BIODIESEL PRODUCTION FROM MICROALGAE BY ENZYMATIC TRANSESTERIFICATION

This work is submitted in fulfillment for the requirements for the degree of Doctor of Technology: Biotechnology in the Faculty of Applied Sciences at Durban University of Technology.

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2014

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Declaration by student

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I declare that the thesis herewith submitted for the degree of Doctor of Technology: Biotechnology at the Durban University of Technology is the result of my own work and has not been previously submitted for a degree at any other University.

_____
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I hereby approve the final submission of the following thesis.

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This____ day_____ of 2014, at the Durban University of Technology.

Dedication

This thesis is dedicated to my beloved family specially my beloved grandfather

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CONTENTS

LIST OF SYMBOLS

Symbols	Expanded form
µm	Micrometers
ASTM	American society for testing and materials
AV	Acid value
BSP	Biomass support particles
C	Conversion
CI	Compression ignition
DCW	Dry cell weight
EC	Enzyme commission
EN	European nations
FAAE	Fatty acid alkyl esters
FAME	Fatty acid methyl esters
FFA	Free fatty acids
g	Grams
h	Hours
kg	Kilograms
L	Liter
M	Moles
mg	Milligrams
min	Minute
ml	Milliliter
rpm	Revolutions per minute
SI	Spark ignition
SV	Saponification value
v/v	Volume/Volume
W	Watt
wt	Weight
Y	Yield

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ABSTRACT

Main focus of this study is to investigate the enzymatic-conversion of microalgal lipids to biodiesel. However, preceding steps before conversion such as drying of microalgal biomass and extraction of lipids were also studied. Downstream processing of microalgae has several challenges and there is very little literature available in this area. *S. obliquus* was grown in the pilot scale open pond cultivation system for biomass production. Different techniques were studied for biomass drying and extraction of lipids from harvested microalgal biomass. Effect of these drying and extraction techniques on lipid yield and quality was assessed. Energy consumption and economic evaluation was also studied.

Enzymatic conversion of microalgal lipids by extracellular and whole cell lipase application was investigated. For both applications, free and immobilized lipases from different sources were screened and selected based on biodiesel conversion. Process parameters were optimized using chosen extracellular and whole cell lipases; also step-wise methanol addition was studied to improve the biodiesel conversion. Immobilized lipase was studied for its reuse. Final biodiesel was characterized for its fuel properties and compared with the specifications given by international standards. Enzymatic conversion of microalgal lipids was compared with the conventional homogeneous acid-catalyzed conversion. Enzymatic conversion and chemical conversion were techno-economically investigated based on process cost, energy consumption and processing steps.

Freeze drying was the most efficient technique, however at large scale economical sun drying could also be selected as possible drying step. Microwave assisted lipid extraction performed better compared to sonication technique. Immobilized *P. fluorescens* lipase in extracellular

application and *A. niger* lipase in whole cell application showed superior biodiesel conversion. The extracellular immobilized *P. fluorescens* lipase showed better biodiesel conversion and yields than the immobilized *A. niger* whole cell lipase. Both the enzyme catalysts showed lower biodiesel conversion compared to conventional chemical catalyst and higher processing cost. However, techno-economic analysis showed that, the reuse potential of immobilized lipases can significantly improve the economics. Fewer purification steps, less wastewater generation and minimal energy input are the benefits of enzymatic route of biodiesel conversion. Microalgae as a feedstock and lipase as a catalyst for conversion makes overall biodiesel production process environmentally-friendly. Data from this study has academic as well as industrial significance. Conclusions from this study form the basis for greener and sustainable scaling-up of microalgal biodiesel production process.

PREFACE

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- SINGH, B., **GULDHE, A.**, RAWAT, I. & BUX, F. 2014. Towards a sustainable approach for development of biodiesel from plant and microalgae. *Renewable and Sustainable Energy Reviews,* 29**,** 216-245. (Impact factor: 5.627)
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- MISRA, R., **GULDHE, A.**, SINGH, P., RAWAT, I. & BUX, F. 2014. Electrochemical microalgae harvesting process by using non-sacrificial carbon electrode: An effective and advance option for biodiesel production. *4th International conference on algal biomass, biofuels and bioproducts.* Santa Fe, New Mexico, USA: Elsevier.

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CHAPTER ONE: INTRODUCTION

1. General introduction

Biodiesel has emerged as an environmentally-friendly and renewable alternative fuel to petroleum-based fuels. Reserves of conventional petroleum-based fuels which are only located in certain parts of the world are rapidly diminishing. Because of ever-increasing prices of petroleum-based fuels and environmental concerns due to emission of toxic compounds on its combustion, many countries across the world are encouraging the use of biodiesel as a transport fuel. Biodiesel can be produced locally using various feedstocks depending upon its availability in a particular region and thus provides energy security. A high oxygen content in biodiesel allows the complete combustion of biodiesel in engines, thus exhaust emissions have lower amounts of particulates, hydrocarbons, gases like carbon monoxide (CO), carbon dioxide (CO_2) and sulphur oxides (SO_x), making this fuel environmentally-friendly (Atabani et al., 2012, Chisti, 2007, Ma and Hanna, 1999, Robles-Medina et al., 2009). Biodiesel, owing to its high flash point of around 150ºC is safe for transport and storage (Balat, 2011, Yusuf et al., 2011). It is a viable option as an alternative to petroleum-based fuels because it can be used in its pure form or can be blended with petroleum fuels, without modification of existing engines or with only minor modifications. The use of biodiesel can be implemented using the same infrastructure and mechanisms which are currently used for petroleum-based fuels (Amaro et al., 2011, Sharma and Singh, 2009, Scott et al., 2010, Yusuf et al., 2011). In countries like the USA, Brazil, Japan, Malaysia, India, Germany, France and Italy, biodiesel blends with petroleum fuels are being employed as transport fuel. For this application, biodiesel has to comply with the specifications

that are widely accepted viz. European Nations (EN14214), and American Society for Testing and Materials (ASTM D6751) (Atabani et al., 2012, Janaun and Ellis, 2010).

