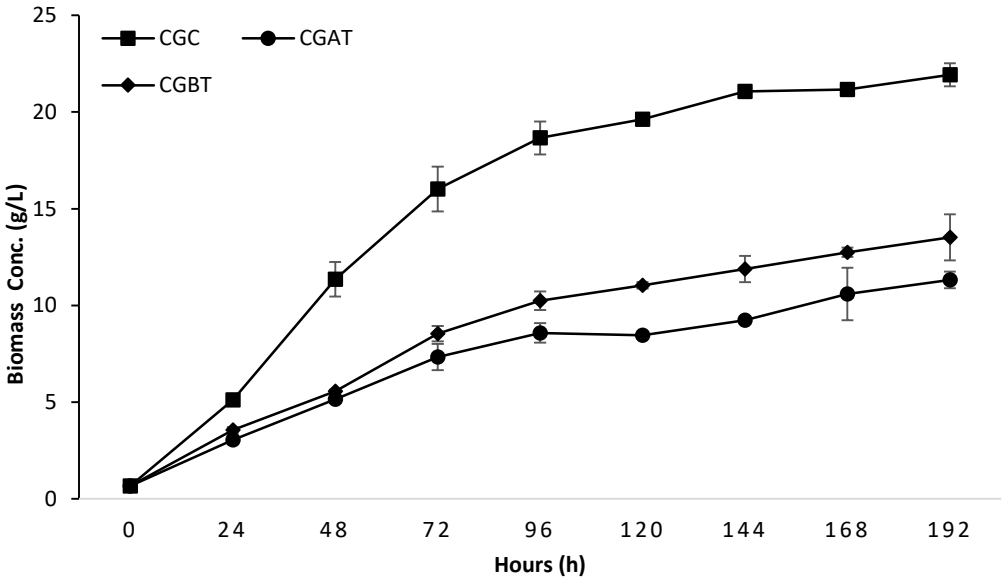



Fig 4.5. Biomass production of *R. toruloides* ATCC 10788 in crude glycerol diluted to various concentrations of methanol. CGC contained 48.2 g/L of glycerol and 1.5% (w/v) of methanol. CGAT and CGBT contained 0.5% (w/v) and 1% (w/v) methanol respectively. Both CGAT and CGBT contained total glycerol to a level of 48.2 g/L consisting of diluted crude glycerol and topped up with pure form

Finally, the biomass and lipid production by this strain in crude glycerol at various time interval was also determined (Fig 4.6). It was found that both the biomass and lipid production increased with time and remained constant after 168h.

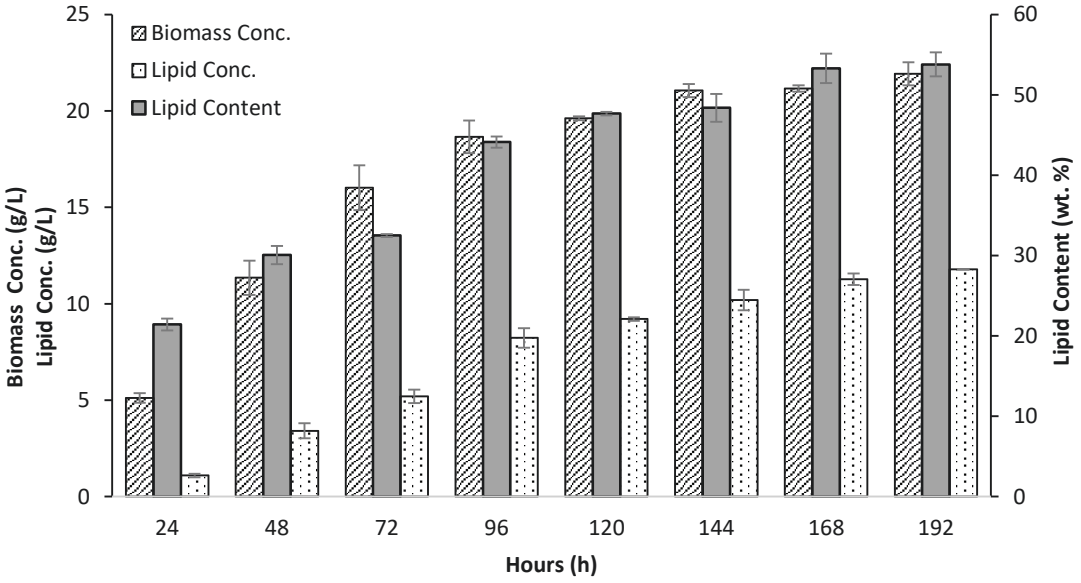



Fig 4.6. Biomass concentration (g/L), lipid concentration (g/L) and lipid content (wt. %) produced by *R. toruloides* ATCC 10788 in crude glycerol CGC media with 48.2% (w/v) of glycerol and 1.5% (w/v) of methanol at various time intervals.

The biomass (i.e. 21.16 g/L) and lipid content (53.28 wt. %) obtained by using this strain at the end of 168 hours was also compared with other oleaginous species used in previous works (Table 4.5). The biomass and lipid content obtained in this study were found to be higher than most previously studied microorganisms under similar shake flask experimental conditions. The ability of this strain to produce more biomass and lipid in crude glycerol than pure glycerol overcomes a major constraint of inhibition caused by impurities present in the crude glycerol.

Table 4.5. Comparison of biomass and lipid content (wt.%) by various oleaginous yeasts in glycerol based media

| Microorganism used | Culture mode | Type of feedstock used | Feedstock composition | | Fermentation time (h) | Biomass Conc. (g/L) | Lipid Conc. (g/L) | Lipid Content (wt.%) | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | Glycerol (wt.%) | Other impurities (wt.%) | | | | | |
| *Rhodosporidium toruloides* AS2.1389 | Shake Flask | Crude glycerol | 32.97% | Methanol 14.89% | 160 | 19.2 | 8.6 | 47.7 | [20] |
| *Rhodosporidium toruloides* AS2.1389 | Batch | Crude glycerol | 32.97% | Methanol 14.89% | 198 | 26.7 | 13.4 | 69.5 | [20] |
| *Rhodosporidium toruloides* Y4 | Fed-Batch | Synthetic crude glycerol | 65% | Methanol 4%<br>Water 27%<br>Salt 4% | 120 | 23.1 | 9.4 | 40.9 | [41] |
| *Rhodosporidium toruloides* Y4 | Shake Flask | Synthetic crude glycerol | 60% | Methanol 6%<br>Salt 6% | 120 | 20.3 | 8.6 | 42.5 | [40] |
| *Rhodosporidium toruloides* NRRLY-27012 | Shake Flask | Crude glycerol | 91-93% | Salt 2-4%<br>Water 2-4%<br>FFA 1% | 195 | 30.1 | - | 40.0 | [39] |
| *Cryptococcus curvatus* ATCC 20509 | Fed-Batch | Crude glycerol | 48.7% | Methanol 22.7%<br>Soap 3%<br>Water 25.6% | 288 | 32.9 | - | 52.9 | [38] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Yarrowia lipolytica* QU21 | Shake flask | Crude glycerol | 82.73% | Methanol 0.008%<br>NaCl 5.15% | 96 | 3.85 | 0.85 | 22.1 | [69] |
| *Candida sp.* LEB-M3 | Shake flask | Crude glycerol | 42.4% | - | 192 | 19.7 | 9.9 | 50.2 | [70] |
| *Kodamaea ohmeri* BY4-523 | Shake flask | Crude glycerol | 40% | Methanol 1-3%<br>Salts 4-5%<br>Water 36-45% | 72 | 10.3 | 5.5 | 53.3 | [42] |
| *Trichosporandoides spathulata* JU4-57 | Shake flask | Crude glycerol | 40% | Methanol 1-3%<br>Salts 4-5%<br>Water 36-45% | 72 | 17.1 | 7.4 | 43.4 | [42] |
| *Rhodosporidium toruloides* ATCC 10788 | Shake Flask | Pure glycerol | - | - | 120 | 7.8 | 2.9 | 37.7 | [43] |
| *Rhodosporidium toruloides* ATCC 10788 | Shake Flask | Pure glycerol* | - | - | 168 | 10.34 | 3.51 | 33.95 | **This study** |
| *Rhodosporidium toruloides* ATCC 10788 | Shake Flask | Crude glycerol † | 44.56% | Methanol 13.86%<br>Ash 10.74%<br>FFA 0.48%<br>Soap 32.97% | 168 | 21.16 | 11.27 | 53.28 | **This study** |

* Pure glycerol media (PGC); † Crude glycerol media (CGC)

### 4.3.4. Effect of other common impurities in crude glycerol on growth and lipid production of *R. toruloides* ATCC 10788

#### 4.3.4.1. Effect of sodium chloride on growth and lipid production

Salts of sodium or potassium are commonly present in crude glycerol from biodiesel industries. This is mainly because basic catalysts such as sodium and potassium hydroxide are generally used during the transesterification of oil into biodiesel [71]. In order to understand the effect of salt on *Rhodosporidium toruloides* ATCC 10788, this strain was grown on minimal salt media with different levels of sodium chloride i.e. 0.5% to 4% (w/v) (Fig. 4.7).

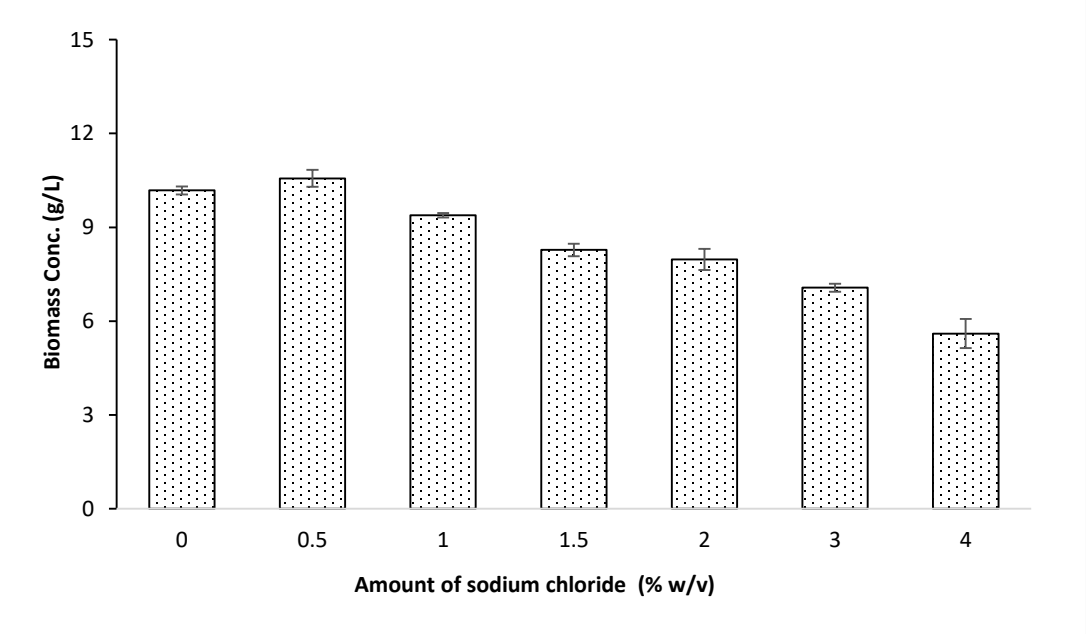



Fig 4.7. Biomass concentration of *R. toruloides* ATCC 10788 obtained at the end of 168 h growth in minimal salt media containing different concentration of sodium chloride (NaCl) and 40 g/L of pure glycerol

From Fig 4.7 it is seen that presence of salt up to 1.5% (w/v) in the media did not reduce the biomass concentration of *R. toruloides* ATCC 10788. Beyond this level of salt, the biomass however reduced gradually. At 4% (w/v), the biomass concentration of this strain reduced by 44.95%. Different strains of *Rhodosporidium toruloides* vary in their ability to withstand salt. Gao et al. (2016) reported an increase in biomass concentration, lipid concentration and lipid content of *R. toruloides* 32489 by 35%, 64% and 21% respectively when 16 g/L (i.e. 1.6 % w/v) of sodium chloride was added to the pure glycerol containing media. At similar levels of sodium

chloride, Xu et al. (2012) reported increase in biomass concentration, lipid concentration and content of *R. toruloides* by 40%, 48% and 20% respectively. Lipid concentration and lipid content of *R. toruloides* ATCC 10788 were also estimated while growing at similar levels of salt in the media (i.e. 1.5% w/v). Unlike previously reported strains, no significant improvement in biomass or lipid concentration were obtained with the use of this strain (Table 4.6).

Table 4.6. Comparison of biomass, lipid concentration and lipid content obtained from *R. toruloides* ATCC 10788 at the end of 168 h when grown on minimal salt media containing some of the major impurities generally present in crude glycerol from biodiesel industry. FAME- Fatty acid methyl ester

| **Media used** | **Media composition** | **Biomass Conc. (g/L)** | **Lipid Conc. (g/L)** | **Lipid Content (wt.%)** |
|---|---|---|---|---|
| MM | Minimal salt media containing 40 g/L of glycerol | 10.18±0.23 | 3.54±0.19 | 34.80±1.12 |
| MMN | MM + 1.5% sodium chloride | 8.21±0.07 | 3.49±0.20 | 42.55±1.36 |
| MMF | MMF + 4% (w/v) FAME | 12.99±0.12 | 5.96±0.17 | 45.91±1.37 |
| MMS | MM + 4% (w/v) soap | 19.70±1.86 | 9.59±0.17 | 48.89±3.75 |

However, most of the microbes commonly used for bioconversion of crude glycerol, do not tolerate even the small amount of salts in the media [72, 73]. Furthermore, it should be noted that crude glycerol contains glycerol in the range of 38-96 wt.% and salt in the range of 5-7 wt.% [1]. This means no more than 1% of salt would be present in the final crude glycerol based minimal media after we dilute the crude glycerol to have a final media glycerol content of 40-60 g/L used in fermentations. Thus, we can conclude that *R. toruloides* ATCC 10788 can grow in most of the crude glycerol obtained from different biodiesel industries without removing salts.

#### 4.3.4.2. Effect of Fatty acid methyl ester (FAME) on the growth and lipid production

Maximum recovery of biodiesel, chemically known as FAME, from its mixture with glycerol is desirable during its production. However, its presence in small quantities in crude glycerol is unavoidable. Depending on the type of downstream processing adopted in a biodiesel industry, the FAME present in crude glycerol varies [20]. Previously, methyl oleate was used as a representative fatty acid methyl ester to understand the effect of FAME on other strains of *Rhodosporidium toruloides* [20, 48]. However, in reality, biodiesel present in crude glycerol consist of methyl esters of varieties of fatty acids rather than one single type. The type of fatty acid present in media have been shown to have an influence on the biomass and fatty acid composition of obtained lipids from oleaginous microbes under study [74, 75]. Thus, in this study, biodiesel prepared from canola oil was used at various concentration (i.e. 0.1, 0.5, 1, 2 and 4 % w/v) to understand its effect on *Rhodosporidium toruloides* ATCC 10788. These levels of biodiesel are higher than previously reported [20, 48]. With increasing levels of FAME in the minimal salt media, the biomass concentration gradually increased and remained constant after 2% (w/v) (Fig. 4.8).

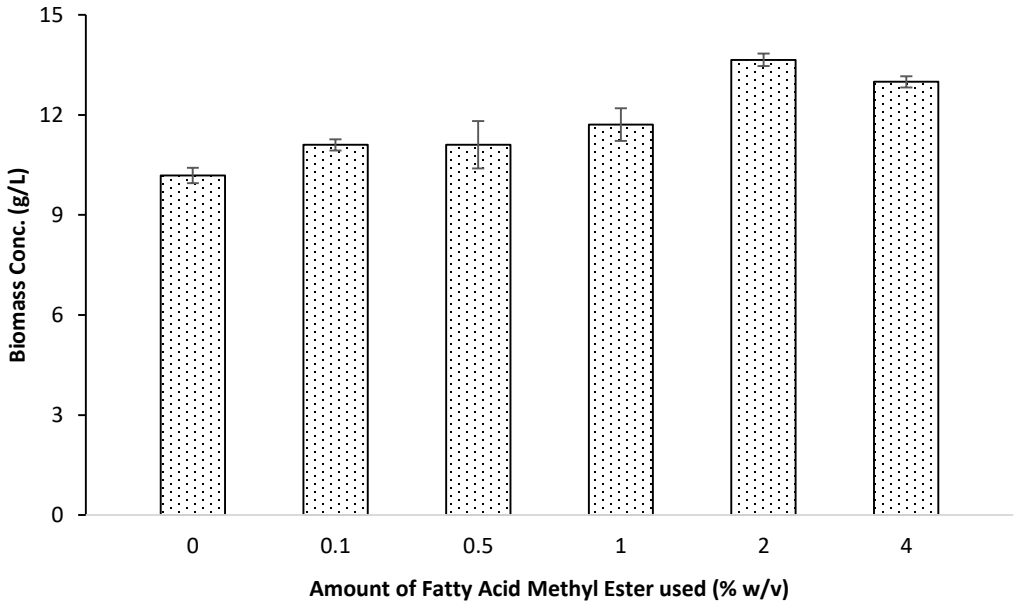



Fig. 4.8. Biomass concentration obtained at the end of 168 h growth of *R. toruloides* ATCC 10788 in grown in minimal salt media with various levels of Fatty Acid Methyl Esters (FAME) with 40 g/L of pure glycerol

The addition of FAME in the range of 0.1% to 4% (w/v) lead to improvement of biomass in the range of approximately 9-28%. The amount of lipid obtained using minimal media with 4% (w/v) FAME was also determined. At this level of FAME, the biomass concentration, lipid concentration and lipid content obtained from this yeast were found to be 12.99 g/L, 6.09 g/L and 46.89 wt.% respectively. These values were approximately 28%, 72% and 35% higher respectively compared to the control experiment with pure glycerol (Table 4.6).

Certain yeast strains have a specialized metabolic pathways to utilize hydrophobic substrates such as alkanes, fatty acids and oils [20, 76, 77]. Hydrophobic compounds also have the capacity to alter the membrane fluidity and enhance the intake of carbon source from the media [78]. It can be assumed that a similar mechanism is employed by *R. toruloides* ATCC 10788 to use FAME as a carbon source or the presence of FAME enhances its ability to take up carbon sources at better rates.

From the above discussion, we can conclude that the strain of *Rhodosporidium toruloides* ATCC 10788 is quite resistant to high levels of FAME. The presence of FAME has been shown to improve the biomass and lipid production of this strain. However, a complete understanding of improvement in biomass production of oleaginous yeast upon the addition of FAME still needs to be understood. Detailed studies to understand its ability to consume FAME as a carbon source is ongoing in our lab and will be reported in future.

#### 4.3.4.3. Effect of Soap on growth and lipid production

In order to understand the effect of soap on *R. toruloides* ATCC 10788, the organism was grown on minimal media containing varying levels of soap ranging from 0.1% to 4% (w/v). With increasing levels of soap in the media, the biomass and lipid concentrations obtained significantly increased. At the end of 168 h, use of soap in the range of 0.1- 4% (w/v) increased the biomass and lipid concentration and lipid content of *R. toruloides* ATCC 10788 by about 23-94%, 43-171% and 16-40% respectively (Fig. 4.9).

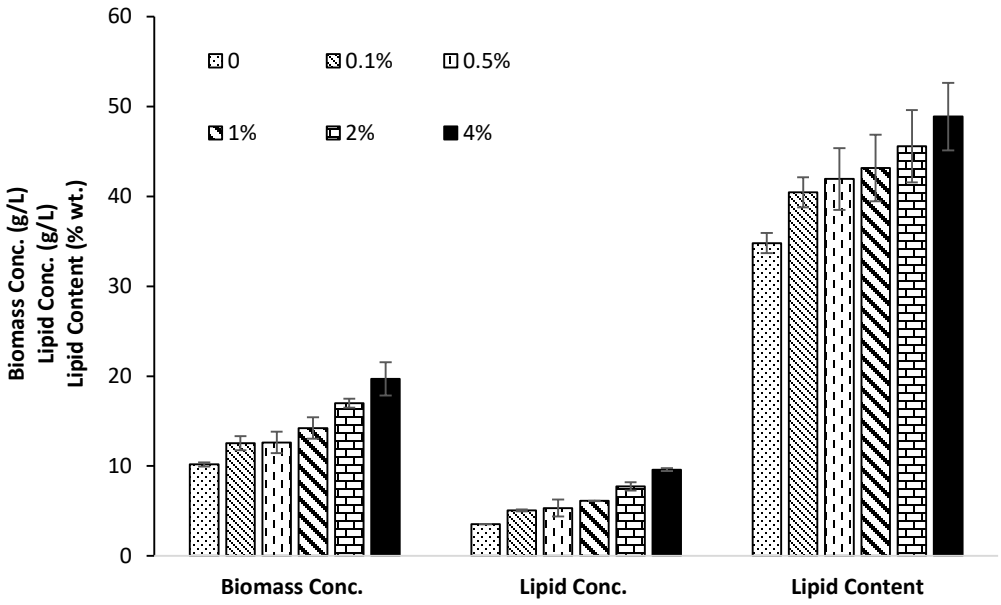



Fig 4.9. Biomass concentration, lipid concentration and lipid content obtained at the end of 168 h growth of R. toruloides ATCC 10788 in minimal salt media containing different levels of soap (0.1% to 4% w/v) and 40 g/L of pure glycerol

Soap is one of the major impurities present in crude glycerol from biodiesel industries [36] . The crude glycerol used in our study contained 32.97 wt.% of soap. The presence of soap in the media have generally been shown to inhibit the growth of microbes used for bioconversion of crude glycerol [33, 48]. Pyle et al. (2008) reported the inhibition of biomass and lipid production by *Schizochytrium limacinum*, a type of oleaginous microbes. However, soap up to 0.2% (w/v) in the media had shown to improve the biomass and lipid of the previously reported strains of *Rhodosporidium toruloides* 32489 [48]. However, beyond this concentration, both the biomass and lipid concentrations obtained from these strains of *Rhodosporidium* began to decrease. The increase of biomass and lipids in the presence of soap was attributed to the facts that soap present in the media increased the membrane permeability of the microbial cells which in turned helped the yeast cells to absorb more carbon source and other nutrients in the media. Similar explanation was provided by Xu et al. (2012) in a report involving *Rhodosporidium toruloides* AS2.1389. However, in the current study, very high levels of soap were tested on the *R. toruloides* ATCC 10788. The explanation provided by previous authors could not completely justify the production of high amount of biomass and lipids obtained in this study. *R. toruloides* ATCC 10788 was able to grow well and produce lipids at even at 4% (w/v) of soap (Table 4.6).

This became apparent when this organism was grown on pure and crude glycerol media with similar levels of glycerol (i.e. 48.2 g/L). In the crude glycerol media, lower amount of glycerol was consumed, at the end of 168 h, but higher amount of biomass and lipids were produced (Table 4.7). This made us realize for the first time that besides glycerol, *R. toruloides* ATCC 10788 was actually consuming the soap present in the crude glycerol used.