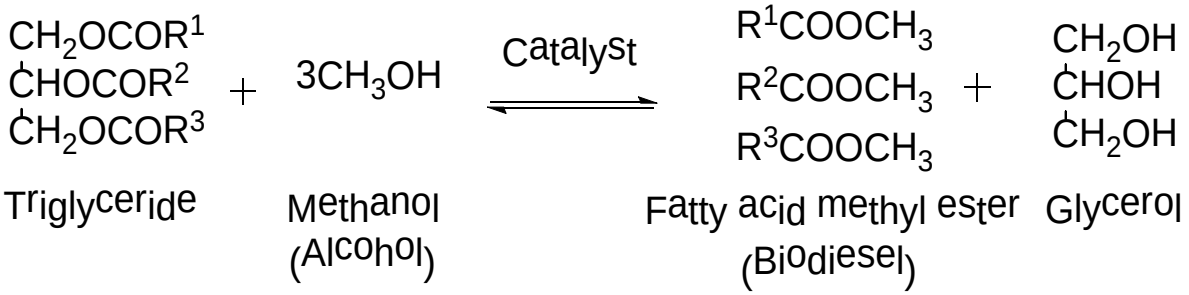
Chemically, biodiesel comprises of mono-alkyl esters of long chain fatty acids (fatty acid alkyl esters, FAAE) derived from natural and renewable sources such as plant (edible and non-edible) oils and animal fats. Natural oils and fats are triglyceride esters of glycerol and three fatty acids molecules (Ma and Hanna, 1999). Transesterification and esterification of plant oils and animal fats is the most widely-used method for biodiesel production (Borugadda and Goud, 2012, Janaun and Ellis, 2010, Yusuf et al., 2011). In the transesterification process, biodiesel is produced by reaction between fatty acids and alcohol in the presence of a catalyst to yield the desired mono-alkyl esters and glycerol (Kim et al., 2013). Various factors such as feedstock, free fatty acid content, concentration and type of catalyst, purity of reactants, temperature, reaction time, selection of acyl acceptor and alcohol to oil molar ratio influence the optimum yield of biodiesel. Sustainability of various feedstocks, catalytic methods, process parameter optimization and improvement of product quality has been investigated by researchers to make the biodiesel production process by transesterification economically-viable and environmentally friendly (Ma and Hanna, 1999, Sharma et al., 2008).

2. Transesterification for biodiesel production

The most common method of biodiesel production is transesterification or alcoholysis of triglyceride oil with alcohol in the presence of a catalyst which yields monoalkyl esters of fatty acids and glycerol (Figure 1.1). This is a three-step reaction in which the triglycerides are converted to diglycerides, diglycerides to monoglycerides and finally monoglycerides to glycerol. A monoalkyl ester of fatty acid is produced in each of the three steps

(Fukuda et al., 2001). Stoichiometrically, three moles of alcohol are needed for conversion of one mole of triglyceride into biodiesel, but a higher amount of alcohol is usually added to drive the reaction in the forward direction (Leca et al., 2010). At industrial scale, biodiesel production by alkaline catalysis using methanol as an acyl acceptor is widely used because of its high yield and economical viability (Sharma et al., 2008).

Figure 1.1: Reaction showing transesterification process. R^1, R^2 and R^3 are alkyl chains



2.1 Feedstocks used in transesterification

A wide range of feedstocks have been investigated for biodiesel production, but only a few of them were found to be successful for commercial implementation at industrial scale of production. Feedstock used for biodiesel production varies with the geographic region depending upon cultivation conditions and its availability. Oil composition, the percentage content per dry biomass, yield per hectare are important criteria for selection of the feedstock. Moreover, the biodiesel properties like oxidation stability and cold filter plugging point are dependent on the type of feedstock used (Ramos et al., 2009). The predominant feedstocks investigated worldwide for the production of biodiesel are edible and non-edible plant oils, animal-derived fats, waste cooking oils and microalgal lipids (Gui et al., 2008, Rawat et al., 2013, Sharma and Singh, 2009).

Biodiesel produced from the edible oil feedstock contribute more than 95% of total biodiesel production. Rapeseed oils (84%) and sunflower oils (13%) are the major contributor as feedstock in biodiesel production from edible oils followed by palm oil (1%) and the remaining from soybean, groundnut, coconut, peanut, corn and canola (2%) (Atabani et al., 2012). Non-edible oil feedstock used for biodiesel production mainly includes Jatropha (*Jatropha curcas),* caster (*Ricinus communis*), karanja (*Milletia pinnata*), tobacco (*Nicotiana tabacum*), mahua (*Madhuca longifolia*), rubber (*Ficus elastica*) and polanga (*Calophyllum inophyllum*) (Gui et al., 2008, Sharma et al., 2008, Tan et al., 2010). Animal-derived products such as tallow, lard and poultry fat have also been explored for their utility as feedstocks for biodiesel production (Dias et al., 2012, Encinar et al., 2011). Waste oil such as used cooking oil, usually from the restaurants and food processing plants; have been utilized as feedstock in production of biodiesel. Utilizing the waste cooking oil as feedstock for development of biodiesel solves its disposal problem thus minimizing the environmental issues (Meng et al., 2008).

Microalgae have recently emerged as one of the most promising feedstocks for biodiesel production. Biodiesel from microalgae is considered to have potential to meet the global demand of transportation fuel, as microalgal growth rate and lipid yield is high as compared to plant oils (Chisti, 2007, Rawat et al., 2013). Developing economically-viable microalgal cultivation, biomass harvesting and lipid extraction and conversion techniques are the present challenges for scaling-up of microalgal biodiesel to industrial scale. Feedstock accounts for the major share in overall production cost of biodiesel. Thus, choice of a cheap, easily available and sustainable feedstock becomes a crucial step towards an economically-viable and sustainable biodiesel production process.

2.2 Catalysts used in transesterification

The homogeneous and heterogeneous chemical catalysts (acid and base), and enzyme catalysts are currently being used to catalyze the transesterification reaction (Table 1.1). Selection of the catalyst, optimization of reaction parameters and overcoming challenges (eg. cost, leaching) for selected catalyst are the important aspects that have been investigated by researchers in order to obtain increased and purified biodiesel yields. Chemical catalysis comprises application of either an acid or a base. Alkaline metal hydroxides such as NaOH and KOH and alkoxides such as CH_3ONa are used as a base catalyst. NaOH is the most commonly used base catalyst, as it is comparatively cheaper and gives a high conversion of triglycerides to FAAE (Helwani et al., 2009). The alkaline catalysis is widely adopted in biodiesel production. The cost of the alkaline catalyst is cheap and a high lipid conversion is obtained. Alkaline catalysis is fast and is performed at moderate temperature ranging from 35 to 70ºC. Free fatty acids (FFA) and water content in the feedstock oil beyond a certain specified limit leads to saponification in alkali-catalyzed reactions. Soap formation due to saponification of FFA not only lowers the yield of FAAE, but also causes difficulty in downstream processing due to formation of emulsions. To overcome saponification, the feedstock containing high FFA could be pretreated with an acid catalyst (Fukuda et al., 2001). For the feedstock containing high FFA content, acidic catalysts are considered a suitable choice as they can catalyze both transesterification and esterification reactions. Among the homogeneous acid catalysts, sulfuric acid is used most often used, while other acids that can be employed are HCl, BF_3 and H_3PO_4. Acid catalysts perform well at higher alcohol to oil molar ratios and concentrations of catalyst. Acid catalysis has been reported to be relatively slower than the alkaline process (Borugadda and Goud, 2012, Helwani et al., 2009, Lam et al., 2010, Vyas et al., 2010). The chemical catalysis