Table 4.7. Biomass concentration, lipid concentration, lipid content, obtained from *R. toruloides* ATCC 10788 at the end of 168 h while growing on varieties of carbon sources at an initial pH of 6.0, agitation 200 rpm and incubation temperature 30 $^0$C for 7 days

| **Carbon source** | **Biomass Conc. (g/L)** | **Lipid Conc. (g/L)** | **Lipid Content (wt.%)** | **Initial Glycerol Conc. (g/L)** | **Glycerol Consumed (g/L)** | **Overall Lipid Yield (wt.%)** |
|---|---|---|---|---|---|---|
| Pure glycerol | 10.34±0.11 | 3.51±0.09 | 33.95±0.87 | 48.2 | 36.12 | 9.70 |
| Crude glycerol | 21.16±0.17 | 11.27±0.14 | 53.28±0.64 | 48.2 | 25.66 | 43.90 |
| Pure glycerol | 10.18±0.23 | 3.54±0.19 | 34.80±1.12 | 40.0 | 37.08 | 9.50 |
| Pure glycerol with 1.5% w/v of methanol | 8.06±0.25 | 2.56±0.1 | 31.79±0.22 | 40.0 | 35.16 | 7.30 |
| Pure glycerol with 3.5% soap | 19.76±0.88 | 10.78±0.29 | 54.57±1.49 | 48.2 | 27.05 | 37.52 |
| Soap 3.5% (w/v) | 12.87±1.47 | 5.40±0.15 | 41.99±1.15 | - | - | - |

In order to confirm this, *R. toruloides* ATCC 10788 was grown in two types of minimal media. The first contained 3.5% soap and 48.2 g/L of glycerol was designated as PGS and the second contained 3.5% of soap as the sole carbon source (PSNG media). The soap content used in PGS and PSNG media were similar to that present in crude glycerol media (CGC). When PGS media was used, 19.76 g/L, 10.78 g/L and 54.57 wt.% of biomass concentration, lipid concentration and lipid yield were obtained respectively at the end of 168 h. Furthermore, the amount of

glycerol consumed in such PGS and CGC media were found to be similar. Similarly, when PSNG media containing 3.5% (w/v) soap was used as a sole carbon source, 12.87 g/L, 5.40 g/L and 41.99 wt.% of biomass, lipid concentration and lipid content respectively were obtained at the end of incubation period. In addition to the explanation provided by previous authors [20, 48], another reason for large improvement of lipids produced with the use of soap might be formation of free fatty acids from soap when the pH of the media was adjusted to 6 to make it suitable for the growth of *R. toruloides* ATCC 10788. Certain microbes have shown to consume the high levels of free fatty acids present in the media by ex-novo pathway and produce lipids [79, 80]. A similar pathway was adopted by *R. toruloides* ATCC 10788 to consume the free fatty acid formed during media preparation due to break down of soap. However, further studies have to be carried out to confirm any one or both of possible routes discussed above for high lipid production from soap by this strain.

Based on above discussion we can conclude that unlike previously reported oleaginous yeasts, *R. toruloides* ATCC 10788 is a robust strain capable of growing at high levels of FAME and soap. Its unique ability to grow on media with only soap as the carbon source makes it an interesting organism to conduct further research. In addition to biodiesel industry, this strain can also be exploited for the treatment of waste effluents from car wash centers, soap manufacturing and textile industries.

### 4.3.5. Characterization of lipids produced

Fatty acids composition of the lipids obtained in these experiments were determined using GC-FID. Previous studies show that lipids from various other strains of *Rhodosporidium toruloides* mainly consists of C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid) and C18:2 (linoleic acid) [20, 68, 76, 81]. Fatty acid composition of lipids from some of the strains of *R. toruloides* is tabulated in Table 4.8. *Rhodosporidium toruloides* ATCC 10788 was also found to have the same major fatty acid components.

Table 4.8. Comparison of fatty acid composition of lipid obtained from *R. toruloides* ATCC 10788 in glycerol media to lipid obtained from various other sources. (C16:0 Palmitic acid; C18:0 Stearic acid; C18:1 Oleic acid; C18:2 Linoleic acid; SFA Saturated fatty acid; UFA Unsaturated fatty acid)

| **Lipid source** | **% C16:0** | **% C16:1** | **% C18:0** | **% C18:1** | **% C18:2** | **% C18:3** | **% Others** | **% SFA** | **% UFA** | **Reference** |
|---|---|---|---|---|---|---|---|---|---|---|
| Palm | 42.70 | - | 2.13 | 39.37 | 10.62 | 0.21 | 4.97 | ~49 | ~51 | [82] |
| Canola | 3.75 | 0.21 | 1.87 | 62.41 | 20.12 | 8.37 | 3.27 | ~7 | ~93 | [82] |
| Corn | 10.34 | - | 2.04 | 25.54 | 59.27 | 1.07 | 1.74 | ~13 | ~87 | [82] |
| *R. toruloides* Y4* | 18.0 | 1.2 | 8.1 | 66.6 | 4.4 | 0.4 | 1.3 | ~27 | ~ 73 | [41] |
| *R. toruloides* Y4 † | 20.1 | - | 12.7 | 55.3 | 11.9 | - | - | ~33 | ~ 67 | [41] |
| *R. toruloides* 32489 † | 22.4 | 2.7 | 5.9 | 20.8 | 39.5 | 6.9 | 1.8 | ~30 | ~70 | [48] |
| *R. toruloides* AS2.1389 † | 29.2 | - | 13.9 | 41.4 | 10.4 | 2.9 | 2.2 | ~ 44 | ~56 | [20] |
| *R. toruloides* NRRL Y-27012† | 35.4 | - | 12.2 | 44 | 2.4 | - | 6 | ~50 | ~50 | [39] |
| *R. toruloides* ATCC 10788* | 18.55±0.17 | - | 11.48±0.02 | 59.7±0.16 | 10.25±0.01 | - | - | ~30 | ~70 | **This study** |
| *R. toruloides* ATCC 10788 † | 24.39±0.26 | - | 16.38±0.13 | 47.16±0.12 | 12.05±0.01 | - | - | ~ 40 | ~ 60 | **This study** |

* Pure glycerol media † Crude glycerol media

When pure glycerol was used as a carbon substrate, oleic acid (a monounsaturated fatty acid) content of lipid produced by *R. toruloides* ATCC 10788 was approximately 60%. Similarly, C16:0 (palmitic acid), C18:0 (stearic acid), and C18:2 (linoleic acid) accounted for about 19%, 11% and 10% of total fatty acid respectively. On the other hand, when crude glycerol substrate was utilized, a decrease in oleic (~ 47%) acid but increase in other fatty acids (i.e. 24% stearic, 16% palmitic and 12% linoleic acid) of the lipid was observed. However, this value of oleic acid (i.e. ~ 47%) was still higher than some of the other strains of *R. toruloides* reported in the past (Table 4.8). The decrease in oleic acid while growing this strain in crude glycerol as compared to that in pure glycerol can be attributed to the various impurities present in crude glycerol. Some oleaginous organisms have been reported to take up fatty acids present in the media and incorporate them into their stored lipid [80, 83]. The fatty acid profiles of the lipids obtained from such organisms are thus influenced by the type of fatty acids present in the media [74, 77, 83–85]. In our study, even though the amount of free fatty acids (FFA) present in the media was quite low (crude glycerol contained only 0.48 wt. % of FFA), it certainly had some influence upon the lipid production profile of *R. toruloides* ATCC 10788. Additionally, other impurities such as fatty acid methyl esters, soap, di-glycerides, mono-glycerides and residual triglycerides can also influence the change in fatty acid compositions [20, 28, 36, 48].

Biodiesel produced from feedstock containing higher amounts of saturated fatty acid (SFA) are expected to have excellent oxidative stability, but fuel properties would be poor at low temperatures. This can lead to problem at lower winter temperatures. On the other hand, if biodiesel is made from a feedstock high in polyunsaturated fatty acid (PUFA), the fuel would be suitable for use even at lower temperature (i.e. suitable for winters) but would have poor oxidative stability and also emit high amounts of nitrogen oxide (a type of greenhouse gas). Thus, biodiesel feedstock (i.e. triglycerides) rich in monounsaturated fatty acid (MUFA) are the most suitable feedstock for biodiesel production [86]. As discussed in the previous paragraphs, the lipid obtained from *R. toruloides* ATCC 10788 is composed of higher percentage of oleic acid, a type of monounsaturated fatty acid. Additionally, the type of fatty acids it contains and oleic acid percentage is also similar to that present in some of the oils which are presently used as a feedstock for biodiesel production (Table 4.8). The ability of *R. toruloides* ATCC 10788 to grow well in the presence of 1.5% (w/v) methanol and take up other components present in

crude glycerol to produce higher amount of biomass and lipid rich in MUFA makes microbial lipid from *R. toruloides* ATCC 10788 a suitable renewable feedstock for the biodiesel industry.

### 4.3.6. Effect of individual impurities on fatty acid composition

Analysis of lipid obtained from *R. toruloides* ATCC10788 grown in the presence of various impurities were also carried out. This study helped us to understand the effect of such impurities on fatty acid profiles of this strain (Table 4.9).

Table 4.9. Comparison of fatty acid profiles of lipids obtained on growth of *R. toruloides* ATCC 10788 on pure and crude glycerol and some of its major impurities. (C16:0 Palmitic acid; C18:0 Stearic acid; C18:1 Oleic acid; C18:2 Linoleic acid; SFA Saturated fatty acid; UFA Unsaturated fatty acid)

| Carbon source | %C16:0 | %C18:0 | %C18:1 | %C18:2 | %SFA | %UFA |
|---|---|---|---|---|---|---|
| Pure glycerol | 18.55±0.17 | 11.48±0.02 | 59.7±0.16 | 10.25±0.01 | 30.03 | 69.97 |
| Crude glycerol | 24.39±0.26 | 16.38±0.13 | 47.16±0.12 | 12.05±0.01 | 40.77 | 59.23 |
| Pure glycerol with 1.5% (w/v) methanol | 18.11±0.51 | 11.54±0.46 | 61.35±1.13 | 9 ±0.16 | 29.65 | 70.35 |
| Pure glycerol with 4% (w/v) NaCl | 16.91±1.95 | 12.98±1.03 | 64.95±3.04 | 5.16±2.13 | 29.89 | 70.11 |
| Pure glycerol with 4% (w/v) FAME | 13.71±0.83 | 11.13±1.03 | 64.75±3.15 | 10.42±5.37 | 24.84 | 75.16 |
| Pure glycerol with 4% (w/v) soap | 24.60±1.65 | 16.51±0.10 | 56.60±0.88 | 2.28±0.87 | 41.11 | 58.89 |

It was found that methanol and sodium chloride had an insignificant amount of effect on the fatty acid composition of *R. toruloides* ATCC 10788. However, presence of fatty acid methyl esters (FAME) in the growth media showed only slight change in the saturation level of the lipid obtained. Addition of 4% (w/v) of FAME in the media showed approximately 8% increase of oleic acid content of the lipid and about 7% increase in overall unsaturation level of thus obtained lipid. A major influence on the fatty acid profile was observed when soap was added into the media. At 4% (w/v) of soap in the media, the saturated and unsaturated fatty acids in the lipid obtained from *R. toruloides* ATCC 10788 were found to be ~41% and ~59%

respectively. This was similar to the fatty acid profile of the lipid obtained by growing this strain in crude glycerol (Table 4.9). Thus, it can be inferred that the soap present in crude glycerol was one of the major contributors in the alteration of fatty acid of the lipid obtained from *R. toruloides* ATCC 10788.

### 4.4. Summary

Characterization of crude glycerol from biodiesel industry showed 44.56 wt. % glycerol and many other impurities including 13.86 wt. % methanol and 32.97 wt. % soap. The oleaginous yeast *Rhodosporidium toruloides* ATCC 10788 converted crude glycerol into microbial lipid and was found to have many advantages over other organisms and strains reported in the literature. Biomass concentration of 21.16 g/L, lipid concentration of 11.27 g/L and lipid content of 53.28 wt. % was obtained by the end of 168 h. When biomass production by this strain on crude glycerol was compared to that on pure glycerol, it was observed that the impurities present in crude glycerol enhanced the biomass growth and the overall lipid production. This proves the robustness of this yeast strain for microbial lipid production on the crude glycerol by-product. The impurities present in crude glycerol helped in enhancing the growth and lipid producing ability of this organism. The growth of this organism on individual impurities of this crude glycerol was also carried out. Methanol and salt (>1.5% w/v) inhibited the growth, whereas FAME and soap improved the biomass and lipid obtained. This strain was also capable of growing with soap as the sole carbon source. This was also the reason for improved biomass and lipid production with crude glycerol compared to pure glycerol. The unique ability of this strain to consume soap makes it a suitable strain for its use in the treatment of effluents laden with soap. Characterization of the lipid using GC-FID indicated that oleic acid (47.16%) is a major fatty acid component. The presence of higher amounts of oleic acid in this lipid make it suitable for its utilization as a biodiesel feedstock. The microbial lipid produced by *R. toruloides* ATCC 10788 can also be used to produce other value added products like bioplastics. However, its use as a substrate for biofuel production is suggested as the converted crude glycerol obtained from the biodiesel industry can easily be fed back as a biodiesel substrate. The integrated process can add to the profits of the biodiesel industry by reducing costs of by-product glycerol transportation and disposal.

**References**


1. Yang F, Hanna M a, Sun R (2012) Value-added uses for crude glycerol-a byproduct of biodiesel production. Biotechnol Biofuels 5:13. doi: 10.1186/1754-6834-5-13
2. Guan G, Kusakabe K, Sakurai N, Moriyama K (2009) Transesterification of vegetable oil to biodiesel fuel using acid catalysts in the presence of dimethyl ether. Fuel 88:81–86. doi: 10.1016/j.fuel.2008.07.021
3. Miao X, Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. Bioresour Technol 97:841–846. doi: 10.1016/j.biortech.2005.04.008
4. Lane J (2016) Biofuels Mandates Around the World : 2016. BiofuelsDigest 1–10.
5. Sorda G, Banse M, Kemfert C (2010) An overview of biofuel policies across the world. Energy Policy 38:6977–6988. doi: 10.1016/j.enpol.2010.06.066
6. Liang MH, Jiang JG (2013) Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. Prog Lipid Res 52:395–408. doi: 10.1016/j.plipres.2013.05.002
7. Azócar L, Ciudad G, Heipieper HJ, Navia R (2010) Biotechnological processes for biodiesel production using alternative oils. Appl Microbiol Biotechnol 88:621–636. doi: 10.1007/s00253-010-2804-z
8. Chaudhari SG, Modi MA, Patel TM, et al (2016) Performance of Small Capacity Compression Ignition Engine Using Jatropha Biodiesel Blends. Int J Res Eng Technol 5:129–133.
9. Pfleger BF, Gossing M, Nielsen J (2015) Metabolic engineering strategies for microbial synthesis of oleochemicals. Metab Eng 29:1–11. doi: 10.1016/j.ymben.2015.01.009
10. Bautista LF, Vicente G (2012) Biodiesel from microbial oil. In: Luque R, Antonio J (eds) Adv. biodiesel Prod. Woodhead Publishing Limited, Cambridge, pp 179–197
11. Meng X, Yang J, Xu X, et al (2009) Biodiesel production from oleaginous microorganisms. Renew Energy 34:1–5. doi: 10.1016/j.renene.2008.04.014
12. Haas MJ (2005) Improving the economics of biodiesel production through the use of low value lipids as feedstocks: Vegetable oil soapstock. Fuel Process Technol 86:1087–1096. doi: 10.1016/j.fuproc.2004.11.004
13. Yen H-W, Chen P-W, Chen L-J (2015) The synergistic effects for the co-cultivation of oleaginous yeast-Rhodotorula glutinis and microalgae-Scenedesmus obliquus on the

biomass and total lipids accumulation. Bioresour Technol 184:148–152. doi: 10.1016/j.biortech.2014.09.113

14. Christophe G, Kumar V, Nouaille R, et al (2012) Recent developments in microbial oils production: A possible alternative to vegetable oils for biodiesel without competition with human food? Brazilian Arch Biol Technol 55:29–46. doi: 10.1590/S1516-89132012000100004
15. Li Y, Zhao Z (Kent), Bai F (2007) High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture. Enzyme Microb Technol 41:312–317. doi: 10.1016/j.enzmictec.2007.02.008
16. Papanikolaou S, Aggelis G (2002) Lipid production by Yarrowia lipolytica growing on industrial glycerol in a single stage continuous culture. Bioresour Technol 82:43–49. doi: 10.1186/s13068-015-0286-z
17. Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 51:1–51. doi: 10.1016/S0065-2164(02)51000-5
18. Koutinas AA, Chatzifragkou A, Kopsahelis N, et al (2014) Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. Fuel 116:566–577. doi: 10.1016/j.fuel.2013.08.045
19. Thevenieau F, Nicaud J-M (2013) Microorganisms as sources of oils. Ocl 20:D603. doi: 10.1051/ocl/2013034
20. Xu J, Zhao X, Wang W, et al (2012) Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast Rhodosporidium toruloides and the individual effect of some impurities on lipid production. Biochem Eng J 65:30–36. doi: 10.1016/j.bej.2012.04.003
21. Schneider T, Graeff-Hönninger S, French WT, et al (2013) Lipid and carotenoid production by oleaginous red yeast Rhodotorula glutinis cultivated on brewery effluents. Energy 61:34–43. doi: 10.1016/j.energy.2012.12.026
22. Chi Z, Zheng Y, Jiang A, Chen S (2011) Lipid production by culturing oleaginous yeast and algae with food waste and municipal wastewater in an integrated process. Appl Biochem Biotechnol 165:442–453. doi: 10.1007/s12010-011-9263-6
23. Vamvakaki AN, Kandarakis I, Kaminarides S, et al (2010) Cheese whey as a renewable

substrate for microbial lipid and biomass production by Zygomycetes. Eng Life Sci 10:348–360. doi: 10.1002/elsc.201000063

24. Raimondi S, Rossi M, Leonardi A, et al (2014) Getting lipids from glycerol: new perspectives on biotechnological exploitation of Candida freyschussii. Microb Cell Fact 13:83. doi: 10.1186/1475-2859-13-83
25. Yen HW, Yang YC, Yu YH (2012) Using crude glycerol and thin stillage for the production of microbial lipids through the cultivation of Rhodotorula glutinis. J Biosci Bioeng 114:453–456. doi: 10.1016/j.jbiosc.2012.04.022
26. Uprety BK, Chaiwong W, Ewelike C, Rakshit SK (2016) Biodiesel production using heterogeneous catalysts including wood ash and the importance of enhancing byproduct glycerol purity. Energy Convers Manag 115:191–199. doi: 10.1016/j.enconman.2016.02.032
27. Johnson DT, Taconi KA (2007) The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ Prog 26:338–348. doi: 10.1002/ep.10225
28. Ardi MS, Aroua MK, Hashim NA (2015) Progress, prospect and challenges in glycerol purification process: A review. Renew Sustain Energy Rev 42:1164–1173. doi: 10.1016/j.rser.2014.10.091
29. Pachauri N, He B (2006) Value-added Utilization of Crude Glycerol from Biodiesel Production: A Survey of Current Research Activities. An ASABE Meet. Present.
30. Garlapati VK, Shankar U, Budhiraja A (2016) Bioconversion technologies of crude glycerol to value added industrial products. Biotechnol Reports 9:9–14. doi: 10.1016/j.btre.2015.11.002
31. Salakkam A, Webb C (2015) The inhibition effect of methanol, as a component of crude glycerol, on the growth rate of Cupriavidus necator and other micro-organisms. Biochem Eng J 98:84–90. doi: 10.1016/j.bej.2015.02.024
32. Raimondi S, Rossi M, Leonardi A, et al (2014) Getting lipids from glycerol: new perspectives on biotechnological exploitation of Candida freyschussii. Microb Cell Fact 13:83. doi: 10.1186/1475-2859-13-83
33. Pott RWM, Howe CJ, Dennis JS (2014) The purification of crude glycerol derived from biodiesel manufacture and its use as a substrate by Rhodopseudomonas palustris to

produce hydrogen. Bioresour Technol 152:464–470. doi: 10.1016/j.biortech.2013.10.094

34. Venkataramanan KP, Boatman JJ, Kurniawan Y, et al Impact of impurities in biodiesel-derived crude glycerol on the fermentation by Clostridium pasteurianum ATCC 6013. doi: 10.1007/s00253-011-3766-5
35. Tang S, Boehme L, Lam H, Zhang Z (2009) Pichia pastoris fermentation for phytase production using crude glycerol from biodiesel production as the sole carbon source. Biochem Eng J 43:157–162. doi: 10.1016/j.bej.2008.09.020
36. Pyle DJ, Garcia RA, Wen Z (2008) Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: Effects of impurities on DHA production and algal biomass composition. J Agric Food Chem 56:3933–3939. doi: 10.1021/jf800602s
37. Papanikolaou S, Fakas S, Fick M, et al (2008) Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. Biomass and Bioenergy 32:60–71. doi: 10.1016/j.biombioe.2007.06.007
38. Liang Y, Cui Y, Trushenski J, Blackburn JW (2010) Converting crude glycerol derived from yellow grease to lipids through yeast fermentation. Bioresour Technol 101:7581–7586. doi: 10.1016/j.biortech.2010.04.061
39. Tchakouteu SS, Kalantzi O, Gardeli C, et al (2015) Lipid production by yeasts growing on biodiesel-derived crude glycerol: Strain selection and impact of substrate concentration on the fermentation efficiency. J Appl Microbiol 118:911–927. doi: 10.1111/jam.12736
40. Yang X, Jin G, Gong Z, et al (2014) Recycling biodiesel-derived glycerol by the oleaginous yeast Rhodosporidium toruloides Y4 through the two-stage lipid production process. Biochem Eng J 91:86–91. doi: 10.1016/j.bej.2014.07.015
41. Uçkun Kiran E, Trzcinski A, Webb C (2013) Microbial oil produced from biodiesel by-products could enhance overall production. Bioresour Technol 129:650–654. doi: 10.1016/j.biortech.2012.11.152
42. 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 Res 6:300–310. doi: 10.1007/s12155-012-9257-4

43. Polburee P, Yongmanitchai W, Lertwattanasakul N, et al (2015) Characterization of oleaginous yeasts accumulating high levels of lipid when cultivated in glycerol and their potential for lipid production from biodiesel-derived crude glycerol. Fungal Biol 119:1194–1204. doi: 10.1016/j.funbio.2015.09.002
44. Lee JJL, Chen L, Shi J, et al (2014) Metabolomic Profiling of Rhodosporidium toruloides Grown on Glycerol for Carotenoid Production during Different Growth Phases. J Agric Food Chem 62:10203–10209. doi: 10.1021/jf502987q
45. Wiebe MG, Koivuranta K, Penttilä M, Ruohonen L (2012) Lipid production in batch and fed-batch cultures of Rhodosporidium toruloides from 5 and 6 carbon carbohydrates. BMC Biotechnol 12:26. doi: 10.1186/1472-6750-12-26
46. Yu X, Zheng Y, Dorgan KM, Chen S (2011) Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresour Technol 102:6134–6140. doi: 10.1016/j.biortech.2011.02.081
47. Turcotte G, Kosaric N (1988) Biosynthesis of lipids by Rhodosporidium toruloides ATCC 10788. J Biotechnol 8:221–237. doi: 10.1016/0168-1656(88)90004-1
48. Gao Z, Ma Y, Wang Q, et al (2016) Effect of crude glycerol impurities on lipid preparation by Rhodosporidium toruloides yeast 32489. Bioresour Technol 218:373–9. doi: 10.1016/j.biortech.2016.06.088
49. Wu S, Zhao X, Shen H, et al (2011) Microbial lipid production by Rhodosporidium toruloides under sulfate-limited conditions. Bioresour Technol 102:1803–7. doi: 10.1016/j.biortech.2010.09.033
50. Meesters PAEP, Huijberts GNM, Eggink G (1996) High-cell-density cultivation of the lipid accumulating yeast Cryptococcus curvatus using glycerol as a carbon source. Appl Microbiol Biotechnol 45:575–579. doi: 10.1007/s002530050731
51. Picse B (2011) Titration of oil for soap content. http://www.picse.net/CD2011/biodiesel/titrationOfOilForSoapContent.html.
52. EPA Method-3051A (2007) Microwave Assisted Acid Digestion of Sediments, Sludges, Soils and Oils. https://www.epa.gov/sites/production/files/2015-12/documents/3051a.pdf. Accessed 27 Sep 2016
53. Hu S, Luo X, Wan C, Li Y (2012) Characterization of crude glycerol from biodiesel plants. J Agric Food Chem 60:5915–5921. doi: 10.1021/jf3008629