process has several disadvantages such as high energy consumption and the downstream process requires removal of inorganic salts and water (used for purification) from biodiesel. Also, chemical catalysis leads to generation of acidic or alkaline wastewater which needs extra processing and energy requirements and acid catalyst like sulfuric acid also causes corrosion of reactor and pipelines.

Table 1.1: Advantages and disadvantages of various catalyst types used in biodiesel synthesis

Catalyst type	Advantage	Disadvantage
Homogeneous alkaline	High yield, low cost, fast reaction rate, medium energy requirement	Saponification of FFA, generation of wastewater, difficult catalyst recovery, purification of products required
Homogeneous acid	Medium yield, conversion of FFA to biodiesel, low cost, medium reaction rate	Generation of wastewater, difficult catalyst recovery, purification of products required, corrosion of equipments, high energy requirement
Heterogenous alkaline	High yield, medium cost, reusability, fast reaction rate, can be used in continuous process	High energy requirement, tedious catalyst preparation, catalyst leaching, saponification of FFA
Heterogenous acid	High yield, reusability, medium cost, fast reaction rate, conversion of FFA to biodiesel, can be used in continuous process	High energy requirement, tedious catalyst preparation, catalyst leaching, corrosion of equipments
Enzyme (immobilized lipase)	High yield, conversion of FFA to biodiesel, low energy requirement, high product purity, reusability, less wastewater generation, can be used in continuous process	Inhibition by alcohols, high cost

The separation procedure in homogeneous catalysis is costly, thus encouraging researchers to develop heterogeneous catalysts. The main advantage of utilizing heterogeneous catalysts is that they can be reused and washing steps are avoided, thus reduces input costs (Vyas et al., 2010). Heterogeneous catalysts can also be used in continuous production of biodiesel. Heterogenous catalysts include solid bases like zeolites, alkaline earth metal oxides (CaO, MgO), hydrotalcites and solid acids like zirconia and alumina and sulfated tin oxide (STO). Heterogenous acid catalysts catalyze both esterification and transesterification simultaneously increasing the product yield when feedstocks with high FFA are used. A constraint is that leaching of solid heterogeneous catalysts during reaction adds impurities in the product. The three phase immiscible system of the heterogeneous catalysis is also mass transfer limiting (Helwani et al., 2009, Lam et al., 2010).

Lipases have been used as an enzyme catalyst in the transesterification (Fjerbaek et al., 2009, Helwani et al., 2009, Robles-Medina et al., 2009, Shimada et al., 2002,). As compared to chemical catalysis, the enzyme catalysis occurs at milder temperature, thus requiring less energy consumption. Enzymes have the capability of converting oils from different sources as they catalyze the conversion of both triglycerides and free FFA by transesterification and esterification respectively. This property of enzymes makes them suitable for conversion of unconventional feedstocks such as waste cooking oil and microalgal oil to biodiesel. If the enzyme is immobilized, it can be used repeatedly, consequently reducing operational cost in production of biodiesel. In enzyme catalysis, separation and purification of product are easier than the chemical process. Biodiesel and glycerol obtained from the enzymatic conversion process are more pure as compared to other catalytic processes. There is less generation of wastewater in enzymatic transesterification which makes the process environmentally friendly

(Bajaj et al., 2010, Fukuda et al., 2001, Shah et al., 2003, Shimada et al., 2002). The product obtained by enzyme catalysis is, however, expensive because of high enzyme cost. Also, enzyme activity is inhibited by the short chain alcohols used as an acyl acceptor in the transesterification process. The development of new techniques to overcome the constraints associated with enzyme catalysis has been addressed by researchers and requires further investigation. Enzyme catalysis is considered to be a greener technology in biodiesel production with its environmentally-benign nature and attractive product purity.

3. Microalgal biodiesel

Microalgal biodiesel is gaining interest due to the numerous advantages associated with it. Microalgae require minimal land and fresh water and sequester CO_2 during its cultivation process. Microalgae are known to produce more lipids per acre of land area than most other oil crops and can be grown on marginal lands addressing the food security concerns (Chisti, 2007). Microalgae are diverse group of organisms growing in varied range of environments. Thus it can be cultivated in any part of world applying various cultivation technologies. Wastewater can also be utilized for its cultivation as a nutrient medium. Microalgae cultivation on wastewater can cater to biomass requirements as well as remove nutrients from the wastewater (Rawat et al., 2011). Lipid quality and fatty acid profile of microalgae has shown that it can be used as a suitable feedstock for biodiesel production. Moreover other constituents apart from lipids e.g. pigments, proteins, carbohydrates in microalgae have commercial importance as nutraceuticals and pharmaceuticals. These other constituents can be produced from microalgae in an integrated biorefinary approach along with biodiesel production (Mutanda et al., 2011b). Lipid extracted algae can also be used as animal and fish feed. Microalgae as a biodiesel

feedstock improves the fresh water and carbon footprint, has capability to cater transport fuel needs, provides local energy security and thus holds promise for greener future.

4. Limitations of microalgal biodiesel

Microalgae derived biodiesel despite of its advantages, facing some technological challenges to reduce environmental adverse impacts and high processing cost. Downstream processing of microalgae has many challenges in terms of technological limitations, cost effectiveness and environmental concerns. Harvesting of biomass, extraction of lipids, conversion of lipids to biodiesel and performance of biodiesel are still bottleneck in sustainable biodiesel production (Li et al., 2011d, Scott et al., 2010). Most of the research work has been focused on upstream strategies of microalgae cultivation, screening of oleaginous strains and enhancement of lipid accumulation. Cost of microalgal biodiesel is biggest challenge in its industrial viability. Microalgal biodiesel production process has high energy input, which needs to be reduced. To make microalgal biodiesel sustainable and economically viable, energy input during the course of biodiesel production should be reduced. Downstream processing like drying of biomass, extraction and lipid conversion needs more attention from the research community to deal with the existing challenges and minimize energy input and environmental concerns.