54. Rukunudin IH, White PJ, Bern CJ, Bailey TB (1998) A modified method for determining free fatty acids from small soybean oil sample sizes. J Am Oil Chem Soc 75:563–568. doi: 10.1007/s11746-998-0066-z
55. Manirakiza P, Covaci A, Schepens P (2001) Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer Extraction Methods. J Food Compos Anal 14:93–100. doi: 10.1006/jfca.2000.0972
56. Bligh EG, Dyer WJ (1959) A Rapid Method of Total Lipid Extraction and Purification. Can. J. Biochem. Physiol. 37:
57. Ichihara K, Fukubayashi Y (2010) Preparation of fatty acid methyl esters for gas-liquid chromatography. J Lipid Res 51:635–40. doi: 10.1194/jlr.D001065
58. Boga DA, Liu F, Bruijnincx PCA, Weckhuysen BM (2016) Aqueous-phase reforming of crude glycerol: effect of impurities on hydrogen production. Catal Sci Technol 6:134–143. doi: 10.1039/c4cy01711k
59. Thompson JC, He BB (2006) Characterization of Crude Glycerol from Biodiesel Production From Multiple Feedstocks. Appl Eng Agric 22:261–265.
60. Hansen CFA, Hernandez AA, Mullan BPB, et al (2009) A chemical analysis of samples of crude glycerol from the production of biodiesel in Australia , and the effects of feeding crude glycerol to growing- fi nishing pigs on performance , plasma metabolites and meat quality at slaughter. 154–161.
61. Glycerine properties Physical Properties of Glycerine and its Solution. http://www.aciscience.org/docs/physical_properties_of_glycerine_and_its_solutions.pdf. Accessed 27 Sep 2016
62. Behera S, Singh R, Arora R, et al (2015) Scope of Algae as Third Generation Biofuels. Front Bioeng Biotechnol 2:1–13. doi: 10.3389/fbioe.2014.00090
63. Nanda M, Yuan Z, Qin W (2014) Purification of crude glycerol using acidification: Effects of acid types and product characterization. Austin Chem Eng 1:1–7.
64. Thanh LT, Okitsu K, Boi L Van, Maeda Y (2012) Catalytic Technologies for Biodiesel Fuel Production and Utilization of Glycerol: A Review. Catalysts 2:191–222. doi: 10.3390/catal2010191
65. Guo F, Fang Z (2011) Biodiesel Production with Solid Catalysts. Biodiesel - Feed

Process Technol. doi: 10.5772/25602

66. Samul D, Leja K, Grajek W (2014) Impurities of crude glycerol and their effect on metabolite production. Ann Microbiol 64:891–898. doi: 10.1007/s13213-013-0767-x
67. Valerio O, Horvath T, Pond C, et al (2015) Improved utilization of crude glycerol from biodiesel industries: Synthesis and characterization of sustainable biobased polyesters. Ind Crops Prod 78:141–147. doi: 10.1016/j.indcrop.2015.10.019
68. Gao Z, Ma Y, Wang Q, et al (2016) Effect of crude glycerol impurities on lipid preparation by Rhodosporidium toruloides yeast 32489. Bioresour Technol 218:373–379. doi: 10.1016/j.biortech.2016.06.088
69. Poli JS, da Silva MAN, Siqueira EP, et al (2014) Microbial lipid produced by Yarrowia lipolytica QU21 using industrial waste: A potential feedstock for biodiesel production. Bioresour Technol 161:320–326. doi: 10.1016/j.biortech.2014.03.083
70. Duarte SH, Ghiselli G, Maugeri F (2013) Influence of culture conditions on lipid production by Candida sp. LEB-M3 using glycerol from biodiesel synthesis. Biocatal Agric Biotechnol 2:339–343. doi: 10.1016/j.bcab.2013.07.001
71. Uprety BK, Dalli SS, Rakshit SK (2017) Bioconversion of crude glycerol to microbial lipid using a robust oleaginous yeast Rhodosporidium toruloides ATCC 10788 capable of growing in the presence of impurities. Energy Convers Manag 135:117–128. doi: 10.1016/j.enconman.2016.12.071
72. Mothes G, Schnorpfeil C, Ackermann JU (2007) Production of PHB from crude glycerol. Eng Life Sci 7:475–479. doi: 10.1002/elsc.200620210
73. Santibáñez C, Varnero MT, Bustamante M (2011) Residual Glycerol from Biodiesel Manufacturing, Waste or Potential Source of Bioenergy: A Review. Chil J Agric Res 71:469–475. doi: 10.4067/S0718-58392011000300019
74. Papanikolaou S, Gortzi O, Margeli E, et al (2008) Effect of Citrus essential oil addition upon growth and cellular lipids of Yarrowia lipolytica yeast. Eur J Lipid Sci Technol 110:997–1006. doi: 10.1002/ejlt.200800085
75. Moustogianni A, Bellou S, Triantaphyllidou I-E, Aggelis G (2014) Alterations in fatty acid composition of Cunninghamella echinulata lipids induced by orange essential oil. Environ Biotechnol 10:1–7. doi: 10.14799/ebms237
76. Easterling ER, French WT, Hernandez R, Licha M (2009) The effect of glycerol as a

sole and secondary substrate on the growth and fatty acid composition of Rhodotorula glutinis. Bioresour Technol 100:356–361. doi: 10.1016/j.biortech.2008.05.030

77. Thevenieau F, Beopoulos A, Desfougeres T, et al (2010) Uptake and Assimilation of Hydrophobic Substrates by the Oleaginous Yeast Yarrowia lipolytica. In: Handb. Hydrocarb. Lipid Microbiol. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 1513–1527
78. Ta TMN, Cao-Hoang L, Phan-Thi H, et al (2010) New insights into the effect of medium-chain-length lactones on yeast membranes. Importance of the culture medium. Appl Microbiol Biotechnol 87:1089–1099. doi: 10.1007/s00253-010-2560-0
79. Yang X, Jin G, Wang Y, et al (2015) Lipid production on free fatty acids by oleaginous yeasts under non-growth conditions. Bioresour Technol 193:557–562. doi: 10.1016/j.biortech.2015.06.134
80. Papanikolaou S, Aggelis G (2003) Selective uptake of fatty acids by the yeast Yarrowia lipolytica. Eur J Lipid Sci Technol 105:651–655. doi: 10.1002/ejlt.200300858
81. Davoli P, Mierau V, Weber RWS (2004) Carotenoids and Fatty Acids in Red Yeasts Sporobolomyces roseus and Rhodotorula glutinis. Appl Biochem Microbiol 40:392–397. doi: 10.1023/B:ABIM.0000033917.57177.f2
82. Zambiazi RC, Przybylski R, Weber Zambiazi M, Barbosa Menonca C (2007) Fatty Acid Composition of Vegetable Oils and Fats. Bol do Cent Pesqui Process Aliment 25:111–120. doi: 10.5380/cep.v25i1.8399
83. Abghari A, Chen S (2014) Yarrowia lipolytica as an Oleaginous Cell Factory Platform for Production of Fatty Acid-Based Biofuel and Bioproducts. Front Energy Res 2:1–21. doi: 10.3389/fenrg.2014.00021
84. Beopoulos A, Nicaud J-M (2012) Yeast: A new oil producer? Oléagineux, Corps gras, Lipides 19:22–28. doi: 10.1051/ocl.2012.0426
85. Beopoulos A, Nicaud J-M, Gaillardin C (2011) An overview of lipid metabolism in yeasts and its impact on biotechnological processes. Appl Microbiol Biotechnol 90:1193–206. doi: 10.1007/s00253-011-3212-8
86. Cao Y, Liu W, Xu X, et al (2014) Production of free monounsaturated fatty acids by metabolically engineered Escherichia coli. Biotechnol Biofuels 7:59.

# Chapter 5

## Use of essential oils from various plants to change the fatty acids profiles of lipids obtained from oleaginous yeasts

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**Abstract**

We studied the ability of seven types of essential oils to alter the fatty acid composition of lipids obtained from an oleaginous yeast *Rhodosporidium toruloides*. Except thyme red essential oil, all other essential oils significantly increased the stearic acid levels of the obtained lipids from this strain. Besides, at specific levels of these essential oils in the media, the obtained lipids were different and unique in their fatty acid composition. Subsequently, we tried to understand the effect of the major monoterpenes present in these essential oils. When *R. toruloides* was grown on limonene, a major monoterpene in orange essential oil, the composition of lipid obtained was found to be quite similar to orange essential oil, proving it has a major role in the change in fatty acid profile of the lipids. Effect of orange essential oil on oleaginous yeast, *C. curvatus* was also carried out. Though the effect of the essential oils and the fatty acid composition were similar, it was found that the reduction of the activity of some enzymes in the metabolic pathways were different. From these results, it can also be concluded that effect of essential oil differs from species to species.

## 5.1. Introduction

Biodiesel is a renewable alternative to fossil based diesel and is commonly produced from vegetable oils by a chemical process called transesterification. During biodiesel production processes, large quantities of glycerol are produced as a by-product. For every 100 kg of biodiesel produced, about 10 kg of glycerol is obtained [1]. The glycerol obtained from biodiesel industries contains a number of impurities and is commonly known as crude glycerol. Recent increase in biodiesel production has caused a glut of glycerol in the market. This in turn has reduced the price of all types of glycerol (pure or crude), making the purification of crude glycerol to its pure form quite uneconomical [2]. Additionally, managing crude glycerol in an environmental friendly way is also a concern for biodiesel producers [2]. Thus, various approaches have been explored to utilize the crude glycerol from biodiesel industries to commercially useful products. One such possible route is its bioconversion to microbial lipids. Microbial lipids obtained from crude glycerol are chemically similar to vegetable oils and have numerous commercial applications [3–5].

Certain microorganisms can accumulate lipids to levels higher than 20% of their dried biomass. Such microorganisms are known as oleaginous microbes and the obtained lipids are known as microbial lipids [6]. Like vegetable oils, microbial lipids contain a mixture of fatty acids attached to a glycerol backbone. Depending on the type of fatty acids present, these lipids have different properties and can be used for a number of applications. For instance, lipid rich in monounsaturated fatty acids (MUFA) is suitable for biodiesel production [7]. Similarly, lipids with specific amount of palmitic (24-26%), stearic (33-38%) and oleic acid (33-37%) can be used as an alternative to cocoa-butter in food industries [8]. Most of the researchers working towards the production of microbial lipids from crude glycerol have discussed its potential use in biodiesel industries [2, 4, 9, 10]. However, tailoring these microbial lipids to possess a specific type of fatty acids would expand its applications to other industries. Addition of certain type of chemical compounds or plant extracts into the growth media of the microorganisms has been reported to alter the fatty acids composition of the lipids obtained [11–16].

Essential oils are volatile, hydrophobic compounds obtained from plant sources. They are mainly made up of terpenic hydrocarbons and oxygenated derivatives. Since essential oils

inhibit the growth of several microorganisms, they are commonly used as a food preservative. Additionally, most essential oils possess analgesic, anti-spasmodic and sedative effects. Hence, essential oils are also used in healthcare industries. Studies to understand the effects of essential oils on different microorganisms have been previously carried out. The ability of these essential oils to change the carbon flux inside the organism towards certain specific type of fatty acids has also been discussed [11–14, 17, 18]. However, quite a number of studies have focused their efforts on the use of essential oils to alter the fatty acid composition of lipids from oleaginous microbes in which lipid contents are considerably high.

Essential oils from various plant sources have been studied for their ability to change the fatty acid composition of oleaginous yeasts *Rhodosporidium toruloides* ATCC 10788 in this paper. In order to understand the roles of active compounds present in essential oils, we also studied the effect of limonene, one of the major monoterpenes present in orange essential oil, on the fatty acid profiles of the lipid obtained from this strain. Understanding the effect of the same essential oil on different oleaginous yeasts is quite important to draw suitable conclusions about its role on the fatty acid production mechanisms within such microorganisms. Thus, the effect of orange essential oil on the fatty acid profile of *Cryptococcus curvatus* was studied. Both these yeasts have shown to grow on varieties of carbon substrates [19–27] and accumulate lipids more than 70 wt.% of their dried biomass weight. The ability of both these oleaginous yeasts to produce lipid by growing on pure and crude glycerol has also been studied [2, 28, 29]. However, to the best of our knowledge, studies involving the use of essential oils to alter the fatty acid profile of *R. toruloides* have not been reported previously. Such studies on this oleaginous strain will help us understand the possibility of using essential oils to alter the chemical composition of the lipids obtained from this robust yeast strain. The tailored lipids obtained from such process can be used for numerous applications. This will expand the application of microbial lipids to other industries and make its production from a cheap carbon substrate like biodiesel crude glycerol, a sustainable and economically beneficial route.

## 5.2. Materials and methods

### 5.2.1. Microorganism and chemicals

The oleaginous microorganisms *R. toruloides* ATCC 10788 and *Cryptococcus curvatus* ATCC 20509 were obtained from American Type Culture Collection (ATCC). For the preparation of seed inocula, they were grown on YPG media (10 g/L yeast extract, 20 g/L peptones and 20 g/L glycerol) for 24 hours in an Innova incubator shaker which was set at a temperature of 30 $^0$C and a speed of 200 rpm. The seed cultures obtained were stored at -80 $^0$C in the presence of pure glycerol (50% v/v). All the essential oils used were purchased from Saffire Blue Inc., Canada. The major components present in these essential oils are shown in Table 5.1.

Table 5.1. Major components of essential oils used in this study as per the supplier's reports

| Sl. No. | Essential oils used | Major components of used essential oil |
|---|---|---|
| 1 | Clove bud essential oil | Eugenol 84.55% |
| 2 | Hyssop essential oil | β-pinene 27%, Isopinocamphon 23.28%, Pinocamphone 13.93% |
| 3 | Thyme red essential Oil | Thymol 47.7%, ϒ-terpinene 10.1%, ρ-cymene 26.3%, Linalool 3.9% |
| 4 | Origanum Essential oil | Carvacrol 69%, Thymol 5% |
| 5 | Cinnamon Bark Organic Essential Oil | Cinnamaldehyde 64.84%, Cinnamile Acetate 2.05%, Eugenol 4.09% |
| 6 | Pine essential oil | α-pinene 41.51%, Camphene 3.70%, β-pinene 23.20%, β-Myrcene 5.60%, Limonene 9.41%, β-phellandrene 2.43% |
| 7 | Orange essential oil | Limonene 93%, Aldehydes 2.68% |

Glycerol (>99%), Limonene (97% pure) and all other chemicals and reagents used in this work were of analytical grade and obtained from Fisher Scientific and Sigma Aldrich (Canada) respectively.

### 5.2.2. Culture conditions

In order to prepare the inocula for shake flask experiments, the yeasts under study were grown in a YPG media (10 g/L yeast extract, 20 g/L peptones and 20 g/L glycerol) at 30 $^0$C in a rotary shaker. The speed of the shaker was maintained at 200 rpm and the cells were grown for 48 hours or until the viable cell count reached about 1 x $10^8$ cells/ml. All the shake flask experiments were carried out in 125 ml Erlenmeyer flasks with 50 ml of minimal media. For the experiments using *R. toruloides* ATCC 10788, the minimal medium was prepared by adopting the method previously described in chapter 4. The minimum media contained (g/l): Glycerol 40.0, $KH_2PO_4$ 1.0, $Na_2HPO_4.12H_2O$ 1.0, $MgCl_2.6H_2O$ 1.5, pH 6.0. Ammonium chloride ($NH_4Cl$) was used as a nitrogen source and 0.7 gm of it was added into the media to obtain an overall C: N molar ratio of 100. The media reported by Meesters et al. (1996) in his experiments was adopted for carrying out the shake flask experiments with *Cryptococcus curvatus* ATCC 20509 [29]. The prepared media contained (g/l): Glycerol 40.0, $KH_2PO_4$ 2.7, $Na_2HPO_4.12H_2O$ 0.95, $MgCl_2.6H_2O$ 0.2, Yeast Extract 0.1, EDTA 0.1, $NH_4Cl$ 0.796, pH 5.5. Both these media were also supplemented with 10 ml/L of trace elements. The supplemented trace elements mixture was prepared by dissolving following chemicals in 1L: $CaCl_2.2H_2O$ 4.0 g, $FeSO_4.7H_2O$ 0.55 g, Citric acid monohydrate 0.52 g, $ZnSO_4.7H_2O$ 0.10 g, $MnSO_4.H_2O$ 0.076 g, and 18M $H_2SO_4$ 100 µl. Sample analyses of the experiments performed in duplicates were carried out and percentage errors were expressed as mean ± standard deviation.

### 5.2.3. Analytical methods

For the determination of biomass concentration, 5 ml of fermentation broth was centrifuged for 10 minutes at 4400 rpm, the supernatant was discarded and wet cells were washed using deionized water and dried overnight at 80 $^0$C. The Bligh-Dyer method was used [30] to estimate the lipid content of these dried biomass. The lipid was further transesterified using the method reported by Ichihara et al (2010) with some modifications [31]. Details of the method has been discussed in chapter 4. The transesterified lipids were then analyzed using a Gas chromatograph (Thermo Scientific, Trace 1300 model, polar column TG WaxMS A; 30 m x 0.25 µm x 0.25 µm) equipped with an FID detector. Parameters reported in chapter 3 were adopted in order to carry out GC analysis. This involved using helium at a flow rate of 1 ml/min as a carrier gas. 1µl of transesterified lipid samples were injected into the GC-FID at an injection split ratio of

50:1. The temperature gradient maintained inside the oven was 100 $^0$C (0.25 min); 30 $^0$C/min, 220 $^0$C (0 mins) and 10 $^0$C/min, 250 $^0$C (3 mins). Air flow of 350 ml/min, $H_2$ at 35 ml/min, and $N_2$ at 40 ml/min were adopted throughout the runs. The methyl esters of individual fatty acids were identified by known peaks of known fatty acid standards mixture including methyl nonadecanoate as the internal standard.

## 5.3. Results and discussion

### 5.3.1. Growth of *R. toruloides* ATCC 10788 in presence of various concentration of essential oils

In order to understand the effects of essential oils, *Rhodosporidium toruloides* ATCC 10788 was grown on various concentration of essential oils in the medium. Each of these essential oils were initially tested in the range of 0.5 to 3 g/L in the media. In the cases where there was no biomass production due to inhibition, usually at essential oils concentration above 1 g/L, the test was further carried out in the range of 0.03 g/L to 1 g/L essential oil. With increasing levels of each of these essential oils in the media, the growth and lipid accumulation ability of this yeast decreased (Table 5.2).

However, the degree of inhibition caused by each of these essential oils varied significantly. Presence of 0.5 g/L of clove, origanum or thyme red essential oils in the growth media reduced the biomass and lipid concentration by more than 90% when compared to the control experiment in which no essential oil was added. More than 45% decrease in biomass and 70% decrease in lipids production were obtained when similar levels of cinnamon and hyssop oil were added into the media. Amongst all the essential oils used, this strain was more tolerant to essential oils from pine and orange. No reduction in biomass and lipid concentration was found at 0.5 g/L of these essential oils in the media. A significant reduction in biomass and the lipids began only when pine essential oil or orange essential oil concentration in the media reached 1 g/L or 2 g/L respectively. It is worth mentioning that even though cinnamon essential oil was had considerable inhibiting effect at higher levels (i.e. beyond 0.2 g/L), up to 0.08 g/L it actually improved the lipid obtained. At 0.08 g/L, approximately 22% increase in lipid concentration was found with the use of cinnamon essential oil (Table 5.2).

Reduction in biomass concentration of microbes upon the addition of different essential oils

have been previously reported by various researchers [14, 32–34]. Its effect on the biomass and lipid profile of oleaginous microbes have also been reported by some authors. In this regard, Papanikolaou et al. (2008) reported reduction of biomass and increase in saturated fatty acids (SFA) levels of the obtained lipid, when orange essential oil from *Citrus sinensis* was added into the growth media of *Y. lipolytica* ACA-DC 50109. However, use of essential oil from same source on the growth of *Cunninghamella echinulata* ATHUM 4411 showed enhanced biomass production and the obtained lipids were rich in unsaturated fatty acids [12]. Thus, it can be concluded that essential oil from same source can act differently on different species.

Table 5.2. Changes in biomass concentration, lipid concentration and lipid content of *R. toruloides* at the end of 168 h fermentation due to addition of essential oils at different concentrations

| Essential oil type | Essential oil Conc. (g/L) | Biomass Conc. (g/L) | Lipid Conc. (g/L) | Lipid Content (wt.%) |
|---|---|---|---|---|
| **Control** | 0.00 | 9.82±0.11 | 3.29±0.18 | 33.54±1.86 |
| **Clove oil** | 0.03 | 8.27±0.22 | 2.56±0.19 | 30.93±2.33 |
| | 0.05 | 6.45±0.71 | 2.01±0.10 | 31.22±1.49 |
| | 0.08 | 5.70±0.14 | 1.88±0.15 | 33.07±2.66 |
| | 0.50 | 0.48±0.01 | 0.03±0.01 | 5.93±2.49 |
| **Origanum oil** | 0.03 | 7.76±2.01 | 2.65±0.15 | 34.12±1.99 |
| | 0.05 | 6.49±0.11 | 2.19±0.21 | 33.84±3.28 |
| | 0.08 | 4.00±0.81 | 0.78±0.20 | 19.43±4.95 |
| | 0.50 | 0.37±0.03 | 0.03±0.01 | 9.16±3.20 |
| **Thyme red oil** | 0.03 | 6.30±0.23 | 1.84±0.09 | 29.28±1.48 |
| | 0.05 | 6.72±0.43 | 2.09±0.04 | 31.04±0.59 |
| | 0.08 | 5.70±0.17 | 0.98±0.19 | 17.22±3.35 |
| | 0.50 | 0.34±0.03 | 0.02±0.01 | 4.95±2.12 |
| **Cinnamon oil** | 0.03 | 10.30±0.21 | 3.98±0.19 | 38.63±1.82 |
| | 0.05 | 10.05±0.03 | 3.69±0.13 | 36.72±1.34 |
| | 0.08 | 10.40±1.06 | 4.00±0.01 | 38.44±0.07 |
| | 0.20 | 8.58±0.95 | 2.41±0.23 | 28.06±2.74 |
| | 0.40 | 7.16±0.66 | 1.58±0.27 | 22.12±3.73 |
| | 0.50 | 5.34±0.04 | 0.98±0.06 | 18.31±1.21 |
| **Hyssop oil** | 0.10 | 7.32±0.09 | 2.55±0.09 | 34.83±1.25 |
| | 0.30 | 6.18±0.04 | 1.54±0.06 | 24.98±1.00 |
| | 0.50 | 5.28±0.10 | 0.89±0.09 | 16.93±1.76 |
| | 1.00 | 2.75±0.16 | 0.43±0.04 | 15.77±1.58 |
| **Pine oil** | 0.50 | 9.33±2.11 | 3.21±0.10 | 34.40±1.10 |
| | 1.00 | 5.69±1.60 | 1.68±0.07 | 29.51±1.14 |
| | 3.00 | 4.42±0.01 | 0.74±0.06 | 16.81±1.26 |
| | 5.00 | 0.74±0.08 | 0.06±0.01 | 7.63±1.20 |
| **Orange oil** | 0.50 | 10.47±0.53 | 3.56±0.41 | 33.94±0.85 |
| | 1.00 | 8.54±0.21 | 2.38±0.19 | 27.89±2.87 |
| | 2.00 | 3.10±1.52 | 0.81±0.03 | 26.05±1.98 |
| | 3.00 | 1.31±0.11 | 0.17±0.04 | 13.13±1.93 |

### 5.3.2. Effects of essential oils on the fatty acid profile of *R. toruloides* ATCC 10788

The fatty acid composition of the obtained lipids in this study were determined using a GC-FID. The lipid samples obtained from *R. toruloides* ATCC 10788, growing in pure glycerol media (control experiment without essential oil), contained approximately 16.08% of palmitic acid (C16:0), 10.25% of stearic acid (C18:0), 59.39% of oleic acid (C18:1) and 14.28% of linoleic acid (C18:2). This makes the total saturated (SFA) and unsaturated fatty acids (UFA) in the lipid to be 26.33% and 73.67% respectively. Since the lipid obtained was predominantly rich in oleic acid , a type of mono-unsaturated fatty acid (MUFA), it is considered quite suitable for biodiesel production [2]. Similar fatty acid composition has been reported previously for different strains of *Rhodosporidium toruloides* [2, 4, 35]. However, addition of seven different essential oils in the media changed the fatty acid profile of the obtained lipids. Except in the case of clove and orange essential oil, the overall saturation of the lipid obtained from *R. toruloides* did not change significantly. However, significant change in individual fatty acid composition of obtained lipids were reported in all the cases (Table 5.3).