5. Context of Research

Limited supply, increasing fossil fuel prices and environmental concerns had led to consideration of biodiesel as an alternate source of energy (Ma and Hanna, 1999, Yusuf et al., 2011). Due to requirement in large amount, biodiesel from vegetable oils, animal fats and waste oils cannot meet the global demand for transport fuel. Microalgae, with high biomass yields and

faster growth rates have the capability to meet the global requirement of transport fuel in the near future (Chisti, 2007). The chemical catalysis process is associated with disadvantages such as difficulty in conversion with high FFA content, high energy consumption, multistep purification and recovery of product and generation of acidic or alkaline wastewater. Enzyme catalysis provides advantages of less energy consumption and easy separation of product from the reaction mixture. There is less wastewater generation in enzymatic transesterification. Lipase is the enzyme which catalyzes the transesterification reaction and catalysis can be done using both extracellular and intracellular (whole cell) lipases in the immobilized form (Bajaj et al., 2010). High cost of enzyme and inhibition of activity by alcohol and glycerol are the main drawbacks in enzyme catalysis which need to be addressed (Bajaj et al., 2010, Tan et al., 2010). The enzymatic approach in microalgal biodiesel production is a novel approach, and therefore, needs to be studied as very little information has been published in this area.

CHAPTER TWO: LITERATURE REVIEW

Microalgal biodiesel is gaining lot of emphasis as a renewable and sustainable alternative energy source. Conversion of lipids to biodiesel using enzyme catalyst has shown promising future as a green conversion technology owing to its pure and high product yield, low energy consumption and less wastewater generation. This chapter includes review of microalgae as a biodiesel feedstock, overview of microalgal biodiesel synthesis process, transesterification for conversion of lipids, application of enzyme as a catalyst, factors influencing enzymatic transesterification and application of enzyme catalysis for microalgal biodiesel synthesis.

1. Biodiesel

The world's energy requirement anticipated for 2030 shall be 50% more than it is today. The transportation sector alone accounts for 30% of the world's total energy consumption and the last three decades have witnessed a steep rise in the number of transportation vehicles worldwide, most of which are private cars (Atabani et al., 2012). The global energy demand in transportation sector is expected to grow by 2% per year. This would lead to 80% higher emissions in terms of green house gas emissions and energy use in comparison to that of 2002 levels (Wilson and Lee, 2012). The combustion of diesel and gasoline derived from crude oil in compression ignition (CI) and spark ignition (SI) vehicles emits carbon dioxide along with hydrocarbons, particulate matter, carbon monoxide, oxides of nitrogen, and other carcinogens that pose direct and indirect dangers to mankind and ecosystems. The worldwide concern regarding the increase in carbon dioxide and other pollutants in the atmosphere and the dwindling reserves of crude oil have led to the development of renewable fuels. Despite the

differences in opinion on the amount of crude oil available its exhaustion in coming years seems inevitable. The renewable sources of energy including solar, wind, ocean, hydropower, geothermal, and biomass have the capability to provide alternative sources of clean energy (Schiermeier et al., 2008). Baring biomass and solar energy, these renewable energy sources are place specific and hence can be utilized only at locations that are suitable for their harnessing. Another source of renewable fuel that has received attention worldwide due to its compatibility as a fuel in a compression ignition (CI) engine is biodiesel. In between 2008 and 2010, the global biodiesel production was 17.6 billion litres, which is estimated to grow up to 41.9 billion litres in 2020 (Aytav and Kocar, 2013).

Biodiesel has been blended with mineral diesel (up to 20%) and has been used as fuel in CI engines, possessing a comparable calorific value to mineral diesel. As biodiesel is compatible with mineral diesel, it can even be used in neat form thus proving an alternate to mineral diesel with few, if any, modifications in CI engines (Fahmi and Cremaschi, 2012). Biodiesel emits lower exhaust emissions on its combustion as compared to mineral diesel and have shown a comparable performance as that with mineral diesel and aviation fuel (Durre and Richard, 2011). Owing to its renewability and sustainability, the production of biodiesel has gradually increased in recent years.

1.1 Feedstocks for biodiesel

Biodiesel is produced from a wide variety of raw materials (comprising of triglycerides as major component) by either esterification or transesterification or both depending on the acid value of the feedstock and the type of catalyst (acid or alkaline) used for its synthesis. A feedstock in the terminology of biodiesel has been well defined by Sun et al. (2010) as "*the*

raw material from which oils and fats are derived". The wide spectrum of feedstocks that can be used to synthesize biodiesel are edible oils (usually, wherever in surplus), non-edible oils, waste cooking oils, waste frying oils, animal or fish waste oil and microalgae oil (dos Reis Albuquerque et al., 2012). Approximately 7% of edible vegetable oils globally have been reported to be diverted to the production of biodiesel in 2007 (Balat, 2011). Borugadda and Goud (2012) reported that among the plant origin oils, edible oil contributes 95% of the feedstock constituent for the production of biodiesel. The advantage of edible oil as a feedstock for production of biodiesel is their low free fatty acid (FFA) content (i.e. >1%) which allow single step transesterification of the oil (Falahati and Tremblay, 2012). Edible oil feedstocks that are used worldwide in synthesis of biodiesel are soybean, rapeseed, palm, and sunflower (Ramos et al., 2009). However, with the shrinking arable land availability and the worldwide concern over the fuel versus food debate, it does not seem justified to divert edible oil for the production of biofuels. The limited availability of the edible oil in many developing and undeveloped nations pose a question mark to the continuance of production of biodiesel from edible oils. Non-edible oils, waste cooking/frying oil, animal fats and microalgal oil contain high FFA (>4%) which has to undergo acid esterification so that they should not saponify with the alkali catalysts. Exploring the possibilities of the non-edible feedstocks, Balat (2011) reports the feasibility of few non-edible plant species to be efficient in the production of biodiesel. These include *Jatropha curcas*, *Madhuca indica*, *Pongamia pinnata* and several other plant species along with the microalgal oil. Singh (2010) has also reported the feasibility of *Pongamia pinnata*, and *Madhuca indica* along with a novel feedstock, *Schleichera triguga* to bear a high potential for conversion to biodiesel with a high yield. However, there is a disadvantage with the usage of some of these feedstocks, as at least a few of them have alternate applications in medicine, soap