From Table 5.3 it is seen that, use of different concentrations of each of these essential oils in the media changed the individual fatty acid composition of the obtained lipid quite significantly. Additionally, the obtained lipids using these essential oils were unique in their fatty acid profiles. For instance, use of 0.08 g/L of clove essential oil improved the stearic (C18:0) content of the lipids by 178.15%, but reduced the oleic acid (C18:1) by 12.03%. However, increase in both of these fatty acids were obtained at similar level with origanum essential oil in the medium. An increase in stearic acid (C18:0) and oleic acid (C18:1) content by 19.22% and 17.90% respectively were obtained when this essential oil was used. On the other hand, use of similar concentration (i.e. 0.08 g/L) of thyme essential oil did not change the C18:0 content but improved the C18:1 levels by 11.06% (Table 5.3).

Table 5.3. Shift in fatty acids composition of *R. toruloides* upon addition of different concentrations of essential oils from various plant sources. C16:0 Palmitic acid; C18:0 Stearic acid; C18:1 Oleic acid; C18:2 Linoleic acid; SFA Saturated fatty acid; UFA Unsaturated fatty acid; ΣC18/ΣC16 Elongase activity; C18:1/C18:0 Δ9 desaturase activity; C18:2/C18:1 Δ12 desaturase activity

| Essential oil name | Conc. of essential oil (g/L) | C16:0 | C18:0 | C18:1 | C18:2 | % SFA | % UFA | ΣC18/ΣC16 | C18:1/C18:0 | C18:2/C18:1 |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | - | 16.08±0.78 | 10.25±0.68 | 59.39±0.10 | 14.28±1.55 | 26.33 | 73.67 | 5.22 | 5.80 | 0.24 |
| Clove essential oil | 0.03 | 16.99±0.10 | 9.21±0.01 | 54.77±0.26 | 19.03±0.17 | 26.20 | 73.80 | 4.89 | 5.95 | 0.35 |
| | 0.05 | 14.12±0.25 | 24.69±4.41 | 55.19±1.69 | 6.01±2.97 | 38.81 | 61.19 | 6.08 | 2.24 | 0.11 |
| | 0.08 | 14.88±0.02 | 28.51±0.04 | 52.24±0.09 | 4.37±0.08 | 43.39 | 56.61 | 5.72 | 1.83 | 0.08 |
| Origanum essential oil | 0.03 | 15.33±0.03 | 11.46±0.11 | 61.37±0.29 | 11.84±0.37 | 26.79 | 73.21 | 5.52 | 5.36 | 0.19 |
| | 0.05 | 12.23±0.42 | 11.76±0.80 | 70.94±0.64 | 5.06±0.59 | 23.99 | 76.01 | 7.18 | 6.03 | 0.07 |
| | 0.08 | 12.64±0.05 | 12.22±0.08 | 70.02±1.03 | 5.13±0.90 | 24.86 | 75.14 | 6.91 | 5.73 | 0.07 |
| Thyme red essential oil | 0.03 | 15.53±0.05 | 8.75±0.12 | 65.00±0.69 | 10.71±0.75 | 24.28 | 75.72 | 5.44 | 7.42 | 0.16 |
| | 0.05 | 16.78±0.87 | 9.88±1.92 | 65.26±0.90 | 8.09±1.89 | 26.65 | 73.35 | 4.96 | 6.61 | 0.12 |
| | 0.08 | 15.94±0.91 | 10.50±2.10 | 65.96±1.00 | 7.59±2.19 | 26.45 | 73.55 | 5.27 | 6.28 | 0.12 |
| Cinnamon essential oil | 0.03 | 15.83±0.29 | 9.93±0.44 | 65.25±0.38 | 9.00±1.12 | 25.76 | 74.24 | 5.32 | 6.57 | 0.14 |
| | 0.05 | 14.52±0.35 | 10.45±0.03 | 68.31±0.19 | 6.72±0.13 | 24.97 | 75.03 | 5.89 | 6.54 | 0.10 |
| | 0.08 | 14.27±1.64 | 10.30±0.35 | 68.08±0.73 | 7.34±0.55 | 24.58 | 75.42 | 6.01 | 6.61 | 0.11 |
| | 0.20 | 14.50±0.02 | 15.57±4.66 | 66.29±3.45 | 3.65±1.19 | 30.07 | 69.93 | 5.90 | 4.26 | 0.06 |
| | 0.40 | 15.10±2.07 | 15.03±1.12 | 64.96±0.11 | 4.91±0.84 | 30.13 | 69.87 | 5.62 | 4.32 | 0.08 |
| Hyssop essential oil | 0.10 | 15.12±0.19 | 9.08±0.20 | 65.41±0.51 | 10.39±0.90 | 24.20 | 75.80 | 5.61 | 7.20 | 0.16 |
| | 0.30 | 14.22±0.02 | 7.12±0.55 | 67.49±0.38 | 11.17±0.19 | 21.34 | 78.66 | 6.03 | 9.48 | 0.17 |
| | 0.50 | 17.53±0.98 | 12.39±0.11 | 65.54±1.89 | 4.55±0.80 | 29.92 | 70.08 | 4.70 | 5.29 | 0.07 |
| | 1.00 | 19.06±3.00 | 12.60±1.85 | 60.93±1.69 | 7.41±3.16 | 31.66 | 68.34 | 4.25 | 4.84 | 0.12 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Pine essential oil | 0.50 | 16.40±2.54 | 12.05±1.36 | 59.48±1.34 | 12.07±0.16 | 28.45 | 71.55 | 5.10 | 4.93 | 0.20 |
| | 1.00 | 16.57±2.46 | 12.11±1.58 | 59.95±2.11 | 11.37±1.23 | 28.68 | 71.32 | 5.03 | 4.95 | 0.19 |
| | 3.00 | 12.22±0.15 | 13.84±1.05 | 67.91±0.73 | 6.03±0.48 | 26.06 | 73.94 | 6.41 | 5.67 | 0.17 |
| Orange essential oil | 0.5 | 18.12±0.10 | 11.50±0.14 | 60.09±0.35 | 10.30±0.11 | 29.61 | 70.39 | 4.52 | 5.23 | 0.17 |
| | 1 | 19.27±0.47 | 12.80±1.74 | 61.11±0.72 | 6.82±1.49 | 32.07 | 67.93 | 4.19 | 4.77 | 0.11 |
| | 2 | 24.99±3.34 | 16.01±0.48 | 49.41±2.57 | 9.58±1.25 | 41.01 | 58.99 | 3.00 | 3.09 | 0.19 |
| | 3 | 41.94±0.52 | 30.83±1.33 | 18.62±0.90 | 8.61±0.09 | 72.77 | 27.23 | 1.38 | 0.60 | 0.46 |
| | 5 | 41.73±0.51 | 36.62±0.28 | 13.98±1.59 | 5.58±1.13 | 78.35 | 19.57 | 1.35 | 0.38 | 0.40 |

Except thyme essential oils, all the essential oils used in this study increased the stearic acid (C18:0) content of the obtained lipids (Table 5.3). Lipids rich in stearic acids are quite suitable for the manufacture of candles, cosmetics, soaps, lubricants, softeners, release agents and pharmaceutical drugs [36]. It is also used in food industries as a defoamer or lubricant [36]. Long chain saturated fatty acids (SFAs) such as palmitic (C16:0), myristic (C14:0) and lauric acid (C12:0) are considered to increase blood cholesterol levels. This in turn will increase the risk for cardiovascular diseases. However, unlike other long chain SFAs, stearic acid shows neutral effect on the blood total and low density lipoprotein (LDL) cholesterol levels. This means its use in food industries are safer when compared to other types of SFAs [37, 38]. Thus, the obtained altered lipids from *R. toruloides* can be a good source of stearic acid for its use in varieties of applications. Furthermore, the tailored lipids can also be used as a substitute of some of the prevailing oils and fats in the market. For instance, at 2 g/L concentrations of orange essential oil in the media, the fatty acid composition of lipid obtained from this strain was quite similar to mowrah fat (Mahuwa butter). Palmitic acid, stearic acid, oleic acid and linoleic acid content of lipid obtained from *R. toruloides* at this concentration of orange essential oil were found to be 24.99%, 16.01%, 49.41% and 9.58% respectively. This makes the total saturated and unsaturated fatty acids to be 41.01% and 58.99% respectively (Table 5.3). Mowrah fat (also known as Mahua butter) which is obtained from the seed of *Bassia latifolia*, plant typical to Indian sub-continent. Chemically, mowrah fat contains 24.1% of palmitic acid, 19.3% of stearic acid, 43.4% of oleic acid and 13.2% of linoleic acid (i.e. approximately 43% of SFA and 57% of UFA). Mowrah fat has number of applications in the food and healthcare sectors (Ramadan et al 2006). Besides using it as a cooking media and a lamp oil, it can also be used as a varnish and a feedstock to produce soap and candles [39–42]. It is also used in the manufacture of creams, lotions, balms, and make-up foundations [43]. Its use for the treatment of skin diseases, cephalalgia and chronic rheumatism has been reported by Panda (2002). Thus, the similarity of produced lipid in this study to mowrah fat, makes it a potential substitute for the latter. Similarly, use of origanum (0.3 g/L) or pine essential oil (3 g/L) into the media improved the oleic acid content of the lipid by 17.90% and 14.35% respectively. As mentioned previously, lipid rich in oleic acid are considered suitable for biodiesel production. Thus, the altered lipid obtained by using origanum or thyme essential oils can be used as a potential feedstock for biodiesel industries.

### 5.3.3. Effect of essential oils on the enzyme activities of fatty acid biosynthesis metabolic pathway of *R. toruloides*

Studies to understand the effect of essential oils on the activities of enzyme activities involved in fatty acid biosynthesis were also carried out in this study. Conversion of C16:0 to C18:0, C18:0 to C18:1 and C18:1 to C18:2 are facilitated by the enzymes elongase, Δ9 desaturase, and Δ12 desaturase respectively as shown in Fig 5.1.

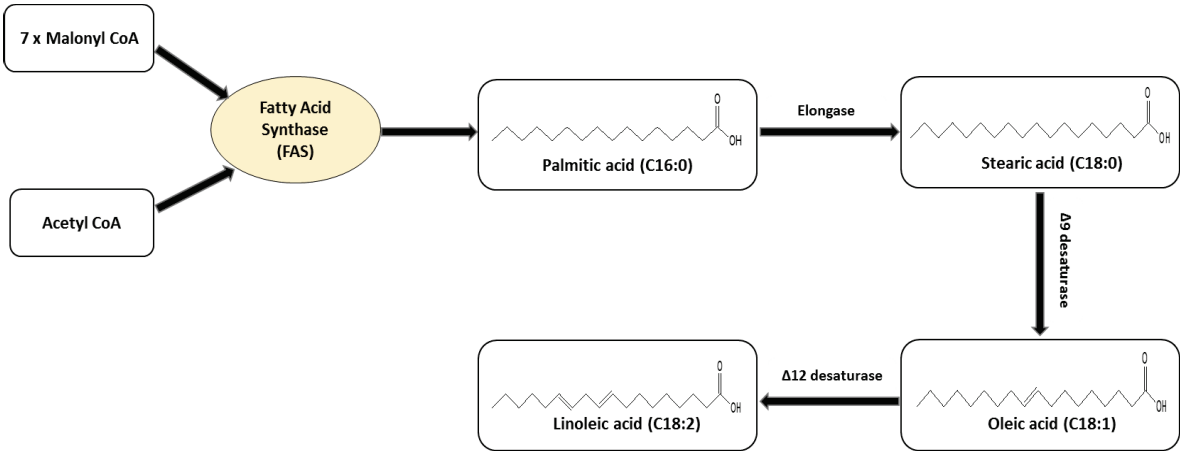


Fig 5.1. Production of palmitic, stearic, oleic and linoleic acids in oleaginous yeast (Adapted from Ratledge 2004)

Values calculated for ΣC18/ΣC16, C18:1/C18:0 and C18:2/C18:1 were used as a parameter to determine the activities of elongase, Δ9 desaturase and Δ12 desaturase enzyme respectively (Table 5.3). ΣC18 and ΣC16 are the sum of total C18 and C16 fatty acids in their saturated and unsaturated forms respectively. These conversions are necessary for the products to be formed as per the metabolic pathways involved (Fig 5.1). Moustogianni et al. (2014) had used the similar approach to determine the activities of elongase, Δ9 desaturase and Δ12 desaturase enzymes involved in fatty acid biosynthesis pathway of oleaginous fungus [12]. Even though these ratios cannot be considered as a true activity of involved enzymes, they can certainly be used as an indicative tool that helps to draw some useful conclusions.

From Table 5.3 it is seen that in the presence of clove, thyme, cinnamon and hyssop, there was no change in the values of ΣC18/ΣC16. Addition of origanum essential oil (>0.03 g/L) or pine

essential oil (3 g/L) into the media improved the ratio of ΣC18/ΣC16. However, with the use of orange essential oil, the value of ΣC18/ΣC16 reduced. At 5 g/L of orange essential oil in the media, value of ΣC18/ΣC16 reduced by 74.14%. Since the values of ΣC18/ΣC16 can be related to the activity of elongase. It can be inferred that origanum and pine essential oil activated the activity of elongase whereas orange essential oil reduced its activity.

Increasing levels of essential oil obtained from clove, cinnamon and orange significantly reduced the value of C18:1/C18:0. For instance, at 0.08 g/L of clove essential oil in the media, 68.45% reduction in the ratio of C18:1 to C18:0 was observed compared to the control experiment. Similarly, with 5 g/L of orange essential oil, 93.45% reduction of this ratio was reported (Table 5.3). Since, C18:1/C18:0 values can also be related to the activity of Δ9 desaturase enzyme, we can conclude that these three essential oils have a negative influence upon the activity of this enzyme. Furthermore, at 0.03 g/L of thyme red essential oil, about 28% increase of C18:1/C18:0 value was observed. However, beyond this concentration, the obtained values were similar to control experiment. Thus, it is difficult to draw any conclusions on the effect of this essential oil upon the activity of Δ9 desaturase enzyme. Similarly, no significant change in C18:1/C18:0 can be seen in Table 5.3, when origanum or pine essential oils were used. Thus, it can be said that these two essential oils did not influence the activity of Δ9 desaturase enzyme.

It can be seen from Table 5.3 that other than clove and orange essential oils, all essential oils used in this work negatively influenced on the ratio of C18:2/C18:1. With the increasing levels of these essential oils in the media, the value of C18:2/C18:1 reduced significantly. The ratio of C18:2 to C18:1 can be used as a parameter to determine the activity of Δ12 desaturase enzyme. Thus, it can be concluded that higher levels of these essential oils in the media lowered the activity of Δ12 desaturase enzyme. Use of 0.03 g/L of clove essential oil increased the value of C18:2/C18:1. Beyond this concentration the value reduced significantly. At 0.08 g/L, 66.67% reduction of this ratio is obtained when compared to control experiments. Similarly, use of orange essential oil up to 2 g/L, reduced the ratio of C18:2 to C18:1. This ratio however increases at higher concentration (i.e. > 2 g/L). At 5 g/L, the value of C18:2/C18:1 increased by 66.67% when compared to control. Due to the ambiguous nature of the results obtained with

the use of these two essential oils, no suitable conclusion can be drawn with respect to their effect on the activity of Δ12 desaturase enzyme. From this we can also infer that at certain specific concentration of essential oil in the media, the carbon flux of the fatty acids is directed towards the production of specific type of fatty acids. Effects of essential oils used in this study on the activities of different enzymes involved in fatty acid biosynthesis of *R. toruloides* ATCC 10788 have been tabulated in Table 5.4. In the table, a comparison of results obtained in this study with previously reported work has also been made.

Table 5.4. Change in fatty acid composition of various microorganisms upon the addition of different types of essential oils and chemical compound; MUFA: Monounsaturated fatty acid; SFA: Saturated fatty acid; UFA: Unsaturated fatty acid; NC: Not Communicated

| Essential oils/ Chemicals compound | Microbes | Carbon source | Remarks | Inferences | References |
|---|---|---|---|---|---|
| *Citrus sinensis* cv *New Hall –citrus aurantium* hybrid plant oil -65% limonene | *Y. lipolytica* ACA-DC 50109 | Glucose | • Enhanced saturated fatty acid<br>• Reduced activity of enzyme elongase and Δ9 desaturase | • Elongase activator<br>• Δ9 desaturase inhibitor | [13] |
| Orange essential oil (*Citrus sinensis*) (optimum at 2 g/l)- 95% limonene | *Cunninghamella echinulata* ATHUM 4411 | Crude glycerol | • Enhanced oleic acid production, a type of MUFA<br>• Increased activity of Δ9 but reduced Δ12 desaturase activity. | • Δ9 desaturase activator<br>• Δ12 desaturase inhibitor | [12] |
| Thymus essential oil | *Thamnidium elegans* CCF 1465 | Crude glycerol | • Thymus essential oil gave same biomass as without essential oil<br>• Enhanced oleic acid production (type of MUFA) | NC | [12] |
| *Hyssopus officinalis* extract | *Aspergillus fumigatus* | Glucose | • 2.5-fold increase in phospholipids<br>• 20% increase in degree of unsaturation | NC | [17] |
| Sesame seed oil (sesamin and lignin compounds) | *Mortierella alpina,* 1S 4 (AKU 3998) | Glucose | • Reduced activity of Δ5 desaturase due to presence of sesamin and lignin compounds in oil<br>• Δ 6, Δ 9 and Δ 12 desaturases activities remained unaffected | • Δ5 desaturase inhibitor | [15] |
| Cyclopropene fatty acids (i.e. sterculic and malvalic fatty acid) | *Candida* sp. 107, *Trichosporon cutaneum* and *Rhodosporidium toruloides* | Glucose | • Reduced activity of Δ9 desaturase thus enhancing the amount of saturated fatty acid in oil<br>• Increased production of stearic acid (a type of SFA) | • Δ9 desaturase inhibitor | [16] |

| | | | | | |
|---|---|---|---|---|---|
| Clove essential oil | *Rhodospordium toruloides* ATCC 10788 | Pure glycerol | • ΣC18/ΣC16- No significant change<br>• C18:1/C18:0- Decreased with increasing levels of essential oil in the media<br>• C18:2/C18:1- Increased by 45.53% at 0.03 g/L and reduced thereafter | • Δ9 desaturase inhibitor | **This study** |
| Origanum essential oil | *Rhodospordium toruloides* ATCC 10788 | Pure glycerol | • ΣC18/ΣC16- Increased with increasing level of essential oil in the media<br>• C18:1/C18:0- No significant change<br>• C18:2/C18:1- Decreased with increasing level of essential oil in the media | • Elongase activator<br>• Δ12 desaturase inhibitor | **This study** |
| Thyme red essential oil | *Rhodospordium toruloides* ATCC 10788 | Pure glycerol | • ΣC18/ΣC16- No significant change<br>• C18:1/C18:0- Increased by 27.93% at 0.03 g/L of essential oil in the media and thereafter values remained similar to the control experiment<br>• C18:2/C18:1- Decreased with increasing level of essential oil in the media | • Δ12 desaturase inhibitor | **This study** |
| Cinnamon essential oil | *Rhodospordium toruloides* ATCC 10788 | Pure glycerol | • ΣC18/ΣC16- No significant change<br>• C18:1/C18:0- No significant change up to 0.08 g/L but reduced gradually beyond this concentration of essential oil in the media<br>• C18:2/C18:1- Decreased with increasing level of essential oil in the media | • Δ9 desaturase inhibitor<br>• Δ12 desaturase inhibitor | **This study** |
| Hyssop essential oil | *Rhodospordium toruloides* ATCC 10788 | Pure glycerol | • ΣC18/ΣC16- No significant change<br>• C18:1/C18:0- Increased up to 0.30 g/L of essential oil in the media and thereafter values remained similar to the control experiment<br>• C18:2/C18:1- Decreased with increasing level of essential oil in the media | • Δ12 desaturase inhibitor | **This study** |

| | | | | | |
|---|---|---|---|---|---|
| Pine essential oil | *Rhodospordium toruloides* ATCC 10788 | Pure glycerol | • ΣC18/ΣC16- No significant change up to 1 g/L but increased by 22.8% at 3 g/L of essential oil in the media<br>• C18:1/C18:0- No significant change<br>• C18:2/C18:1- Decreased gradually with increasing level of essential oil in the media | • Elongase activator<br>• Δ12 desaturase inhibitor | **This study** |
| Orange essential oil | *Rhodospordium toruloides* ATCC 10788 | Pure glycerol | • ΣC18/ΣC16- Decreased with increasing levels of essential oil in the media<br>• C18:1/C18:0- Decreased with increasing levels of essential oil in the media<br>• C18:2/C18:1- Decreased up to 2 g/L of essential oil in the media but increased significantly beyond that | • Elongase inhibitor<br>• Δ9 desaturase inhibitor | **This study** |
| Limonene essential oil | *Rhodospordium toruloides* ATCC 10788 | Pure glycerol | • ΣC18/ΣC16- Decreased with increasing levels of essential oil in the media<br>• C18:1/C18:0- Decreased with increasing levels of essential oil in the media<br>• C18:2/C18:1- Decreased up to 2 g/L of essential oil in the media but increased significantly beyond that | • Elongase inhibitor<br>• Δ9 desaturase inhibitor | **This study** |
| Orange essential oil | *Cryptococcus curvatus* ATCC 20509 | Pure glycerol | • ΣC18/ΣC16- Decreased with increasing levels of essential oil in the media<br>• C18:1/C18:0- Decreased with increasing levels of essential oil in the media<br>• C18:2/C18:1- Increased with increasing levels of essential oil in the media | • Elongase inhibitor<br>• Δ9 desaturase inhibitor<br>• Δ12 desaturase activator | **This study** |

Essential oils from various plant sources have shown to alter the activities of elongase and desaturase enzymes of fatty acid metabolism pathway. The change in activities of such enzymes involved in fatty acid metabolism would in turn change the fatty acid composition of lipids obtained from oleaginous microorganisms [12–14, 17, 18, 34]. Furthermore, the effect of essential oil is species specific and thus acts differently on different microorganisms (Table 5.3). For instance, from Table 5.3 it is seen that use of orange essential oil (obtained from *Citrus sinensis*) increased the saturated fatty acid (SFA) levels of the lipid obtained from *Y. lipolytica* ACA-DC 50109. This was attributed to the fact that the used essential oil reduced the activity of Δ9 desaturase enzyme present inside this yeast [13]. However, use of essential oil from the same source into the growth media of *Cunninghamella echinulata* ATHUM 4411 enhanced the activity of Δ9 desaturase enzyme subsequently making the obtained lipid quite rich in monounsaturated fatty acid (MUFA) [12]. Hence, it can be concluded that the effect of essential oil is specific to species types. In order to support these facts further, we then decided to see the influence of the major component of orange essential oil on *Rhodosporidium toruloides* ATCC 10788. We subsequently also carried out an experiment to understand the effect of one of the essential oil used here (i.e. orange essential oil) on another oleaginous yeast, *Cryptococcus curvatus* ATCC 20509.