manufacture and other useful applications. These plant derived oils also have a low energy density and are not economical in present scenario as compared to mineral diesel. The non-edible oils are utilized in the pharmaceutical and soap industries and have other applications (Sharma et al., 2008). The waste cooking/frying oil and waste animal/fish oil are too scanty to support a large or medium scale biodiesel plant. Algae as a feedstock for biodiesel are in the preliminary stages of research but have shown immense potential to provide alternative and a new generation feedstock. The lipid productivity of microalgae with respect to the dry weight of biomass can be 15 to 300 times greater than that derived from plants (Xin et al., 2010). However, their cultivation and extraction of oil along with optimization of the important parameters (light, pH, temperature, nutrients) for a high yield of oil with low cost is presently a challenge.

A constant supply of the feedstock is a major challenge for the commercialization of biodiesel. The feedstock is also a major contributor towards the total production cost of biodiesel. The overall cost of the feedstock may vary depending on the availability and other alternative uses of feedstock. Ahmad et al. (2011) estimated the contribution of feedstock in production cost of biodiesel to be 75%. Demirbas (2007) reported the feedstock to contribute 80% of the total production cost, whereas, Baroi et al. (2013) reported the contribution of feedstock to be 88% of the total production cost of biodiesel. Thus, feedstock selection and strategies to utilize a low cost feedstock production may be a step towards the economical production of biodiesel. Biodiesel feedstocks are categorized as first, second and third generation. The first generation feedstocks comprise of edible plant oils. The second-generation feedstocks include non-edible plant oils, and waste cooking/frying oil. The oil derived from algae and other microorganisms are being considered to be third generation feedstocks.

2. Microalgae as biodiesel feedstock

Biodiesel from microalgae can meet the global demand of transport fuel as microalgae growth rate and lipid yield is high. The growth rate of microalgae is high; some strains show doubling time as rapid as 3.5 hours and some microalgal species can accumulate up to 20-70% of lipid per dry weight, under suitable conditions. Microalgae reduce greenhouse effect by absorbing around 1.83 kg of CO_2 per kg microalgal biomass (Brennan and Owende, 2010). Photosynthetic efficiency of microalgae is better than the higher plants. Aerial productivity (per hectare oil yield) of microalgae is very high compared to other crops (Chisti, 2007, Janaun and Ellis, 2010, Moazami et al., 2011) (Table 2.1).

Table 2.1: Comparison of per hectare oil production from different sources (Chisti, 2007)

Source of oil	**Oil Yield (liter/hectare)**	**Land area needed (M hectare)[a]**
Corn	172	846
Soybean	446	326
Canola	1190	122
Jatropha	1892	77
Coconut oil	2689	54
Palm oil	5950	24
Microalgae containing 70% oil (by weight) in biomass	136900	2
Microalgae containing 30% oil (by weight) in biomass	58700	4.5

[a] For meeting 50% of all transport fuel needs of the United states.

Microalgae do not need productive agricultural land for their cultivation. Microalgal cultivation requires significantly less water quantities than the conventional crops which require copious amounts of water for irrigation. Microalgae can be effectively grown on municipal domestic wastewater as substrate (Mutanda et al., 2011a, Schenk et al., 2008). Apart from the lipids, microalgae produce other value added co-products like proteins, pigments, animal feed and fertilizer (Khan et al., 2009).

Chlamydomonas, Scenedesmus, Dunaliella, Chlorella, Nannochloropsis, Isochrysis, Nannochloris, Neochlori, Porphyridium and Nitzschia are the species known for accumulating substantial amounts of lipids and fast growth rates (Amaro et al., 2011, Chisti, 2007, Scott et al., 2010). In addition, *Chlamydomonas* sp. is amenable to genetic manipulation to enhance lipid productivity potential (Schenk et al., 2008, Tabatabaei et al., 2011). *Botryococcus braunii* contains over 50% of lipid per unit weight of dry biomass (Chisti, 2007, Scott et al., 2010). The fatty acid profile of microalgal oil is suitable for synthesis of biodiesel (Huang et al., 2010, Scott et al., 2010). Microalgal biodiesel is non toxic and contains no sulphur as compared with petroleum-based fuels therefore has minimal impact on environmental pollution (Chisti, 2007, Scott et al., 2010). The fuel properties of biodiesel produced from microalgae are comparable to those of conventional diesel fuel. These properties comply with the American society for testing and materials (ASTM) standards for biodiesel quality (Miao and Wu, 2006).

2.1 Microalgae to biodiesel: A multistep process

Biodiesel production from microalgae is a multistep process. The process includes steps like cultivation, harvesting and dewatering of microalgal biomass, extraction of lipids from biomass and conversion of microalgal lipids to biodiesel.

2.1.1 Cultivation and harvesting of microalgae

Photoautotrophic cultivation of microalgae is widely accepted and an economically viable method for large scale microalgal biomass production (Chen et al., 2011a). Microalgae require light, carbon dioxide, inorganic nutrients and water for their growth. Microalgae are either cultivated in open raceway ponds or in photobioreactors for biomass and lipid production (Rawat et al., 2013). Less energy input and easy maintenance are attractive features of open raceway ponds. Open raceway ponds are suitable for the cultivation of a few microalgae strains such as *Chlorella* sp., *Scenedesmus* sp. There are high chances of contamination since it is an open system exposed to the weather elements. Also there is a disadvantage of water evaporation in open raceway ponds (Chisti, 2007, Khan et al., 2009, Schenk et al., 2008). Multispecies cultivation is possible in open raceway ponds. Controlling of temperature and growth conditions in open ponds is technically difficult compared to photobioreactors (Mutanda et al., 2011b). Photobioreactors can give good biomass yield with proper aeration, mixing and light control. Photobioreactors are suitable for single species cultivation as chances of contamination are minimal. Photobioreactor cultivation is energy intensive thus expensive technique and technically difficult for scaling up (Chisti, 2007, Brennan and Owende, 2010, Schenk et al., 2008). Tubular photobioreactor is the most widely used design for microalgae cultivation. In a photobioreactors, there is shear stress to microalgal cultures and also some wall growth which needs to be cleaned (Mutanda et al., 2011b, Raehtz, 2009). The choice of a technique for cultivation and production of microalgal biomass with high quantity of lipids depends upon microalgal strain, optimal culture conditions, land area availability, quality and quantity of natural light and production scale.

Harvesting of microalgal biomass is a crucial step in commercial production of microalgae. The harvesting method to be employed is determined by both microalgal strain and the cultivation procedure. Different harvesting methods in use are flocculation, filtration, flotation and centrifugation or any combination of these methods. Flocculation can be done by adding inorganic or organic coagulants or by electrolytic process by neutralizing the negatively charged microalgal cells (Brennan and Owende, 2010, Chen et al., 2011a). Aluminum sulfate and cationic polymers are used as chemical flocculants. The filtration technique is suitable for harvesting of relatively large sized microalgae (>70 μm) like *Spirullina*. Microalgae like *Chlorella*, *Dunaliella* and *Scenedesmus* which are approaching bacterial dimensions (<30 μm) cannot be harvested using filtration technique (Brennan and Owende, 2010). Centrifugation is a reliable and rapid method but is energy intensive and expensive (Taher et al., 2011).

2.1.2 Microalgal biomass drying and lipid extraction

Drying of harvested biomass is necessary to increase the viability of biomass for lipid extraction. Drying can be done by natural sun drying or using sophisticated techniques like freeze drying, drum drying, spray drying and fluidized bed drying (Chen et al., 2009a). Sun drying is a cheap but slow process. All other techniques are expensive out of which freeze drying is widely used for drying of microalgal biomass (Brennan and Owende, 2010, Chen et al., 2009a). Extraction of lipids from microalgae is carried out by using either physical or chemical methods or by using a combination of these methods. A physical method uses an expeller press to extract oil from biomass. Physical methods are low yielding and the instrumentation requires constant maintenance (Pokoo-Aikins et al., 2009). Disrupting the cell wall followed by organic solvent extraction is a widely used method for oil extraction. Cell disruption techniques like

grinding, bead-beating, autoclaving; sonication, microwave and osmotic shock are coupled with solvent extraction for better lipid yield (Chen et al., 2009a). Industrial grade n-hexane is the most used solvent for extraction of microalgal oil because it is not expensive. Other solvents commonly used include chloroform, methanol, and isopropanol (Halim et al., 2011, Halim et al., 2012). Solvents are toxic in nature and thus create environmental problems. Novel environmentally friendly approaches like subcritical water and supercritical carbon dioxide are being tested for extraction of oil from microalgae (Singh and Olsen, 2011). The only disadvantage of these novel methods is their high cost considering that sophisticated instrumentation and particular pressure and temperature conditions are required (Amaro et al., 2011, Taher et al., 2011). Extracted lipids are then subjected to transesterification for conversion to biodiesel. The biomass left after extraction of oil can be used as animal feed or for biogas production (Chisti, 2007).

2.1.3 Conversion of microalgal lipids to biodiesel

Microalgal lipids are converted into biodiesel by transesterification process. Widely used catalyst for transesterification process is alkaline catalyst like NaOH. However, microalgal lipids are known to have high FFA content, which leads to saponification if alkaline catalyst is used. Balasubramanian et al. (2013) reported high FFA content in *Nannochloropsis* sp. ranging from 4-19 mg $FFA.g^{-1}$ of dry biomass. Miao and Wu (2006) reported acid value of 8.97 mg $KOH.g^{-1}$ for *Chlorella protothecoides* oil. Feedstock lipids with high FFA content can be converted to biodiesel applying two strategies viz. two step esterification and transesterification process or use of catalyst which can catalyze simultaneously esterification and transesterification. In two step process acid value of feedstock lipids is lowered by esterification using acid catalyst followed by alkaline transesterification to produce FAAE. Chen et al. (2012) studied two step conversion

processes for high free fatty acid containing microalgal oil. In their study they subjected *Dinoflagellate, Scenedesmus* sp. and *Nannochloropsis* sp. oil first to acid esterification catalyzed by sulphuric acid followed by transesterification catalyzed by potassium hydroxide (KOH). In this process they found initial acid value of microalgal oils ranging from 17-46 mg $KOH.g^{-1}$ was reduced to below 2 $mgKOH.g^{-1}$. Fatty acid methyl ester (FAME) yields obtained after this two step conversion process were 90.1, 78.3 and 70.4% for *Dinoflagellate, Scenedesmus* sp. and *Nannochloropsis* sp. respectively. This two step process is associated with several constraints, which makes it less attractive for commercial implementation. Neutralization and washing steps are required to remove trace catalyst and purification of products. These extra steps not only make whole process cumbersome but also generate excess wastewater, which needs to be treated.

Applying acid catalysts which can simultaneously catalyze esterification and transesterification is another strategy to treat high FFA containing microalgal lipids (Lam and Lee, 2013). Sulphuric acid is widely used homogeneous acid catalyst for conversion of microalgal lipids to biodiesel (D'Oca et al., 2011, Lam and Lee, 2013). Lam and lee (2013) applied sulphuric acid as a homogeneous acid catalyst for conversion of high viscosity microalgal lipids from *Chlorella vulgaris*. To achieve 95% FAME content they found that high methanol to oil molar ratio (180:1) is required, while after using tetrahydrofuran as a co-solvent methanol molar ratio required is reduced to 60:1. Catalyst concentration required was also high (35 wt%) which is reduced to 21 wt% after using co-solvent. Heterogeneous catalysts have shown many advantages over the conventional homogenous catalysts. Heterogeneous acid catalysts are suitable choice for microalgal lipids conversion. Carrero et al. (2011) studied hierarchical zeolites as heterogeneous catalysts for conversion of *Nannochloropsis gaditana*

lipids. Among the zeolites tested they found that h-Beta zeolites showed highest catalytic activity at temperature 115°C, owing to its improved porosity which makes acid sites accessible for lipid molecules. Despite of its advantages chemical catalysis has several drawbacks like high energy requirements, generation of wastewater, corrosion of piping and reactors, extra purification steps (Helwani et al., 2009). High energy requirement and environmental concerns associated with chemical catalysts can be overcome by use of biocatalyst. Enzyme lipase can be applied for conversion of microalgal lipids. However there are very few studies available for microalgal biodiesel synthesis using enzymatic transesterification. Enzymatic transesterification for microalgal lipids, which is a sustainable and green conversion technology, needs attention from researchers.