### 5.3.4. Effect of limonene on the growth and lipid profile of *R. toruloides* ATCC 10788

Amongst all the essential oils used in this study, orange essential oil was one whose chemical composition mainly consisted of one specific type of monoterpenes. Limonene was the major monoterpenes present and makes up 93% of the total constituents of the orange essential oil used in this study (Table 5.1). It was felt that understanding the effect of pure limonene on *R. toruloides* ATCC 10788 would help us draw suitable conclusion on the role of monoterpenes present in such essential oils on the fatty acid profiles of this strain.

In order to carry out this study, *R. toruloides* ATCC 10788 was grown in minimal media with increasing levels of limonene (i.e. 1 to 5 g/L). With increased levels of limonene in the media (1 to 5 g/L), biomass and lipid concentration, and lipid content reduced within the range of 15.67%- 82.26%, 33.78%- 97.60% and 21.75%- 86.37% respectively (Fig 5.2).

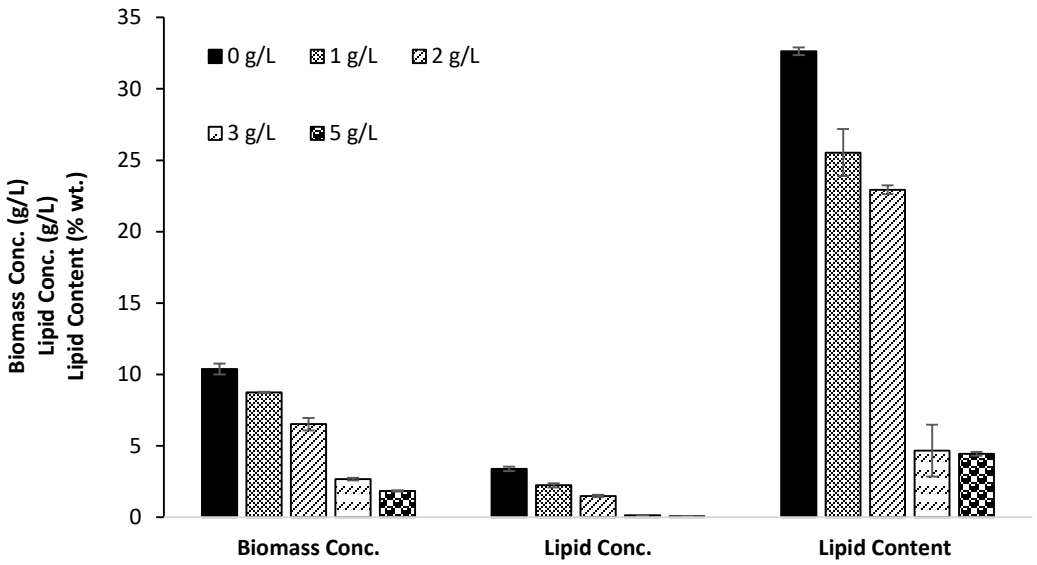


Fig 5.2. Biomass and lipid concentration (g/L) and lipid content (wt.%) of *R. toruloides* ATCC 10788 at the end of 168 h in the presence of various concentration of limonene

Results obtained at the end of fermentation were quite similar to that of orange essential oil. However, better biomass and lipid concentration were obtained with limonene compared to orange essential oil. For example, use of 2 g/L of limonene in the media reduced the biomass and lipid concentration by 37.13% and 55.92% respectively when compared to control experiment. In contrast, use of similar levels of orange essential oil reduced the biomass and lipid concentration by 68.43% and 75.38% respectively. The higher inhibition of biomass and lipid can be attributed to the fact that besides limonene, various other active components (such as myrcene, pinene, sabinene, etc.) are also present in orange essential oil [13, 46]. Such components present in orange essential oil also inhibited the growth of this yeast.

Fatty acid profiles of microbial lipids obtained from *R. toruloides* ATCC 10788 by using limonene were also determined. Like orange essential oil, the amount of oleic acid (C18:1) and linoleic acid (C18:2) decreased with the increasing levels of limonene in the media whereas palmitic (C16:0) and stearic acid (C18:0) increased gradually. Increase of C16:0 and C18:0 fatty acids contributed towards the increase in overall saturated fatty acid content of the obtained lipids (Table 5.5).

Table 5.5. Change in fatty acid compositions of lipid obtained from *Rhodosporidium toruloides* ATCC 10788 at the end of 168 h upon the addition of various concentration of limonene; SFA: Saturated fatty acid; UFA: Unsaturated fatty acid

| **Limonene (g/L)** | **% C16:0** | **%C18:0** | **%C18:1** | **%C18:2** | **%SFA** | **%UFA** | **ΣC18/ΣC16** | **C18:1/C18:0** | **C18:2/C18:1** |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 16.08±0.78 | 10.25±0.68 | 59.39±0.10 | 14.28±1.55 | 26.33 | 73.67 | 5.22 | 5.80 | 0.24 |
| 1 | 18.53±0.28 | 13.72±1.09 | 59.94±1.13 | 7.81±0.25 | 32.25 | 67.75 | 4.40 | 4.37 | 0.13 |
| 2 | 21.59±0.58 | 15.03±0.25 | 54.48±0.60 | 8.91±0.23 | 36.61 | 63.39 | 3.63 | 3.63 | 0.16 |
| 3 | 28.94±0.64 | 17.75±3.65 | 19.73±3.49 | 33.59±0.80 | 46.68 | 53.32 | 2.46 | 1.11 | 1.70 |
| 5 | 27.37±0.65 | 15.43±1.85 | 19.67±0.12 | 37.53±2.62 | 42.80 | 57.20 | 2.65 | 1.27 | 1.91 |

From Table 5.5 it is seen that with increasing levels of limonene in the media, the values of ΣC18/ΣC16 and C18:1/C18:0 gradually reduced. Since, ΣC18/ΣC16 and C18:1/C18:0 can be considered as parameters to determine the activities of elongase and Δ9 desaturase enzyme, it can be said that limonene inhibited the activities of these two enzymes. As discussed in section 5.3.3., similar effect on the activity of these enzymes were observed when orange essential oil was used. From Table 5.3 and 5.5 it is seen that the values of ΣC18/ΣC16 and C18:1/C18:0 were lower when orange essential oil was used when compared to limonene. This was due to the fact that besides limonene, other components present in orange essential oil also had negative influence on the activities of elongase and Δ9 desaturase enzyme. This was also the reason for higher increase in total saturated fatty acids of obtained lipids when orange essential oil was used as compared to limonene at similar level. For instance, at 5 g/L of limonene total saturated fatty acid increased by 62.55% while use of similar level of orange essential oil increased the total saturation by 197.56% (Table 5.3 and 5.5). Furthermore, up to a certain level of limonene (1 g/L), the values of C18:2/C18:1 reduced but thereafter it gradually increased (Table 5.5). Similar trend was obtained when orange essential oil was used (Table 5.3). Thus, it can be concluded that limonene present in orange essential oil was the major component to alter the activities of enzyme involved in fatty acid biosynthesis of *R. toruloides* ATCC 10788 and subsequently change the fatty acid profiles of the obtained lipids.

Use of active compounds present in various plant extracts to understand its effect on the fatty acid profile of oleaginous microbes has been previously studied by some researchers [15, 16]. Moreton (1985) studied the effects of methyl sterculate, a major component present in the seed oils of *Malvaceae* and *Sterculiaceae* families, on the lipid profile of *Candida* sp. 107. The author found out that the addition of this chemical into the growth media of this yeast would reduce the activity of Δ9 desaturase enzyme subsequently increasing the saturated fatty acid (SFA) content of the obtained lipids [16]. Similarly, Shimizu et al. (1991) added the active components found in sesame oil (i.e. sesamin and related lignin compounds) in the growth media of *Mortierella alpine.* Presence of such components in the media, reduced the activity of Δ5 desaturase enzyme which is involved in polyunsaturated fatty acid biosynthesis pathway. Due to this, lower levels of γ-linolenic acid (C18:3) but increased arachidonic acid (20:4) content were found in the altered lipid [15]. These studies show that essential oils and their components

need to be chosen appropriately, depending on the change in fatty acid required.

### 5.3.5. Effect of orange essential oil on the growth and lipid profile of *C. curvatus* ATCC 20509

In order to understand the effect of orange essential oil on another oleaginous species, *Cryptococcus curvatus* ATCC 20509 was grown for 72 hours in a pure glycerol containing media with varying concentrations of this extract (0.5 g/L -5 g/L). Like *R. toruloides*, not much change in biomass and lipid production ability occurred on the addition of 0.5 g/L of essential oil. However, increasing levels of essential oil from 1 to 5 g/L lowered the biomass and lipid concentration and lipid content of this strain in the range of 2.70% - 95.26%, 25.92% - 99.59% and 23.96% - 91.60% respectively (Fig 5.3).

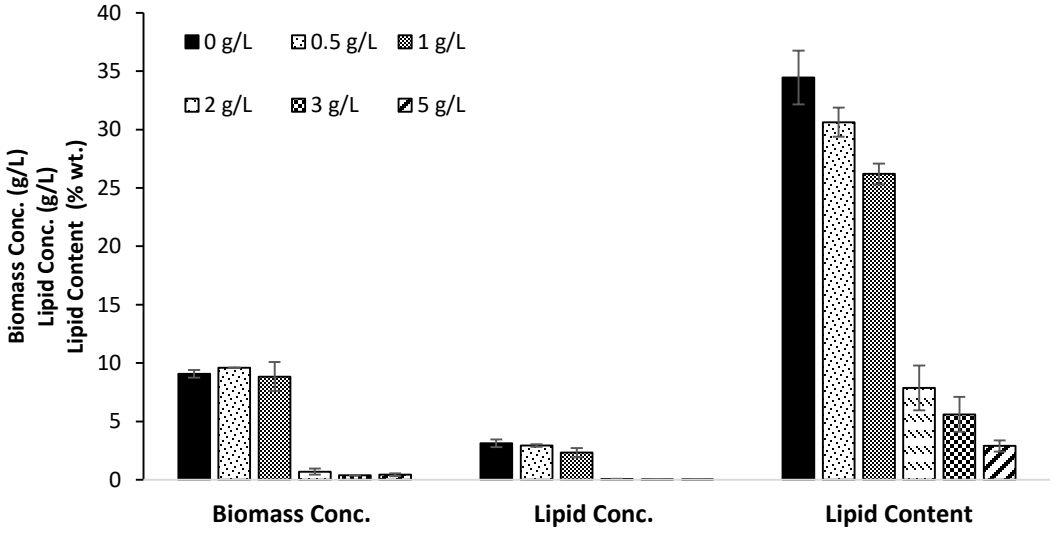


Fig 5.3. Biomass and lipid concentration (g/L) and lipid content (wt.%) of *C. curvatus* ATCC 20509 at the end of 72 h in presence of various concentration of orange essential oil

Fatty acid analysis of lipid obtained from *Cryptococcus curvatus* ATCC 20509 while growing in pure glycerol control media resulted on 43.84% of SFA and 56.16% of UFA. It contained 26.34% of C16:0, 17.50% of C18:0, 46.20% of C18:1 and 9.96% of C18:2. Changes in fatty acid profile of the lipids due to orange essential oil were also quite similar to *R. toruloides*. Increasing levels of essential oils in the media increased the overall saturation of the obtained

lipids (Table 5.6).

From Table 5.6 it is seen that when essential oil concentration in the media increased from 1 to 5 g/L, oleic acid (C18:1) content of the lipid gradually decreased. However, significant improvement in palmitic acid (C16:0) and stearic acid (C18:0) content of the lipids were obtained. As it can be seen in Table 5.3, that similar trends were obtained with *R. toruloides* ATCC 10788 but the obtained values in that case were quite high. For instance, use of 5 g/L of orange essential oil into the media of *C. curvatus* improved the palmitic acid (C16:0), stearic acid (C18:0) and saturated fatty acid (SFA) content of the lipid by 42.06%, 47.71% and 44.32% respectively (Table 5.6). When similar level of essential oil was used with *R. toruloides*, there was approximately 160% increase in palmitic acid (C16:0), 258% increase in stearic acid (C18:0) and 197.56% increase in total saturated fatty acid content of the obtained lipid (Table 5.3). Furthermore, the linoleic acid (C18:2) content of the obtained lipids from *R. toruloides* gradually lowered with increasing levels of essential oil in the media. However, with *C. curvatus* no significant change of linoleic acid was observed up to 2 g/L of essential oil in the medium. However, beyond 2 g/L of essential oil in the media, the C18:2 levels of the obtained lipids started to increase gradually (Table 5.3 and 5.6). Thus, it is obvious from the above discussion that the use of essential oil even from the same source can affect the fatty acids profiles differently depending on organisms used.

Additionally, from Table 5.6 we can also see that, with increasing levels of essential oil in the medium, ΣC18/ΣC16 and C18:1/C18:0 values of lipid from *C. curvatus* decreased. At 5 g/L of essential oil, the values of ΣC18/ΣC16 and C18:1/C18:0 obtained with *C. curvatus* reduced by 40.36% and 67.42% respectively but the value of C18:2/C18:1 increased by 195.45% as compared to the control experiment. From this we can say that the used essential oil reduced the activity of elongase and Δ9 desaturase enzyme but enhanced the Δ12 desaturase activity of this strain. Similar effects on the activities of elongase and Δ9 desaturase was obtained with *R. toruloides* on the use of this essential oil. However, an ambiguous result was obtained for this strain with respect to the effect of orange essential oil on the activity of Δ12 desaturase enzyme. Furthermore, compared to *C. curvatus*, lower values of ΣC18/ΣC16 and C18:1/C18:0 were obtained in case of *R. toruloides* at similar levels of orange essential oil. From this we can conclude that orange essential oil had higher inhibition effect on the activity of elongase and Δ9

desaturase enzyme present in the fatty acid biosynthesis pathway of *R. toruloides*. This was also the reason for higher palmitic acid (C16:0) and stearic acid (C18:0) contents in the altered lipids from this strain when compared to *C. curvatus*. From above discussion, we can conclude that the effect of essential oil is specific to the type of species under study.

Table 5.6. Change in fatty acid compositions of lipid obtained on growing *Cryptococcus curvatus* ATCC 20509 for 72 h with different concentrations of orange essential oil. SFA: Saturated fatty acid; UFA: Unsaturated fatty acid

| Orange Essential Oil (g/L) | %C16:0 | %C18:0 | %C18:1 | %C18:2 | %SFA | %UFA | ΣC18/ΣC16 | C18:1/C18:0 | C18:2/C18:1 |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 26.34±0.29 | 17.50±0.40 | 46.20±0.88 | 9.96±0.20 | 43.84 | 56.16 | 2.80 | 2.64 | 0.22 |
| 0.5 | 30.91±1.65 | 11.29±1.03 | 46.55±0.86 | 11.25±0.24 | 42.20 | 57.80 | 2.24 | 4.12 | 0.24 |
| 1 | 33.28±0.67 | 12.55±0.10 | 43.46±0.42 | 10.71±0.15 | 45.84 | 54.16 | 2.00 | 3.46 | 0.25 |
| 2 | 39.94±0.89 | 27.23±0.36 | 23.14±0.37 | 9.69±0.89 | 67.17 | 32.83 | 1.50 | 0.85 | 0.42 |
| 3 | 34.73±2.31 | 26.19±0.77 | 26.81±1.32 | 12.27±1.76 | 60.92 | 39.08 | 1.88 | 1.02 | 0.46 |
| 5 | 37.42±1.11 | 25.85±0.34 | 22.22±0.06 | 14.51±0.71 | 63.27 | 36.73 | 1.67 | 0.86 | 0.65 |

## 5.4. Conclusion

Effect of essential oils from seven different plant sources were studied on the fatty acid profile of the lipids from *Rhodospordium toruloides* ATCC 10788. All essential oils used inhibited the biomass production but to different degrees. Most of the essential oils enhanced the stearic acid content of the lipids, which has a number of applications in food, pharmaceuticals and cosmetic industries. At specific levels of these essential oils in the media, the obtained lipids were quite unique in their fatty acids composition. Thus, such strains can be tailored in a desirable way by varying the concentration of essential oil in the media to induce them to produce the desired products. The effect of limonene, a major monoterpene present in orange essential oil, on this yeast was also carried out. The obtained results were quite similar to orange essential oil proving that monoterpenes play important components in altering the fatty acid profile of this strain. The other minor components present in essential oils also played similar roles. The effect of orange essential oil on another oleaginous yeast, *Cryptococcus curvatus* ATCC 20509, showed effects similar to *R. toruloides*. However, the degree of reduction in activities of elongase and Δ9 desaturase enzyme involved in the metabolic biosynthesis pathways, were quite different in the two yeasts. Thus, it can be concluded that effect of essential oil is species specific and can be used to produce desirable marketable products.

**References**


1. Uprety BK, Chaiwong W, Ewelike C, Rakshit SK (2016) Biodiesel production using heterogeneous catalysts including wood ash and the importance of enhancing byproduct glycerol purity. Energy Convers Manag 115:191–199. doi: 10.1016/j.enconman.2016.02.032

2. Uprety BK, Dalli SS, Rakshit SK (2017) Bioconversion of crude glycerol to microbial lipid using a robust oleaginous yeast Rhodosporidium toruloides ATCC 10788 capable of growing in the presence of impurities. Energy Convers Manag 135:117–128. doi: 10.1016/j.enconman.2016.12.071

3. Uprety BK, Reddy JV, Dalli SS, Rakshit SK (2017) Utilization of microbial oil obtained from crude glycerol for the production of polyol and its subsequent conversion to polyurethane foams. Bioresour Technol 235:309–315. doi: 10.1016/j.biortech.2017.03.126

4. Xu J, Zhao X, Wang W, et al (2012) Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast Rhodosporidium toruloides and the individual effect of some impurities on lipid production. Biochem Eng J 65:30–36. doi: 10.1016/j.bej.2012.04.003

5. Pyle DJ, Garcia RA, Wen Z (2008) Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: Effects of impurities on DHA production and algal biomass composition. J Agric Food Chem 56:3933–3939. doi: 10.1021/jf800602s

6. Meng X, Yang J, Xu X, et al (2009) Biodiesel production from oleaginous microorganisms. Renew Energy 34:1–5. doi: 10.1016/j.renene.2008.04.014

7. Cao Y, Liu W, Xu X, et al (2014) Production of free monounsaturated fatty acids by metabolically engineered Escherichia coli. Biotechnol Biofuels 7:59. doi: 10.1186/1754-6834-7-59

8. Lipp M, Anklam E (1998) Review of cocoa butter and alternative fats for use in chocolate—Part A. Compositional data. Food Chem 62:73–97. doi: 10.1016/S0308-8146(97)00160-X

9. Raimondi S, Rossi M, Leonardi A, et al (2014) Getting lipids from glycerol: new perspectives on biotechnological exploitation of Candida freyschussii. Microb Cell Fact 13:83. doi: 10.1186/1475-2859-13-83

10. Garlapati VK, Shankar U, Budhiraja A (2016) Bioconversion technologies of crude glycerol to value added industrial products. Biotechnol Reports 9:9–14. doi: 10.1016/j.btre.2015.11.002

11. Moustogianni A, Bellou S, Triantaphyllidou I, Aggelis G (2015) Feasibility of raw glycerol conversion into single cell oil by zygomycetes under non-aseptic conditions. Biotechnol Bioeng 112:827–831. doi: 10.1002/bit.25482

12. Moustogianni A, Bellou S, Triantaphyllidou I, Aggelis G (2014) Alterations in fatty acid composition of Cunninghamella echinulata lipids induced by orange essential oil. Environ Biotechnol 10:1–7. doi: 10.14799/ebms237

13. Papanikolaou S, Gortzi O, Margeli E, et al (2008) Effect of Citrus essential oil addition upon growth and cellular lipids of Yarrowia lipolytica yeast. Eur J Lipid Sci Technol 110:997–1006. doi: 10.1002/ejlt.200800085

14. Helal GA, Sarhan MM, Abu Shahla ANK, Abou El-Khair EK (2007) Effects ofCymbopogon citratus L. essential oil on the growth, morphogenesis and aflatoxin production ofAspergillus flavus ML2-strain. J Basic Microbiol 47:5–15. doi: 10.1002/jobm.200610137

15. Shimizu S, Akimoto K, Shinmen Y, et al (1991) Sesamin is a potent and specific inhibitor of Δ5 desaturase in polyunsaturated fatty acid biosynthesis. Lipids 26:512–516. doi: 10.1007/BF02536595

16. Moreton RS (1985) Modification of fatty acid composition of lipid accumulating yeasts with cyclopropene fatty acid desaturase inhibitors. Appl Microbiol Biotechnol 22:41–45. doi: 10.1007/BF00252154

17. Ghfir B, Fonvieille JL, Koulali Y, et al (1994) Effect of essential oil of Hyssopus officinalis on the lipid composition of Aspergillus fumigatus. Mycopathologia 126:163–7.

18. Aggelis G, Komaitis M (1999) Enhancement of single cell oil production by Yarrowia lipolytica growing in the presence of Teucrium polium L. aqueous extract. Biotechnol Lett 21:747–749. doi: 10.1023/A:1005591127592

19. Li YH, Liu B, Zhao ZB, Bai FW (2006) Optimized culture medium and fermentation conditions for lipid production by Rhodosporidium toruloides. Chin J Biotechnol 22:650–6.

20. Hassan M, Blanc PJ, Granger L-M, et al (1996) Influence of nitrogen and iron limitations on lipid production by Cryptococcus curvatus grown in batch and fed-batch culture. Process Biochem 31:355–361. doi: 10.1016/0032-9592(95)00077-1

21. Wiebe MG, Koivuranta K, Penttilä M, Ruohonen L (2012) Lipid production in batch and fed-batch cultures of Rhodosporidium toruloides from 5 and 6 carbon carbohydrates. BMC Biotechnol 12:26. doi: 10.1186/1472-6750-12-26

22. Liang Y, Jarosz K, Wardlow AT, et al (2014) Lipid production by Cryptococcus curvatus on hydrolysates derived from corn fiber and sweet sorghum bagasse following dilute acid pretreatment. Appl Biochem Biotechnol 173:2086–2098. doi: 10.1007/s12010-014-1007-y

23. Gong Z, Shen H, Zhou W, et al (2015) Efficient conversion of acetate into lipids by the oleaginous yeast Cryptococcus curvatus. Biotechnol Biofuels 8:189. doi: 10.1186/s13068-015-0371-3

24. Fei Q, O'Brien M, Nelson R, et al (2016) Enhanced lipid production by Rhodosporidium toruloides using different fed-batch feeding strategies with lignocellulosic hydrolysate as the sole carbon source. Biotechnol Biofuels 9:130. doi: 10.1186/s13068-016-0542-x

25. Chi Z, Zheng Y, Jiang A, Chen S (2011) Lipid Production by Culturing Oleaginous Yeast and Algae with Food Waste and Municipal Wastewater in an Integrated Process. Appl Biochem Biotechnol 165:442–453. doi: 10.1007/s12010-011-9263-6

26. Ling J, Nip S, Shim H (2013) Enhancement of lipid productivity of Rhodosporidium toruloides in distillery wastewater by increasing cell density. Bioresour Technol

146:301–309. doi: 10.1016/j.biortech.2013.07.023

27. Yu X, Zheng Y, Xiong X, Chen S (2014) Co-utilization of glucose, xylose and cellobiose by the oleaginous yeast Cryptococcus curvatus. Biomass and Bioenergy 71:340–349. doi: 10.1016/j.biombioe.2014.09.023

28. Liang Y, Cui Y, Trushenski J, Blackburn JW (2010) Converting crude glycerol derived from yellow grease to lipids through yeast fermentation. Bioresour Technol 101:7581–7586. doi: 10.1016/j.biortech.2010.04.061

29. Meesters PAEP, Huijberts GNM, Eggink G (1996) High-cell-density cultivation of the lipid accumulating yeast Cryptococcus curvatus using glycerol as a carbon source. Appl Microbiol Biotechnol 45:575–579. doi: 10.1007/s002530050731

30. Bligh EG, Dyer WJ (1959) A Rapid Method of Total Lipid Extraction and Purification. Can. J. Biochem. Physiol. 37:

31. Ichihara K, Fukubayashi Y (2010) Preparation of fatty acid methyl esters for gas-liquid chromatography. J Lipid Res 51:635–40. doi: 10.1194/jlr.D001065

32. Matasyoh JC, Wagara IN, Nakavuma JL, Kiburai AM (2011) Chemical composition of Cymbopogon citratus essential oil and its effect on mycotoxigenic Aspergillus species. African J Food Sci 5:138–142.

33. Helal GA, Sarhan MM, Abu Shahla ANK, Abou El-Khair EK (2006) Effects of Cymbopogon citratus L. essential oil on the growth, lipid content and morphogenesis of Aspergillus niger ML2-strain. J Basic Microbiol 46:456–469. doi: 10.1002/jobm.200510106

34. Helal GA, Sarhan MM, Abu Shahla ANK, Abou El-Khair EK (2006) Effect of Cymbopogon citratus L. essential oil on growth and morphogenesis of Saccharomyces cerevisiae ML2-strain. J Basic Microbiol 46:375–386. doi: 10.1002/jobm.200510084

35. Gao Z, Ma Y, Wang Q, et al (2016) Effect of crude glycerol impurities on lipid preparation by Rhodosporidium toruloides yeast 32489. Bioresour Technol 218:373–379. doi: 10.1016/j.biortech.2016.06.088

36. Stearate (2015) Applications and Uses of Stearic Acid. https://www.yameiaspartame.com/applications-and-uses-of-stearic-acid/. Accessed 16 Mar 2017

37. Beef-facts (2007) Stearic acid-A unique saturated fat. Cattlemen's Beef Board

38. Grundy SM (1994) Influence of stearic acid on cholesterol metabolism relative to other long-chain fatty acids. Am J Clin Nutr 60:986S–990S.

39. Hiwale S (2015) Mahua (Bassia latifolia Roxb.). In: Sustain. Hortic. Semiarid Dry Lands. Springer India, New Delhi, pp 255–261

40. Awasthi YC, Bhatnagar SC, Mitra CR, Mitra1 CR Chemurgy of Sapotaceous Plants: Madhuca Species of India. Source Econ Bot 29:380–389.

41. Tiwari S, Saxena M, Tiwari SK (2002) Preparation and characterization of penta alkyds based on mahua oil. J Sci Ind Res (India) 61:110–116.

42. Ramadan MF, Sharanabasappa G, Parmjyothi S, et al (2006) Profile and levels of fatty acids and bioactive constituents in mahua butter from fruit-seeds of buttercup tree [Madhuca longifolia (Koenig)]. Eur Food Res Technol 222:710–718. doi: 10.1007/s00217-005-0155-2

43. Ramadan MF, Moersel J-T (2016) Mowrah butter : Nature's novel fat. inform 17:

44. Panda H (2002) Bassia Latifolia, Roxb. Med. Plants Cultiv. Their Uses

45. Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. Biochimie 86:807–815. doi: 10.1016/j.biochi.2004.09.017

46. Verzera A, Trozzi A, Dugo G, et al (2004) Biological lemon and sweet orange essential oil composition. Flavour Fragr J 19:544–548. doi: 10.1002/ffj.1348

# Chapter 6

## Utilization of microbial oil obtained from crude glycerol for the production of polyol and its subsequent conversion to polyurethane foams

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*** Uprety BK**, Reddy JV, Dalli SS, Rakshit SK (2017) Utilization of microbial oil obtained from crude glycerol for the production of polyol and its subsequent conversion to polyurethane foams. Bioresour Technol 235:309–315.

**Abstract**

We have demonstrated possible use of microbial oil in biopolymer industries. Microbial oil was produced from biodiesel based crude glycerol and subsequently converted into polyol. Fermentation of crude glycerol in a batch bioreactor using *Rhodosporidium toruloides* ATCC 10788 produced 18.69 g/L of lipid at the end of 7 days. The microbial oil was then chemically converted to polyol and characterized using FT-IR and $^{1}$H-NMR. For comparison, canola oil and palm oil were also converted into their respective polyols. The hydroxyl numbers of polyols from canola, palm and microbial oil were found to be 266.86, 222.32 and 230.30 (mg KOH/g of sample) respectively. All the polyols were further converted into rigid and semi-rigid polyurethanes (maintaining the molar -NCO/-OH ratio of 1.1) to examine their suitability in polymer applications. Conversion of microbial lipid to polyurethane foam also provides a new route for the production of polymers using biodiesel based crude glycerol.

## 6.1. Introduction

Fossil resources are used for various applications including energy generation, running transportations, polymer and plastic synthesis. Dependency on such resources and its derivatives have led to various socio-economic and environmental problems. This includes global warming, deforestation, accumulation of non-biodegradable substances, contamination of natural resources and disputes among the countries [1, 2]. In order to overcome these issues, there is considerable focus on the use of alternative renewable energy sources. Biodiesel is one such renewable fuel obtained via transesterification of vegetable oils. It can be blended in various concentrations with fossil based diesel and be used with conventional diesel engine, without any modification. In the past decade, production of biodiesel has increased throughout the world [3].

However, with an increase in biodiesel production, there is also a rise in the accumulation of the by-product glycerol. During the transesterification of vegetable oils into biodiesel, approximately 10% wt. of glycerol is also produced. The glycerol obtained during the biodiesel production process, also known as crude glycerol, contains many impurities. Crude glycerol from biodiesel plant requires numerous purification steps before it can become suitable for direct commercial applications. Due to the buildup of crude glycerol in the past decade, there has been slump in the prices of pure glycerol [1]. Thus, carrying out an expensive downstream processing of crude glycerol to obtain its purer form has become uneconomical, mainly for the small and medium scale biodiesel producers. However, the glycerol needs to be diverted to value added products instead of causing environmental problems. Researchers have thus focussed on producing various value added components from crude glycerol in order to add revenues for the biodiesel industries [4, 5]. Among many such production processes that are being evaluated, bioconversion of crude glycerol to microbial oils or single cell oils (SCO) has gained considerable attention [4]. The term “single cell oils” were initially coined to include the lipids (rich in triacylglycerol) obtained from heterotrophs such as yeast and fungus [6]. Ratledge and Wynn (2002) reported the maximum theoretical lipid yield from glycerol to be 30% (w/w) [7]. Use of various types of yeasts and fungi to convert glycerol into lipids or microbial oil was previously reported by various authors [8–10]. Since the fatty acid characteristics of obtained microbial oils were similar to vegetable oils, all the reported works had stressed its use for the

production of biodiesel. However, exploring other applications of obtained microbial oils from such sources will make the overall conversion process more economically feasible and sustainable.

At present, production of varieties of polyols and polymers is mainly dependent on fossil resources [11–13]. Presently, only 1% of plastic produced are obtained from biological resources mainly vegetable oils. However, the global market of bioplastic is increasing every year and is expected to grow from 4.2 million tonnes in 2016 to 6.1 million tonnes by 2021 [14]. Even though only a very small portion of current plastic market is occupied by bio-based counterpart, the use of renewable sources for polymer production over fossil resources has certainly contributed in reducing the negative effects that fossil based chemicals would cause on environment. Polyurethanes are an important class of thermoplastic and thermoset polymers as it can be used in various fields [15]. Its production from vegetable oils have been reported by various authors [16–20]. Even though polymers (including polyurethanes) from vegetable oils are environmentally friendly, it has some major drawbacks. Firstly, using vegetable oils for polymers production is dependent on many factors including availability of huge arable lands for its cultivation, labor cost, climatic condition of the region and increase in prices of cooking oils and consequently on food security. Secondly, it has longer production lifecycle and higher costs than fossil resources [21]. Thus, an alternative cheaper renewable feedstock addressing these would be of great interest to the present scientific communities and oleo-chemical industries.

In this study, a novel route to produce polyols from crude glycerol has been reported by using a combination of biological and chemical processes. Firstly, crude glycerol was converted into microbial oil by using an oleaginous yeast *Rhodosporidium toruloides* ATCC 10788. The obtained microbial oil was further converted into its polyol via a chemical reaction. Suitability of obtained polyol for polyurethane (PU) production was also examined by producing two different polyurethane foams (i.e. rigid and semi-rigid). For comparison, polyurethane foams production from canola and palm oil were also carried out under similar reaction conditions. Utilization of such a potential environmental waste (i.e. crude glycerol) to produce polyurethane will help fix the carbon present in crude glycerol. This in turn reduces the release of greenhouse

gases to the atmosphere, thus making the whole process a green pathway. To the best of our knowledge, production of polyols from oil obtained from heterotrophic organisms (i.e. yeast or fungal) is not reported so far. Additionally, the uniqueness of our work lies in the fact that a complete conversion of crude glycerol into polyol via the production of microbial oil is demonstrated here.

## 6.2. Materials and methods

### 6.2.1. Microorganism and chemicals

The oleaginous yeast *R. toruloides* ATCC 10788 used in this work to produce microbial oil was obtained from American Type Culture Collection (ATCC). It was grown in YPG media (10 g/L yeast extract, 20 g/L peptones and 20 g/L glycerol) at 30 $^0$C for 24 hours maintaining a shaker speed of 200 rpm. For long term use, the culture was mixed with pure glycerol (1:1 ratio) and stored at -80 $^0$C. Refined, bleached and deodorized (RBD) palm oil was obtained from Saffire Blue Inc. (Canada). Refined pure canola oil was bought at local store located in Thunder Bay, Canada. Crude glycerol containing 44.56 %wt. glycerol, 13.86 %wt. methanol, 32.97 %wt. of soap (as sodium oleate), 0.82 %wt. of water and 4.38 %wt. of fatty acid methyl esters (FAME) was obtained from a biodiesel producer in Ontario (Canada). Tegostab B 8404 was a kind gift from Evonik Canada Inc. All other chemicals and reagents used in the experiments were analytically pure and obtained from Fisher Scientific, Canada.

### 6.2.2. Production of microbial oil from crude glycerol

Microbial oil from crude glycerol was produced using an oleaginous yeast, *R. toruloides* ATCC 10788. Fermentation conditions as described earlier in chapter 4 was adopted in this study. For the preparation of the inoculum, the yeast was grown in a YPG media (10 g/L yeast extract, 20 g/L peptones and 20 g/L glycerol) inside a rotary shaker maintained at 200 rpm, 30 $^0$C for 48 hours until viable cell count reached ~ 1 x $10^8$ cells/ml. The minimal media was prepared by using distilled water and maintained a pH of 6.0. It contained 1.0 g/L $KH_2PO_4$, 1.0 g/L $Na_2HPO_4.12H_2O$, 1.5 g/L $MgCl_2.6H_2O$ and 10 ml/L of trace elements. The trace elements mixture was prepared by mixing 4.0 g $CaCl_2.2H_2O$, 0.55 g $FeSO_4.7H_2O$, 0.52 g Citric acid. $H_2O$, 0.10 g $ZnSO_4.7H_2O$, 0.076 g $MnSO_4.H_2O$, and 100 µl 18M $H_2SO_4$ in 1 L of distilled water. Crude glycerol and ammonium chloride ($NH_4Cl$) were added in the media to obtain the overall

C: N molar ratio of 100.

Shake flask experiments were carried out in 125 ml Erlenmeyer flask with a working volume of 50 ml. Similarly, larger scale production of microbial oil was carried out in 1L bioreactor (Sartorius, Biostat A) using 750 ml of minimal media, containing crude glycerol as a carbon source. Fermentation was carried out at 30 $^0$C, 300 rpm stirrer speed and 1.2 vvm aeration. The pH of the media was maintained at 6 by automatic addition of 1 M NaOH or 1 M HCl. Sample analyses were performed in duplicates and percentage errors were expressed as mean ± standard deviation.

### 6.2.3. Analytical methods

Quantitative determination of methanol and glycerol in crude glycerol were done by using an Agilent 1260 infinity HPLC equipped with Aminex ion-exchange Biorad column (HPX-87H, 300 x 7.8mm) and a Refractive Index (RI) detector. 5mM Sulfuric Acid was used as a mobile phase and ran through the column at a flow rate of 0.60 ml/min. Column and detector temperatures were maintained at 50 $^0$C throughout the analysis [1].

In order to estimate the dry weight of biomass produced, 5 ml of fermentation broth was centrifuged for 10 minutes at 4400 rpm. The supernatant was then discarded and wet cells were washed using deionized water and dried overnight at 80 $^0$C. Lipid accumulated inside the biomass was estimated by using a modified Bligh-Dyer method [22]. Extracted lipid was trans-esterified using the method reported by Ichihara and Fukubayashi (2010). The transesterified solution lipid samples were then analyzed using GC-FID using methyl nonadecanoate as an internal standard. 1 µl of sample mixture was injected into Gas chromatography (Thermo Scientific, Trace 1300 model, polar column TG WaxMS A; 30 m x 0.25 µm x 0.25 µm) equipped with FID detector. Helium at 1 ml/min (as a carrier gas), air at 350 ml/min, $H_2$ at 35 ml/min, and $N_2$ at 40 ml/min were used throughout the run. Sample injection split ratio of 50:1 was kept during the injection. The temperature gradient maintained in the oven were 100 $^0$C (0.25 min), 30 $^0$C/min, 220 $^0$C (0 mins) and 10 $^0$C/min, 250 $^0$C (3 mins). Quantitative determination of each types of fatty acids in the lipid samples were done by comparing its peaks with the peaks of known fatty acid standards mixture including methyl nonadecanoate which

was used as an internal standard.

### 6.2.4. Production and characterization of polyols

Production of polyols from vegetable oils (Canola oil and Palm oil) and microbial oil were carried out by adopting the slightly modified method used by Saifuddin et al. (2010) for palm oil based polyol production. The overall conversion involved two separate reaction steps, including (i) Epoxidation of oils and (ii) Conversion of epoxidized oils into polyols via oxirane ring opening.

#### 6.2.4.1. Epoxidation of oils

Epoxidation of oils were carried out chemically as follows. 10 grams of oils were weighed and transferred into a 250 ml Erlenmeyer flask. To this, 11 grams of ethyl acetate was added followed by 0.9 grams of formic acid. 11 ml of hydrogen peroxide was then added dropwise (to control reaction temperature) to this solution by using a burette. The solution mixture was then heated on a heating plate at 60 $^0$C for 6 hours with constant stirring speed of 900 rpm. Percentage oxirane values were measured after each hour using the method described by Paquot (1979) involving titration of fat in benzene solution by hydrogen bromide acetic solution. At the end of reaction time, the aqueous layer was removed and oil layer was washed with distilled water in the ratio of 1:1. The residual solvents and water in the resulting oil layer was removed by drying at 140 $^0$C for about 3 hours.

#### 6.2.4.2. Production and characterization of polyol from epoxidized oils

Polyols were produced by taking 10 g of epoxidized oils and heating it on a hot plate until the temperature of the oils reached 85 $^0$C. To this warm solution, 1 g of phosphoric acid was added and was stirred at 300 rpm for 1 hour. At the end of reaction time, the acid values and hydroxyl values of the obtained polyols were determined using the method described by Paquot (1979). FT-IR analysis of obtained polyols were also carried out using Bruker Tensor 37 FTIR spectrophotometer, fitted with Pike Miracle ATR accessory containing ZnSe crystal. The background was recorded prior to placing the sample. The spectra were obtained by placing samples on the crystal and applying a light pressure using the Miracle micrometre clamp. For further confirmation of ring opening, proton NMR analysis of microbial oil (MO), epoxidized

microbial oil (EMO) and its polyol (MOP) were also carried out. Approximately 5 mg of samples were dissolved in 1 ml of deuterated chloroform and transferred into 5mm NMR tubes and analyzed using INOVA-500 Varian-NMR instrument.

### 6.2.4.3. Preparation and characterization of polyurethane

The rigid and semi rigid polyurethane were prepared from the polyols produced by adopting the method of Narine et al (2007) and Guo et al (2000) with slight modifications. The polyurethane foams were prepared by mixing the formulated polyols with toluene di-isocyanate (TDI) keeping a -CNO/-OH molar ratio of 1.1 [16, 25]. Formulated polyols were prepared by mixing the polyols with other additives (i.e. catalysts, co-catalyst and surfactant) as described in Table 6.1.

Table 6.1. The polyol formulation used to mix with toluene di-isocyanate (TDI) to maintain an isocyanate index of 1.1 to produce polyurethanes

| Sl. No. | Chemical | Role of chemical | Mass of chemicals (g) |
|---|---|---|---|
| 1 | Polyol | Monomer | 100 |
| 2 | DBTDL | Main catalyst | 1 |
| 3 | Tegostab B-8404 | Surfactant | 2 |
| 4 | DMEA | Co-catalyst | 1 |
| 5 | Water* | Blowing agent | 2 |

*Water is excluded during the preparation of rigid polyurethane

In order to produce polyurethanes, the formulated polyol was mixed with di-isocyanate (TDI) maintaining the isocyanate (-NCO) index of 1.1. For the preparation of semi-rigid polyurethane foams, 2 grams of water per 100 grams of polyols was added as a blowing agent. However, for the preparation of rigid foams, water was excluded. The amount of TDI to be reacted with 100 gm of polyols during the formulation of polyurethanes was calculated by using the formula [26];

$$\text{Amount of TDI needed (g)} = \left(\frac{OH\ value}{561} + \frac{m}{9}\right) \text{ x AE x } \frac{NCO\ index}{p}$$

Where, “OH value” represents hydroxyl value of the polyol used (mg KOH/g), “m” is the mass

(g) of water, NCO index is the ratio of -NCO/ -OH (i.e. 1.1 in this study), "AE" is the amine equivalent of toluene di-isocyanate (TDI) and "p" is the percentage purity of TDI used (95%).

The obtained polyurethanes were also analyzed using a Scanning Electron Microscope (SEM) to study the morphology and microstructures at cellular level. The prepared polyurethanes were placed on a stub and were carbon coated before being observed by SEM (Hitachi Su-70 Schottky Field Emission; Resolution is 1.0nm @15 kv/ 1.5 nm at 1 kv).

## 6.3. Results and discussion

### 6.3.1. Bioconversion of crude glycerol into microbial oil

Oleaginous yeast *Rhodosporidium toruloides* ATCC 10788 was utilized to produce microbial oil from crude glycerol. Methanol is one of the major impurities present in the crude glycerol by-product obtained from the biodiesel industries [3]**.** Preliminary work carried out in our lab showed that the growth of this strain is inhibited by the presence of methanol in a media [3]. Our studies also showed that reduction in significant amount of biomass and lipid concentration was observed when methanol concentration in the media was above 15 g/L. However, no such inhibition was observed when this strain was grown in a media containing crude glycerol diluted to get similar concentration of methanol (i.e. 15 g/L). Comparison of biomass and lipid production by *R. toruloides* in media containing pure glycerol (PG media), crude glycerol (CG media) and pure glycerol with methanol (GM media) is shown in Table 6.2. CG media was prepared by diluting the crude glycerol in such a way that total methanol concentration in the media was 15 g/L. The amount of glycerol in this media was 48.2 g/L. PG and GM media were used as controls for the CG media. PG contained 48.2 g/L of pure glycerol whereas GM media contained 15 g/L of methanol in addition to 48.2 g/L of pure glycerol.

From Table 6.2, it is seen that presence of methanol in the media inhibited the growth of the organism, whereas the use of crude glycerol improved both the growth and lipid production ability of this strain. Almost double the biomass concentration and triple the lipid concentration were obtained with crude glycerol (CG) media compared to PG media. When crude glycerol media was used, biomass concentration, lipid concentration and lipid content reached 23.63 g/L, 13.86 g/L and 58.66 % wt. respectively at the end of incubation period (i.e. 7 days). The higher

production of biomass and lipid in crude glycerol media (CG) even in presence of methanol was mainly due to other components in crude glycerol. Some of the other components in crude glycerol, such as soap, salt, FAME and free fatty acid, supported the growth of this strain in such a way that the effect of methanol on this organism was either nullified or overshadowed. Details on robustness of this strain and its ability to consume various impurities in crude glycerol has been discussed elsewhere [3]. Similar results were reported by authors while growing different strains of *R. toruloides* in crude glycerol media containing various concentration of impurities [10, 27, 28]. In order to obtain higher quantities of microbial lipids required for polyol production, batch fermentation was carried out for 7 days in a 1L bioreactor. Total biomass concentration, lipid concentration and lipid content at the end of incubation were found to be 27.48 g/L, 18.69 g/L and 68.03 %wt. respectively. This increase in biomass and lipid concentration when compared to flask experiments was due to better control over pH of the media, improved oxygen supply and better access to different growth factors present in the media.