2.2 Lipid content and characterization

The lipid constituent in the microalgal oil is made up of saponifiable as well as non-saponifiable matter. The neutral lipids comprises of the saponifiable matter viz. triglycerides and free fatty acids. The total lipid constitutes of neutral lipids, pigments, phospholipids and glycolipids (Li et al., 2011b). Despite neutral and polar lipids being able to be converted to biodiesel, neutral lipids are considered to be the best substrate for production of FAAE as they can be easily transesterified (Doan et al., 2011). The focus in recent times has been a high lipid accumulation in the cells of microalgae species. Various studies have been done by researchers to explore the species with a high lipid accumulating potential as the lipid content varies significantly among the microalgal species. However, not all species have shown a high amount for the fatty acid accumulation and biomass productivity. The oil content is found to be highest in *Botryococcus braunii* (25 to 75%), but its productivity is poor. The commonly grown microalgae (species of *Chlorella*, *Scenedesmus*, *Nannochloropsis*, *Isochrysis*, *Dunaliella*,

Nannochloris, *Neochloris*, *Phaeodactylum*, *Nitzschia* and *Porphyridium*) have oil content in the range 20 to 50% (Malcata, 2011). Abou-Shanab et al. (2011) isolated 45 microalgae from a freshwater lake and on the basis of their morphology studied the lipid accumulating potential of five species amongst them. The lipid content potential varied significantly for the selected species and possessed a comparatively low content of 26% (of dry weight) for *Chlorella vulgaris* and a high content of 51% (of dry weight) for *Chlamydomonas pitschmannii*. Chinnasamy et al. (2010) demonstrated that only about 63.9% of the algal oil extracted could be converted to biodiesel. The final recovery of the FAAE further reduced to 38.7% due to loss during base transesterification and purification of the product. There exists a trade-off between the productivity of microalgae and its lipid accumulating capacity (Ahmad et al., 2011). Thus, when environmental conditions are controlled for high lipid accumulation, the microalgae spend energy to form lipids thus compromising with its productivity. Similarly, high biomass productivity in microalgae is achieved when no stress is induced for high lipid accumulation (Ahmad et al., 2011). Hence, a balance between the lipid production and biomass growth in microalgae has to be maintained for on optimum production of oil. Microalgal lipids have shown high amount of saponifiable contents as well as high amount of free fatty acids. Microalgal lipids have high saponification value ranging from 170-220 $mgKOH.g^{-1}$ (Da Rós et al., 2012, Lai et al., 2012b, Miao and Wu, 2006).

The chain length of the fatty acid and degree of unsaturation present in the feedstock lipids influences the fuel properties of the biodiesel (Nogueira, 2011). Microalgal lipids have shown lipid profile which is suitable for the biodiesel production (Abd El Baky et al., 2012, Gao et al., 2013). Microalgal lipids have shown palmitic (16:0) palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic acid (18:3) as major contributing fatty acids (Breuer et

al., 2012, Cha et al., 2011). Saturated fatty acids present in lipids influence cetane number and oxidative stability, while polyunsaturated fatty acids have influence on cold temperature properties (Ramos et al., 2009). It becomes necessary to choose feedstock with suitable lipid profile with striking balance in saturated and unsaturated fatty acids. Presence of highly unsaturated fatty acid could render biodiesel properties off specification. European standard for biodiesel EN 14214 specifies 12% as a maximum limit for linolenic acid methyl esters (Gouveia et al., 2009). Polyunsaturated fatty acids are highly susceptible for oxidative cleavage due to number of factors such as temperature, irradiation and nutrient stress (Borges et al., 2011, Gao et al., 2013). Table 2.2 depicts the lipid content and fatty acid profile of some microalgal strains.

Table 2.2: Lipid content and fatty acid profile of some microalgal strains

Microalgal strain	**Lipid content (%)**	**Fatty acid composition (%)**									**Ref.**
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	SFA	MUFA	PUFA	
Chlorella vulgaris	17.3	14.55	1.18	10.51	23.62	13.8	32.1	25.06	24.8	45.9	(Talebi et al., 2013)
Scenedesmus sp.	30-36	18.42	2.31	3.43	49.64	11.30	8.26	21.85	51.95	22.82	(Chen et al., 2012)
Nannochloropsis sp.	-	28.83	32.93	0.98	21.16	2.24	-	35.18	54.09	8.57	(Chen et al., 2012)
Chlamydomonas reinhardtii	18.9	23.77	1.94	4.41	19.73	6.58	25.49	18.18	22.88	32.07	(Talebi et al., 2013)
Chlorococcum sp	7.1	19	4	3	63	4	-	-	-	-	(Halim et al., 2011)
Monoraphidium sp. FXY-10	56.80	22.6	-	1.2	-	4.2	42.2	23.8	-	68	(Yu et al., 2012)
Neochloris oleoabundans	10-15	23-30	0.6-3.5	0.9-11	30-43	18-23	5-12	-	-	-	(Levine et al., 2011)
Selenastrum capricornutum	27.08	45.62	5.96	-	4.27	11.75	-	≈ 48	≈ 10	≈ 17	(Song et al., 2013)

3. Transesterification for biodiesel production

The transesterification reaction for the synthesis of biodiesel involves reaction of oils or fat as triglycerides with a lower group alcohol (preferably methanol or ethanol due to their high reactivity owing to shorter chain length) in presence of an acid or alkali catalyst. Esterification is the process adopted for the conversion of FFA content in oils to fatty acid alkyl ester (FAAE) in presence of an acid catalyst. Hence, with feedstock containing high FFA content, a two step process comprising of esterification followed by transesterification is usually adopted (Meher et al., 2006). The preference of methanol over ethanol as alcohol is due to the higher reactivity and lower cost of the former. To enhance the rate of reaction, a catalyst (homogeneous or heterogeneous) is compulsorily added. The only case where the catalyst is usually not added is when the esterification/transesterification reaction is done at supercritical conditions (Sharma et al., 2008). A supercritical method is the technique by which, at a high temperature and pressure, the dielectric constant of the reactant (alcohol in the present case) is reduced to facilitate the formation of a single phase as compared to the usual two phases of oil and alcohol (Santana et al., 2012). The advantage with supercritical method is that the purification or separation steps are not required (Santana et al., 2012). However, the process requires high energy input due to high temperature requirement and is not economically feasible. Biocatalyst for transesterification has promising potential because of high and pure product yield, low energy consumption and environmental benefits due to less wastewater generation.