Table 6.2. Biomass concentration, lipid concentration and lipid content obtained by growing *R. toruloides* ATCC 10788 for 7 days in different media containing 48.2 g/L of glycerol

| **Fermentation conditions** | **Fermentation type** | **Biomass Conc. (g/L)** | **Lipid Conc. (g/L)** | **Lipid content (% wt.)** |
|---|---|---|---|---|
| In pure glycerol (PG media) | Flask | 11.86±1.85 | 4.62±0.69 | 39.01±5.86 |
| In pure glycerol with 15 g/L of methanol (GM media) | Flask | 9.78±1.76 | 3.36±0.38 | 34.38±3.93 |
| In crude glycerol (CG media) | Flask | 23.63±2.92 | 13.86±1.48 | 58.66±6.26 |
| In crude glycerol (CG media) | Batch | 27.48±0.93 | 18.69±1.46 | 68.03±5.30 |

The lipid obtained was further characterized using GC-FID using the parameters of Uprety et al. (2016). Palmitic, stearic, oleic, linoleic and linolenic acids were the major fatty acids in the obtained lipid. Comparison of fatty acid profile of the obtained microbial lipid to other commonly used vegetable oils showed it to be similar to palm oil with regards to its total saturated and unsaturated fatty acid content (Table 6.3).

From Table 6.3 it is seen that, even though, palmitic (C16:0) and stearic acid (C18:0) contents of obtained lipid are not similar to palm oil, the oleic (C18:1) and linoleic acid (C18:2) contents of both the oils are quite similar. Carbon-carbon double bonds present in the vegetable oils (e.g. C18:1 and C18:2) are the starting point for the production of polyols by epoxidation and oxirane ring opening [31]. Thus, double bonds or the degree of unsaturation of the oils are the major factors deciding its suitability for polyol production. Since, the degree of unsaturation of the obtained microbial oil was similar to palm oil, we decided to treat it in a similar manner as the latter to produce polyol and subsequently convert it to polyurethane foams.

Table 6.3. Fatty acid profile of varieties of oils used in this study. SFA: Saturated fatty acid; UFA: Unsaturated fatty acid

| Oil source | C16:0 (%) | C18:0 (%) | C18:1 (%) | C18:2 (%) | C18:3 (%) | Others (%) | SFA (%) | UFA (%) | References |
|---|---|---|---|---|---|---|---|---|---|
| Palm | 42.70 | 2.13 | 39.37 | 10.62 | 0.21 | 4.97 | ~49 | ~51 | [29] |
| Canola | 3.75 | 1.87 | 62.41 | 20.12 | 8.37 | 3.48 | ~7 | ~93 | [29] |
| Corn | 10.34 | 2.04 | 25.54 | 59.27 | 1.07 | 1.74 | ~13 | ~87 | [29] |
| Sunflower | 6.20 | 2.80 | 28.0 | 62.2 | 0.16 | 0.64 | ~9 | ~91 | [30] |
| Rapeseed | 4.60 | 1.70 | 63.3 | 19.6 | 1.20 | 9.60 | ~6 | ~94 | [30] |
| ***R. toruloides* ATCC 10788** | **24.39±0.26** | **16.38±0.13** | **47.16±0.12** | **12.05±0.01** | **-** | **-** | **~ 40** | **~ 60** | **This study** |

### 6.3.2. Conversion of oils into polyols and its characterization

Polyol production was carried out using a method described by Saifuddin et al. (2010) that was slightly modified and using canola oil, palm oil and microbial oil as starting material. The overall conversion process was carried out in two separate reaction steps (i) Epoxidation of oils and (ii) Polyols formation via oxirane ring opening of epoxidized oils as described in materials and methods. Epoxidation of oils is a process where an epoxy group is added to the double bond present in the fatty acid chains of vegetable oils [32]. Generally, vegetable based oils do not contain hydroxyl groups required for polymer synthesis. Thus, for their conversion into polyols, they are chemically modified to introduce hydroxyl groups into their structures, mainly at the carbon-carbon double bonds and ester linkages. Addition of hydroxyl groups to the fatty acid chains of vegetable oils can be done by using various methods including ozonolysis, thiol-ene coupling, transesterification and amidation, epoxidation followed by oxirane ring opening and hydroformylation followed by hydrogenation [31]. However, production of polyols by epoxidation and its subsequent ring opening is the most common method reported [31–36]. During the epoxidation of oils, the double bonds present in fatty acid chains of triglycerides are converted into epoxy group or oxirane rings. Epoxidation can be carried out in various ways, including chemical epoxidation, epoxidation using acid ion exchange resin (AIER), epoxidation using enzymes and epoxidation using metal catalyst [32]. Similarly, various ring opening agents such as alcohols, acids and hydrogen can be used to obtain polyols from epoxidized oils. Characteristics of polyols varies with the type of ring opening agents used [31]. In our study, epoxidation was carried out by reacting oils with peroxyacid (generated in situ via reaction between hydrogen peroxide and formic acid) under conventional heating of 85 $^{0}$C. The epoxidized oils were then converted into their respective polyols by using phosphoric acid as a ring opening agent.

The percentage oxirane is a measure of formation of epoxy group in the fatty acid chain of triglycerides (oil). Thus, for each of these oils, percentage oxirane values were measured as a function of time until constant values were obtained. The change in oxirane values of different oils with increase in epoxidation time are shown in Fig 6.1. It can be seen that the oxirane values of all the three types of oils used in this work increased with time and remained almost constant at the end of 6$^{th}$ hour. This was mainly because of the process conversion of epoxy groups from

the double bonds present in the fatty acid chains of the oils. The oxirane values of canola oil, palm oil and microbial oil at the end of 6 hours were 1.17%, 0.91% and 0.75% respectively (Fig 6.1).

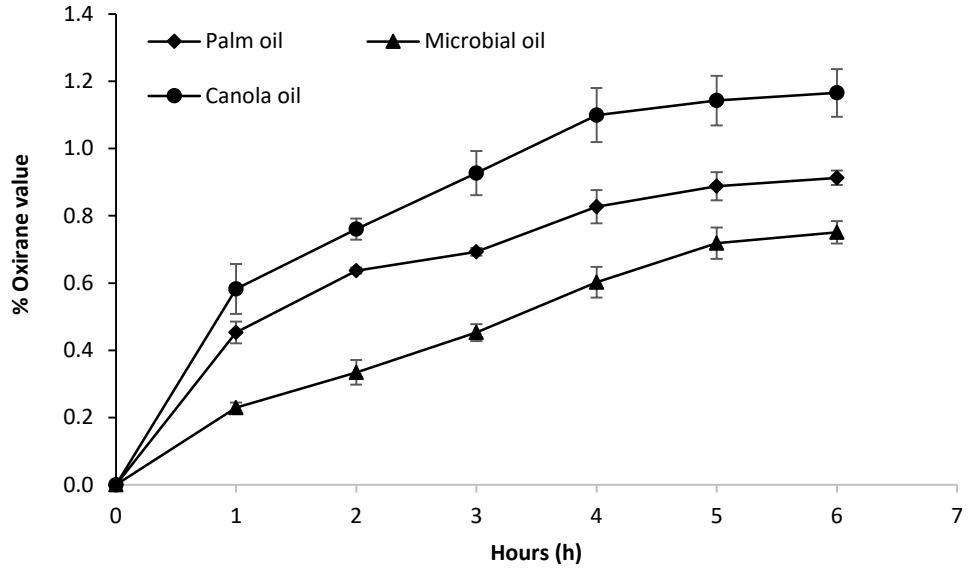



Fig 6.1. Change in percentage oxirane values of various types of oils during the epoxidation process

The higher % oxirane value for canola oil when compared to oil from palm and yeast was because of the higher degree of unsaturation of canola oil compared to the other two oils. Oils with large degree of unsaturation have larger potential to undergo epoxidation reaction as this reaction involves conversion of double bonds in oils into epoxy groups [32]. Since the degree of unsaturation of microbial oil was similar to palm oil, the percentage oxirane value for these two oils were also similar.

Hydroxyl number is the measure of content of free hydroxyl groups present in any chemical substance. The hydroxyl number of the obtained polyols were determined before it was converted into polyurethane foams. The hydroxyl number of polyols obtained from canola oil, palm oil and microbial oil were found to be 266.86, 222.32 and 230.30 (mg KOH/g of sample) respectively. The obtained hydroxyl numbers are similar to the previously reported values of polyols obtained from various vegetable oils [16, 37]. Table 6.4 shows hydroxyl numbers of vegetable oil based polyols reported by different authors recently.

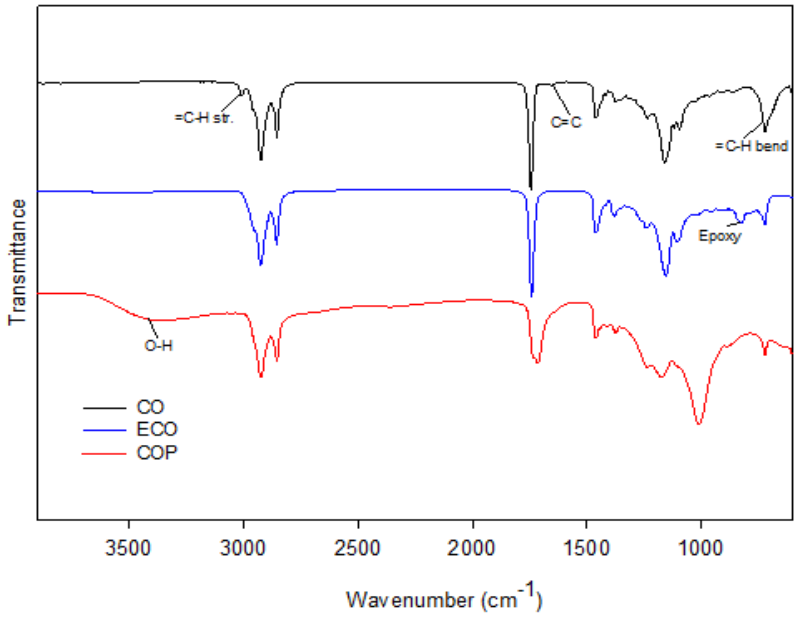
Table 6.4. Comparison of hydroxyl value of microbial oil with other vegetable oils obtained via epoxidation and oxirane ring opening

| Oil type | Ring opening Agent used | Hydroxyl value (mg KOH/g of sample) | References |
|---|---|---|---|
| Canola | Methanol | 173.6 | [35] |
| Palm oil | Hexamethylene glycol | 110 | [17] |
| Palm oil | Phthalic acid | 70-80 | [38] |
| Soybean oil | HCl | 197 | [36] |
| Soybean oil | 1,2-propanediol | 289.31 | [37] |
| Soybean oil | HBr | 182 | [36] |
| Linseed oil | Methanol | 247.8 | [35] |
| Sunflower oil | Methanol | 177.8 | [35] |
| Rapeseed oil | Diethylene glycol | 114-196 | [39] |
| Canola oil | Phosphoric acid | 266.86 | **This study** |
| Palm oil | Phosphoric acid | 222.32 | **This study** |
| Microbial oil | Phosphoric acid | 230.30 | **This study** |

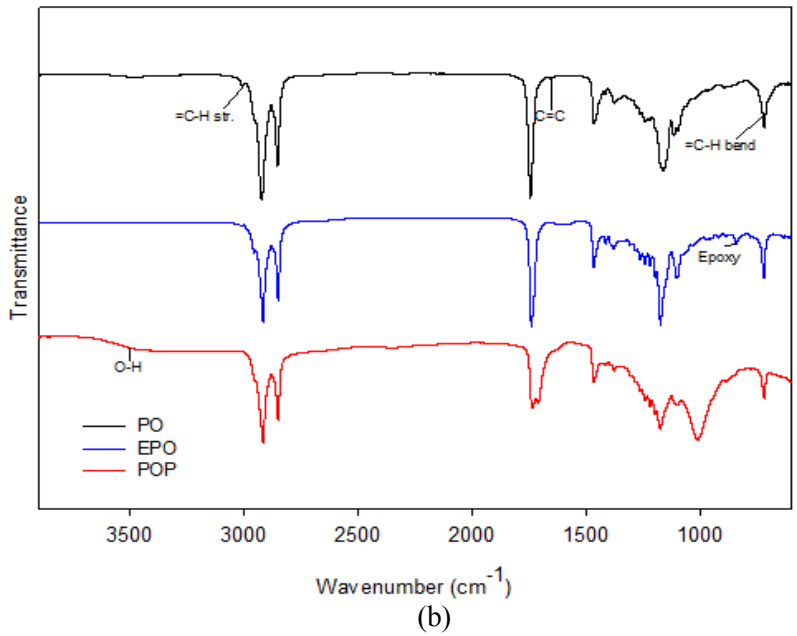
From the table, it is seen that the hydroxyl value of canola oil and palm oil based polyols obtained using a similar method as in our study were in the ranges of 150-275 and 70-110 (mg KOH/g of sample) respectively. The obtained hydroxyl value of canola based polyol (266.86 mg KOH/g of sample) in our study lies in the range of previously reported values. However, the obtained hydroxyl value of palm oil based polyol (222.32 mg KOH/g of sample) is little higher than previously reported values for palm oil based polyols. This is attributed to the fact that different ring opening agents and reaction conditions used in those studies [17, 38]. Additionally, the hydroxyl value of produced microbial oil based polyol in this study (i.e. 230.30 mg KOH/g of sample) is similar to that of palm oil based polyol (i.e. 222.32 mg KOH/g of sample). Thus, it can be concluded that the microbial oil can be successfully converted into polyol and the latter is similar in quality to that obtained from palm oil.

The conversion of oils into their respective polyols were confirmed by carrying out FT-IR analysis of the oils and the obtained polyols. Various authors have reported IR band typical to epoxy group in the range of 823-915 $cm^{-1}$ [37, 40, 41]. Presence of such bands in IR chromatogram of epoxidized canola, palm and microbial oils at 827, 844 and 850 $cm^{-1}$ respectively, confirmed the formation of the epoxy ring. The hydroxyl group attached to the

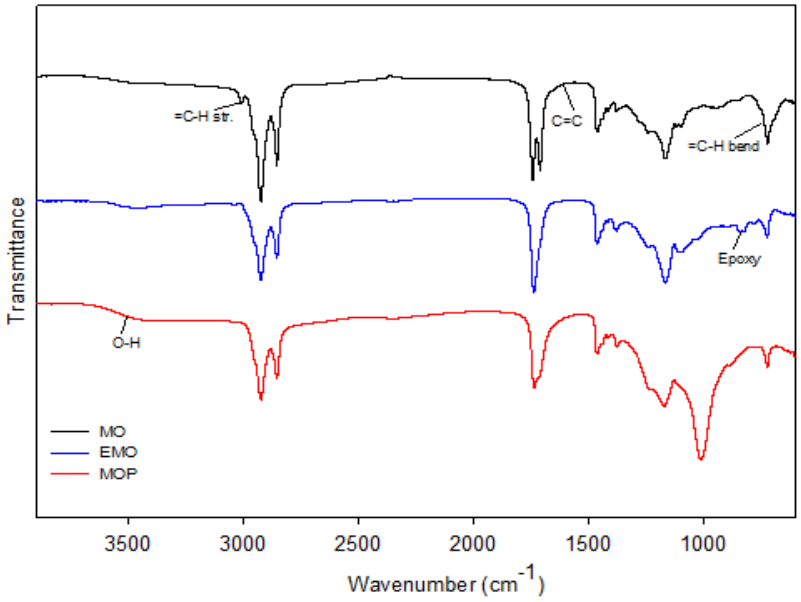
polyol gives the band in the range of 3200-3600 $cm^{-1}$ [42, 43]. Presence of such bands at approximately 3400 $cm^{-1}$ in case of all the produced polyols confirmed the attachment of hydroxyl group to the polyols. Unsaturated bonds in fatty acids chains of oils show characteristic bands in the range of 3010-3100, 675-1000 and 1620-1680 $cm^{-1}$ attributed to =C-H stretch, =C-H bend, and C=C stretch respectively. Its presence in IR spectra of oils but not in polyols showed the complete conversion of oils into its respective polyols (Fig 6.2a, 6.2b, 6.2c).




(a)




(b)




(c)

Fig 6.2. Comparison of FT-IR analysis of (a) canola oil (CO), epoxidized canola oil (ECO) and its polyol (COP) (b) palm oil (PO), epoxidized palm oil (EPO) and its polyol (POP) (c) microbial oil (MO), epoxidized (EMO) and its polyol (MOP)

Additionally, the efficiency of the conversion process was also determined by carrying out $^{1}$H-NMR of microbial oil and its polyol. In the spectra of microbial oil, presence of an alkene proton signals of –CH=CH– at around 5.30 ppm indicated the presence of alkene group in the produced oil. Upon epoxidation of oil, small peaks appeared at 2.9 ppm which is attributed to the epoxy group formed. The typical peaks for epoxy group is generally found in the range of 2.8-3.2 ppm [37, 41]. Similarly, after the ring opening of epoxidized oil, the peaks at around 5.30 ppm and 2.9 ppm disappeared and a singlet appeared at around 3.1 due to proton attached to hydroxyl group. This confirmed the complete conversion of microbial oil into polyol (Fig 6.3).

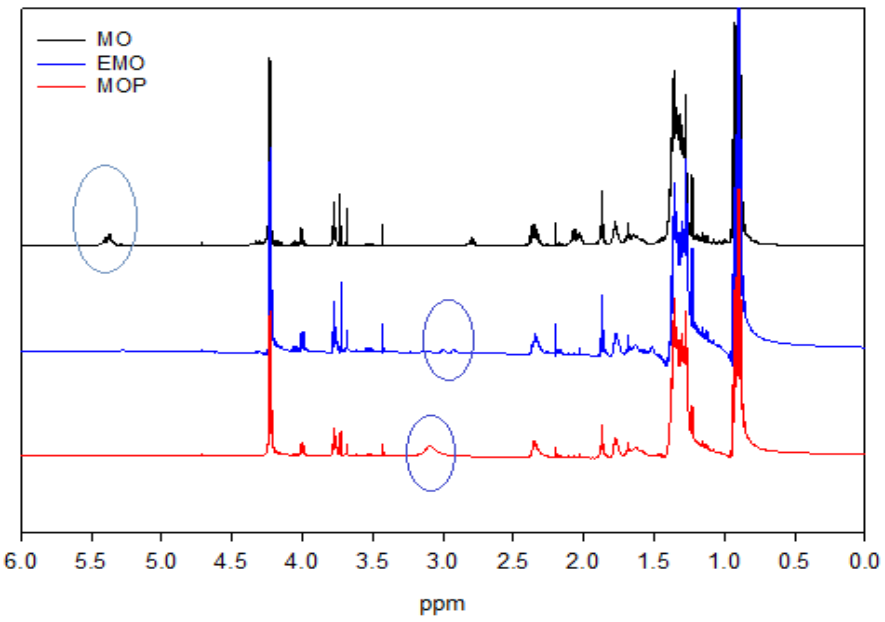



Fig 6.3. $^{1}$H-NMR of microbial oil (MO), epoxidized microbial oil (EMO) and microbial oil polyol (MOP)

### 6.3.3. Production of polyurethanes from polyols and its characterization

Different methods to produce polyurethanes from vegetable oil based polyols have been reported [16, 44]. Production of polyurethane foams are based on the principle of reacting the polyol with isocyanate. The polymerization reaction between polyol (-OH group) and isocyanate (-NCO group) give rise to the formation of polyurethanes [45]. Theoretically, equal amounts of monomers (i.e. polyols and isocyanate) are required for the complete polymerization reaction. However, in practice an isocyanate is always used in excess to compensate the amount of moisture generally present in polyols [45]. Similarly, in any given formulation, the ratio between NCO and OH group is called as NCO- index. For the production of polyurethanes, NCO index in between 1-1.1 is mostly recommended [46]. In order to confirm the suitability of obtained polyols for polymer formation, rigid and semi-rigid polyurethanes were prepared using previously reported methods [19, 43] with some modifications (Fig 6.4). In this study, NCO index of 1.1 was used to obtained the polyurethanes.

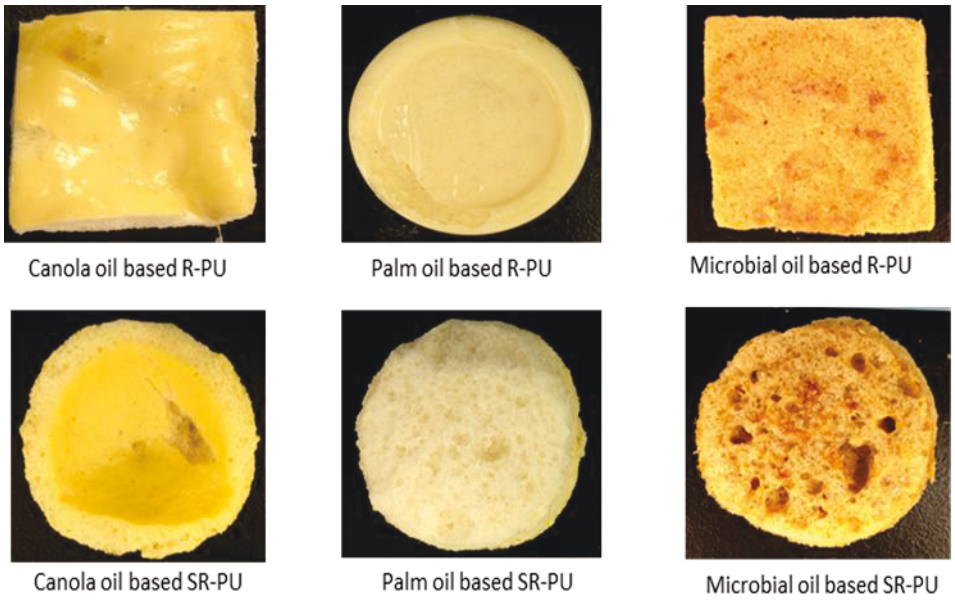
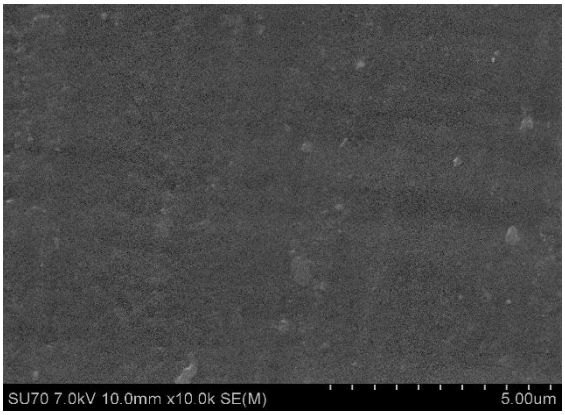
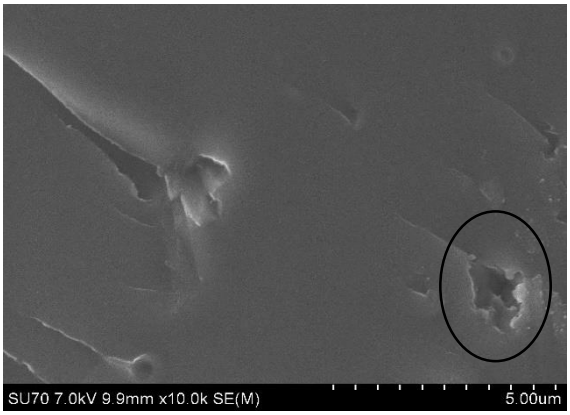



Fig 6.4. Various types of polyurethanes obtained from canola, palm and microbial oil. R-PU: Rigid Polyurethane; SR-PU: Semi-rigid Polyurethane

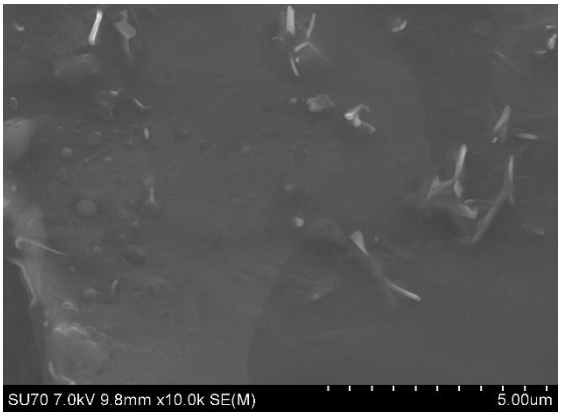
SEM analysis of the polyurethanes prepared were also carried out in order to study the cellular and morphological differences between these two types of PU foams. All the prepared rigid polyurethane foams surfaces were smooth with hardly any holes on them. However, many non-uniforms holes were present on the surfaces of prepared semi-rigid polyurethanes as expected. The formation of holes was due to the escaping carbon-dioxide formed during the reaction between water and di-isocyanate used to prepare polyurethanes (Fig 6.5).



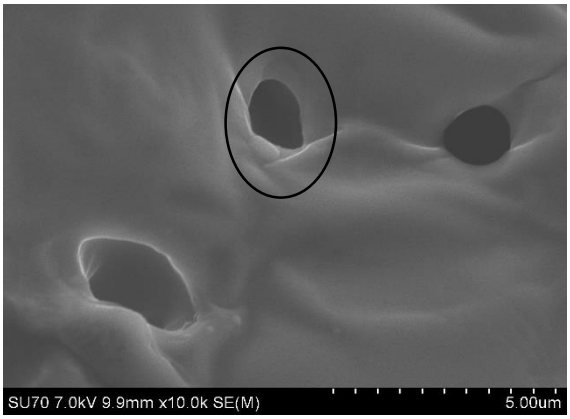
a. Canola oil rigid polyurethane



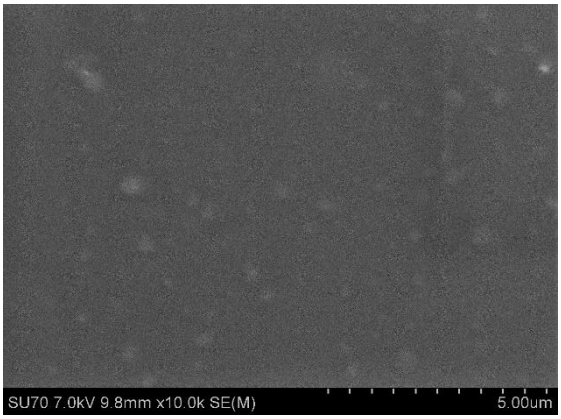
b. Canola oil semi-rigid polyurethane



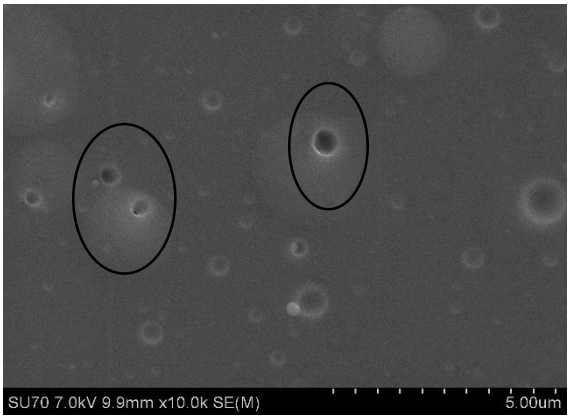
c. Palm oil rigid polyurethane



d. Palm oil semi-rigid polyurethane



e. Microbial oil rigid polyurethane



f. Microbial oil semi-rigid polyurethane

Fig 6.5. SEM analysis of various types of rigid and semi-rigid polyurethanes obtained from oils. Canola oil (a, b); palm oil (c, d); microbial oil (e, f)

## 6.4. Summary

Biomass concentration of 27.48 g/L and lipid concentration of 18.69 g/L were obtained by batch fermentation of crude glycerol using *R. toruloides*. The obtained microbial lipid was converted into polyol and characterized. For comparison, polyols produced from canola and palm oil were also converted under the similar reaction conditions. The hydroxyl numbers of polyols from canola, palm and microbial oil were found to be 266.86, 222.32 and 230.30 (mg KOH/g of sample) respectively. Rigid and semi-rigid types of polyurethanes were produced using the obtained polyols. This confirms the possibility of using microbial oil as a feedstock in the polymer industries.

**References**


1. Uprety BK, Chaiwong W, Ewelike C, Rakshit SK (2016) Biodiesel production using heterogeneous catalysts including wood ash and the importance of enhancing byproduct glycerol purity. Energy Convers Manag 115:191–199. doi: 10.1016/j.enconman.2016.02.032
2. Rittmann BE (2008) Opportunities for renewable bioenergy using microorganisms. Biotechnol Bioeng 100:203–212. doi: 10.1002/bit.21875
3. Uprety BK, Dalli SS, Rakshit SK (2017) Bioconversion of crude glycerol to microbial lipid using a robust oleaginous yeast Rhodosporidium toruloides ATCC 10788 capable of growing in the presence of impurities. Energy Convers Manag 135:117–128. doi: 10.1016/j.enconman.2016.12.071
4. Garlapati VK, Shankar U, Budhiraja A (2016) Bioconversion technologies of crude glycerol to value added industrial products. Biotechnol Reports 9:9–14. doi: 10.1016/j.btre.2015.11.002
5. Ardi MS, Aroua MK, Hashim NA (2015) Progress, prospect and challenges in glycerol purification process: A review. Renew Sustain Energy Rev 42:1164–1173. doi: 10.1016/j.rser.2014.10.091
6. Ratledge C (2013) Microbial oils : an introductory overview of current status and future prospects. Oilseeds Fats Crop Lipids. doi: 10.1051/ocl/2013029
7. Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 51:1–51. doi: 10.1016/S0065-2164(02)51000-5
8. 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 Res 6:300–310. doi: 10.1007/s12155-012-9257-4
9. Marchand K, Lubitz WD, Nicol RW (2013) Utilization of Biodiesel Derived Crude Glycerol by Fungal Isolates for Biomass and Single Cell Oil Production. J Biobased Mater Bioenergy 7:415–419. doi: 10.1166/jbmb.2013.1367
10. Xu J, Zhao X, Wang W, et al (2012) Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast Rhodosporidium toruloides and the individual effect of some impurities on lipid production. Biochem Eng J 65:30–36. doi:

10.1016/j.bej.2012.04.003

11. Katiyar V, Tripathi N, Patwa R, Kotecha P (2015) Environment Friendly Packaging Plastics. In: Alavi S, Thomas S, Sandeep KP, et al (eds) Polym. Packag. Appl. Taylor & Francis, Canada, p 115
12. Imre B, Pukánszky B (2013) Compatibilization in bio-based and biodegradable polymer blends. Eur Polym J 49:1215–1233. doi: 10.1016/j.eurpolymj.2013.01.019
13. Bradley R. (1998) Renewable Energy: Not Cheap, Not Green? Strateg Plan Energy Environ 17:15–21. doi: http://dx.doi.org/10.1080/10485236.1998.10530522
14. Bioplastic market (2016) Bioplastic market data. In: Eur. bioplastics. http://www.european-bioplastics.org/market/.
15. Zia KM, Bhatti HN, Ahmad Bhatti I (2007) Methods for polyurethane and polyurethane composites, recycling and recovery: A review. React Funct Polym 67:675–692. doi: 10.1016/j.reactfunctpolym.2007.05.004
16. Kong X, Liu G, Curtis JM (2012) Novel polyurethane produced from canola oil based poly(ether ester) polyols: Synthesis, characterization and properties. Eur Polym J 48:2097–2106. doi: 10.1016/j.eurpolymj.2012.08.012
17. Pawlik H, Prociak A (2012) Influence of Palm Oil-Based Polyol on the Properties of Flexible Polyurethane Foams. J Polym Environ 20:438–445. doi: 10.1007/s10924-011-0393-2
18. Saifuddin N, Chun Wen O, Wei Zhan L, Xin Ning K (2010) Palm Oil Based Polyols for Polyurethane Foams Application. Proc. Int. Conf. Adv. Renew. Energy Technol.
19. Narine SS, Kong X, Bouzidi L, Sporns P (2007) Physical properties of polyurethanes produced from polyols from seed oils: I. Elastomers. JAOCS, J Am Oil Chem Soc 84:55–63. doi: 10.1007/s11746-006-1006-4
20. Hu YH, Gao Y, De Wang N, et al (2002) Rigid polyurethane foam prepared from a rape seed oil based polyol. J Appl Polym Sci 84:591–597. doi: 10.1002/app.10311
21. Liang MH, Jiang JG (2013) Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. Prog Lipid Res 52:395–408. doi: 10.1016/j.plipres.2013.05.002
22. Manirakiza P, Covaci A, Schepens P (2001) Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh &

Dyer Extraction Methods. J Food Compos Anal 14:93–100. doi: 10.1006/jfca.2000.0972

23. Ichihara K, Fukubayashi Y (2010) Preparation of fatty acid methyl esters for gas-liquid chromatography. J Lipid Res 51:635–40. doi: 10.1194/jlr.D001065
24. Paquot C (1979) Standard Methods for the Analysis of Oils , Fats and Derivatives, 6th ed. Thiais
25. Kong X, Liu G, Qi H, Curtis JM (2013) Preparation and characterization of high-solid polyurethane coating systems based on vegetable oil derived polyols. Prog Org Coatings 76:1151–1160. doi: 10.1016/j.porgcoat.2013.03.019
26. Wang RM, Zheng SR, Zheng YP (2011) Molding technology of sandwich structure composites. In: Wang RM, Zheng SR, Zheng YP (eds) Polym. matrix Compos. Technol., 1st ed. Woodhead Publishing Limited, Cambridge, pp 321–353
27. Gao Z, Ma Y, Wang Q, et al (2016) Effect of crude glycerol impurities on lipid preparation by Rhodosporidium toruloides yeast 32489. Bioresour Technol 218:373–379. doi: 10.1016/j.biortech.2016.06.088
28. Uçkun Kiran E, Trzcinski A, Webb C (2013) Microbial oil produced from biodiesel by-products could enhance overall production. Bioresour Technol 129:650–654. doi: 10.1016/j.biortech.2012.11.152
29. Zambiazi RC, Przybylski R, Weber Zambiazi M, Barbosa Menonca C (2007) Fatty Acid Composition of Vegetable Oils and Fats. Bol do Cent Pesqui Process Aliment 25:111–120. doi: 10.5380/cep.v25i1.8399
30. Orsavova J, Misurcova L, Ambrozova JV, et al (2015) Fatty Acids Composition of Vegetable Oils and Its Contribution to Dietary Energy Intake and Dependence of Cardiovascular Mortality on Dietary Intake of Fatty Acids. Int J Mol Sci 16:12871–90. doi: 10.3390/ijms160612871
31. Li YY, Luo X, Hu S (2015) Polyols and Polyurethanes from Vegetable Oils and Their Derivatives. In: Li YY, Luo X, Hu S (eds) Bio-based Polyols and Polyurethanes, 1st ed. Springer International Publishing, pp 15–43
32. Saurabh T, Patnaik M, Bhagt SL, Renge VC (2011) Epoxidation of Vegetable Oils : a Review. Int J Adv Eng Technol 2:491–501.
33. Pan X, Webster DC (2012) New biobased high functionality polyols and their use in

polyurethane coatings. ChemSusChem 5:419–429. doi: 10.1002/cssc.201100415

34. Miao S, Wang P, Su Z, Zhang S (2014) Vegetable-oil-based polymers as future polymeric biomaterials. Acta Biomater 10:1692–1704. doi: 10.1016/j.actbio.2013.08.040
35. Zlatanic A, Lava C, Zhang W, Petrović ZS (2004) Effect of Structure on Properties of Polyols and Polyurethanes Based on Different Vegetable Oils. J Polym Sci Part B Polym Phys 42:809–819. doi: 10.1002/polb.10737
36. Guo A, Cho Y, Petrović ZS (2000) Structure and properties of halogenated and nonhalogenated soy-based polyols. J Polym Sci Part A Polym Chem 38:3900–3910. doi: 10.1002/1099-0518(20001101)38:21<3900::AID-POLA70>3.0.CO;2-E
37. Dai H, Yang L, Lin B, et al (2009) Synthesis and Characterization of the Different Soy-Based Polyols by Ring Opening of Epoxidized Soybean Oil with Methanol, 1,2-Ethanediol and 1,2-Propanediol. J Am Oil Chem Soc 86:261–267. doi: 10.1007/s11746-008-1342-7
38. Ang KP, Lee CS, Cheng SF, Chuah CH (2014) Synthesis of palm oil-based polyester polyol for polyurethane adhesive production. J Appl Polym Sci 131:1–8. doi: 10.1002/app.39967
39. Rojek P, Prociak A (2012) Effect of Different Rapeseed-Oil-Based Polyols on Mechanical Properties of Flexible Polyurethane Foams Piotr. J Appl Polym Sci. doi: 10.1002/app.36500
40. Nikolic G, Zlatkovic S, Cakic M, et al (2010) Fast fourier transform IR characterization of epoxy GY systems crosslinked with aliphatic and cycloaliphatic EH polyamine adducts. Sensors 10:684–696. doi: 10.3390/s100100684
41. Zhang J, Tang JJ, Zhang JX (2015) Polyols Prepared from Ring-Opening Epoxidized Soybean Oil by a Castor Oil-Based Fatty Diol. 2015:
42. De Vasconcelos Vieira Lopes R, Loureiro NPD, Pezzin APT, et al (2013) Synthesis of polyols and polyurethanes from vegetable oils-kinetic and characterization. J Polym Res. doi: 10.1007/s10965-013-0238-x
43. Guo A, & Ivan J&, Petrovic Z (2000) Rigid polyurethane foams based on soybean oil. J Appl Polym Sci 77:467–473.
44. Kong X, Narine SS (2007) Physical properties of polyurethane plastic sheets produced

from polyols from canola oil. Biomacromolecules 8:2203–2209. doi: 10.1021/bm070016i

45. Pinto ML (2010) Formulation, preparation, and characterization of polyurethane foams. J Chem Educ 87:212–215. doi: 10.1021/ed8000599

46. Szycher M (2013) Flexible and Semiflexible foams. In: Szycher M (ed) Szycher's Handb. Polyurethanes, 2nd ed. Taylor & Francis, Florida, pp 181–255

# Chapter 7

## Summary and Future Recommendations

## 7.1. Summary

In this this thesis, we explored ways to economize the overall biodiesel production process by studying cheaper more effective catalysts and the use of the crude glycerol by-product for the production of microbial lipids. We also discussed and demonstrated the possible use of the microbial lipids produced as a biodiesel feedstock and the production of polyurethane foams.

At the end of experiments directed towards specific objective 1, we found that as compared to unanchored heterogeneous catalysts, attaching calcium oxide (a type of heterogeneous catalyst, CaO) to aluminum oxide (used as an inert support, $Al_2O_3$) improves the purity of both the biodiesel and glycerol obtained on transesterification of oils. When methanolysis of Refined, Bleached and Deodorized (RBD) palm oil were carried out using calcium oxide 90.11% biodiesel conversion and 81.84% glycerol yield was obtained. In contrast, with the use of the supported catalyst, $CaO/Al_2O_3$, the amount biodiesel conversion and glycerol yields were found to be 86.38% and 79.32% respectively. Although the methanolysis by using calcium oxide showed higher biodiesel and glycerol conversion, the FAME and glycerol purity (97.66% and 96.36% respectively) obtained using $CaO/Al_2O_3$ were higher as compared to using un-anchored CaO (96.75% and 92.73% respectively). Thus, CaO attached to alumina, which simultaneously produces a purer form of by-product glycerol, can be used for production of value added compounds. Similarly, use of wood ash from two different sources (i.e. birch bark and fly ash from wood pellet) as basic catalysts produced biodiesel and glycerol with a purity in the range of 88.06% - 99.92% and 78.18% -88.23% respectively. This showed that wood ash from different sources can potentially be used as a cheap renewable heterogeneous catalyst for biodiesel production.

In order to address specific objective 2 of this thesis, crude glycerol obtained from biodiesel industry was initially characterized and then used to test the robustness of an oleaginous yeast *Rhodosporidium toruloides* ATCC 10788. The crude glycerol contained 44.56 wt.% glycerol and other impurities. Methanol (13.86 wt.%) and soap (32.97 wt.%) were the predominant impurities in the glycerol used. Use of such glycerol samples to grow *Rhodosporidium toruloides* ATCC 10788 enhanced the biomass and lipids obtained from this strain. Biomass

concentration of 21.16 g/L, lipid concentration of 11.27 g/L and lipid content of 53.28 wt. % were obtained at the end of 168 h fermentation. The biomass and lipids obtained were approximately double and triple the amounts compared to control experiments where similar level of pure glycerol was used as the carbon source. We then tested the effects of some of the individual major impurities present in crude glycerol used. Methanol and sodium chloride (>1.5%) present in crude glycerol inhibited the growth of this strain whereas FAME and soap present improved the biomass and lipid obtained. Since crude glycerol had comparatively lower FAME content (4.38 wt.%) as compared to soap, we realized that the latter was the major contributor for enhanced overall lipid production by this strain. The strain used was capable of growing on soap as a sole carbon source. 5.40 g/L of lipids was obtained when 3.5% (w/v) of soap was used as substrate. Lipids obtained from *R. toruloides* ATCC 10788 are high in oleic acid content. The presence of higher amounts of oleic acid, a type of mono-unsaturated fatty acids (MUFA), make it suitable for its use as a feedstock for the production of biodiesel. Use of robust strains like *R. toruloides* ATCC 10788 to produce microbial lipids from crude glycerol can brings profits to the biodiesel industry as such processes can easily be integrated into existing plants.

We subsequently studied the use of essential oils from seven plant sources on the metabolism of lipids produced (objective 3). All the essential oils inhibited R. toruloides ATCC 10788 biomass production to different degrees. The fatty acid profiles of the obtained lipids from this strains were also affected differently. Most of the essential oils enhanced the stearic acid content of the lipids. Stearic acid has numerous applications in food, pharmaceuticals and cosmetic industries. At specific levels of these essential oils in the media, the obtained lipids were quite unique in their fatty acids composition. For example, use of 2 g/L of orange essential oil in the growth medium produced lipids with fatty acid composition similar to mahua butter which has lots of commercial applications. Similarly, addition of origanum (0.3 g/L) or pine essential oil (3 g/L) into the media improved the oleic acid content of the lipid by 17.90% and 14.35% respectively. Such lipids are considered suitable for biodiesel production. Most of the essential oils used were found to inhibit $\Delta 9$ and $\Delta 12$ desaturase enzymes present in the fatty acids biosynthesis pathways of this strain. This was the reason for increased amount of stearic acids

in most of the lipids obtained. Mono-terpenoids in essential oils were the major components responsible for bringing about such changes in the fatty acids composition of the lipids. One of the essential oils, orange essential oil, used in the study of *R. toruloides* ATCC 10788 was also tested on another oleaginous yeast, *Cryptococcus curvatus* ATCC 20509. Orange essential oil reduced the activities of elongase and Δ9 desaturase enzyme involved in lipid metabolism in both the yeast strains but to different degrees**.** Hence, it was concluded that effect of essential oil is specific to the species used and the addition of such essential oils into the growth media can help produce desirable marketable products.

Batch fermentation of crude glycerol using *Rhodosporidium toruloides* ATCC 10788 in a bioreactor produced a biomass concentration of 27.48 g/L and lipid concentration of 18.69 g/L at the end of 168 h. The microbial lipid obtained was used for conversion into polyol, a precursor to polyurethane production (objective 4). Epoxidation and oxirane ring opening reactions were used for these purposes. The polyols were further characterized using FT-IR and $^{1}$H-NMR. Polyols from canola and palm oil were also produced under the similar reaction conditions to compare with microbial lipid based polyol. The hydroxyl numbers of polyols from canola, palm and microbial oil were found to be 266.86, 222.32 and 230.30 (mg KOH/g of sample) respectively. These values were similar to previously reported polyols obtained from vegetable oils using similar conversion methods. Rigid and semi-rigid types of polyurethanes were then synthesized using the obtained polyols from these oils. The produced polyurethanes can potentially be used for various commercial applications.

### 7.2. Future recommendations

Based on the results obtained from this thesis study the following recommendations for future study are suggested:

1. Biodiesel and glycerol conversion obtained using birch ash reported in this thesis were 88.06% and 75.01% respectively. These values can be improved by further optimization of the process. We have also found that ash from different sources show different degrees of catalytic activity. Hence ash from different plant sources can be can be tested for higher catalytic activities. Besides being using as a catalyst, ash has been used as a support to to

catalyze various chemical reactions [1–3]. Some experiments are already being designed in our lab to explore the possibility of attaching a heterogeneous catalyst calcium oxide onto ash. Use of such supports can reduce the issue of leaching commonly associated with heterogeneous catalysts and lead to higher catalytic activity compared to presently used support inert compounds such as aluminum oxides.

2. A detailed study on ability of *Rhodosporidium toruloides* ATCC 10788 to consume soap should be carried out. The potential application of this strain to treat effluents rich in soap from car wash centers, soap manufacturing and textile industries can also be studied. Besides lipids, red yeasts such as *Rhodosporidium toruloides* ATCC 10788 are known to simultaneously produce carotenoids, a low volume-high value product. Some initial experiments with this strain to produce such compounds on crude glycerol are being carried out in our laboratory.

3. The Δ9 desaturase gene is responsible for coding Δ9 desaturase enzyme which convert stearic acid (C18:0) to oleic acid (C18:1) in the fatty acid biosynthesis metabolism of *Rhodosporidium toruloides*. Similarly, Δ12 desaturase gene is responsible for coding Δ12 desaturase enzyme which convert oleic acid (C18:1) to linoleic acid (C18:2). Future studies on metabolic engineering using molecular techniques to simultaneously overexpress Δ9 desaturase gene and knockout Δ12 desaturase gene present inside this yeast can be done. This will certainly enhance oleic acid content of the lipids making it suitable for biodiesel production and other such applications.

4. Future studies could also involve the optimization and scale up of developed methods for the conversion of crude glycerol to polyurethane via the formation of microbial lipids. Physical and spectroscopic characterization of polyurethane (PU) foams should also be carried out. PU foams resistant to fire can be produced by introducing certain halogen groups (e.g. bromide or chloride) to the polyols. This can potentially lead to the use of crude glycerol for a value-added product and fixing the carbon content of this by-product.

**References**


1. Jain D, Hada R, Rani A (2013) Surface Modification of Fly Ash for Active Catalysis. J Catal 2013:1–9. doi: 10.1155/2013/723957
2. Khatri C, Mishra MK, Rani A (2010) Synthesis and characterization of fly ash supported sulfated zirconia catalyst for benzylation reactions. Fuel Process Technol 91:1288–1295. doi: 10.1016/j.fuproc.2010.04.011
3. Jain D, Khatri C, Rani A (2010) Fly ash supported calcium oxide as recyclable solid base catalyst for Knoevenagel condensation reaction. Fuel Process Technol 91:1015–1021. doi: 10.1016/j.fuproc.2010.02.021