3.1 Catalysts for transesterification

3.1.1 Chemical catalysts for transesterification

The catalyst plays an important role in the esterification or transesterification of lipids. The catalysts that have been used for the esterification and transesterification of plant oils include homogeneous as well as heterogeneous chemical catalysts (Helwani et al., 2009, Lam et al., 2010, Sharma et al., 2011b). The mechanism of esterification/transesterification from the homogeneous and heterogeneous catalysts is similar. Homogeneous base catalysis for transesterification is a three step process which starts with the formation of alkoxide from the alcohol. The nucleophilic alkoxide attacks the carbonyl carbon group in the triglyceride to form a tetrahedral intermediate. In the second step, intermediate formed reacts with an alcohol to redevelop the alkoxide. The rearrangement of the intermediates leads to formation of diglycerides and FAAE. In the last two steps, two molecules of FAAE are formed when diglyceride and monoglyceride are converted to monoglyceride and glycerol, respectively. In acid catalysis, the carbonyl group of the triglyceride gets protonated which leads to carbocation. The protonated carbon is attacked by an alcohol that results in formation of an intermediate. In the next step the intermediate eliminates glycerol that results in formation of an ester while the catalyst is regenerated (Endalew et al., 2011, Meher et al., 2006). In the heterogeneous Lewis basic catalyzed reaction (CaO and MgO) formation of the alkoxide group is pertinent. In general, the Brönsted acid group of catalysts is found to be active mainly in esterification reactions and Lewis acid catalysts are found to be more active in transesterification reactions (Di Serio et al., 2007). A high biodiesel yield with feedstocks of acid value <4.0 $mgKOH.g^{-1}$ amount to almost complete conversion (100%) has been reported by the researchers using homogeneous base catalysts. The biodiesel yields with heterogeneous base catalysts are also high

amounting to >98%, although the reaction rate is comparatively slow compared to those catalyzed with homogeneous catalysts. The reason is due to three phase system in the heterogeneous catalyzed reaction which causes a mass transfer limitation during reaction. In the heterogeneous catalyzed reaction, the adsorption of reactants (oil and alcohol) and desorption of products [FAAE (biodiesel), di-, mono-glyceride, and glycerol] should occur on the surface of the solid (acid or base) catalyst at a considerable rate (Endalew et al., 2011). The advantage using the heterogeneous catalyst is its easy separation from the products and production of glycerol of high purity (98%) as compared to that with homogeneous derived catalyst (80%) (Atadashi et al., 2012).

Though, homogeneous catalysts are few, there is a vast range of the solid (heterogeneous) catalysts that have been explored to catalyze the transesterification reaction. Common homogeneous base catalysts used in transesterification include sodium hydroxide, potassium hydroxide, and sodium methoxide. Sulphuric acid is utilized as homogeneous acid catalyst for esterification. The application of the homogeneous acid and base catalysts in biofuel industry have numerous disadvantages e.g. corrosion of the reactors, storage tanks, and engines (Lam et al., 2010). In addition, their removal from the biofuels through aqueous quenching results in the formation of stable emulsions and soap and are energy intensive (Wilson and Lee, 2012). The heterogeneous catalysts utilized in synthesis of biodiesel are grouped as solid acid and solid base catalyst. Solid acid catalyst includes a wide range of chemicals viz. resins, tungstated and sulfated zirconia, polyaniline sulphate, heteropolyacid, metal complexes, sulfated tin oxide, zeolite, acidic ionic liquids, and others have been used by researchers (Sharma et al., 2011a). Solid base catalysts also include a wide range of catalysts viz. calcium oxide, hydrotalcite (also called layered double hydroxide), alumina and zeolites (Sharma et al., 2011b). Removal of

leached constituents of heterogeneous catalysts is a major issue in synthesis of biodiesel. While a large number of heterogeneous catalysts have been utilized for the synthesis of biodiesel from the plant based oil, the activity of several of these catalysts on the microalgal based oil is yet to be tested (Sharma et al., 2011a).

3.1.2 Biocatalyst for transesterification

Enzyme lipases are employed as catalyst for biodiesel synthesis. Enzyme catalyst can be effectively used for low quality feedstocks with high free fatty acid content. Lipases catalyze both esterification as well as transesterification. Quality and yield of product is high. Enzymes have shown a significant catalytic efficiency in the conversion of oil to biodiesel with purity ranging from 90–96%. A high purity has been attributed to the "polishing" cycles where the contaminant also gets converted to biodiesel. However, the enzymatic mode of catalysis suffers from constraints. Besides it being costly, a high alcohol concentration can cause irreversible damage to the enzyme (Fedosov and Xu, 2011).

There is immense scope for usage of enzymes as catalyst in the synthesis of biodiesel from microalgae derived oil. The enzymes have constrains in the current scenario in its application as catalyst for synthesis of biodiesel. However, there are possibilities of improvement in the process, where enzymatic methods can be adopted at relatively low temperatures and atmospheric pressure which will lead to reduction in consumption of energy. Enzymes pose a high selectivity and specification and can lead to a high conversion of oil to biodiesel resulting in a high quality product. When an enzyme (particularly lipase) is being used as a catalyst, methanol has to be added in step-wise manner to avoid the lipase deactivation. There have been

limited studies on the conversion of microalgal oil to biodiesel, either using chemical or enzymatic catalyst (Lai et al., 2012a) (Table 2.3).

Table 2.3: Various catalysts used for conversion of microalgal oil

Microalgae	Catalyst	Reaction conditions				Biodiesel yield (Y)/ conversion (C) %	Ref.
		Catalyst loading (%wt/oil wt)	Molar ratio Alcohol : Oil	Temp (°C)	Time (Hours) Stirring (RPM)		
Chlorella protothecoides	Sulfuric acid	100	56:1	30	4, 160	Y≈60	(Miao and Wu, 2006)
Oedogonium sp.	Sodium hydroxide	-	-	-	3, 300	Y>90	(Hossain et al., 2008)
Spirogyra sp.	Sodium hydroxide	-	-	-	3, 300	Y>90	(Hossain et al., 2008)
Nannochloropsis oculata	Al_2O_3 supported CaO	2	30:1	50	4, 1100	Y=97.5	(Umdu et al., 2009)
Chlorella protothecoides	*Candida* sp. 99-125 sp. lipase	30	3:1	38	12, 180	C=98.15	(Xiong et al., 2008)
Chlorella vulgaris ESP-31	*Burkholderia* lipase	1203.11 U/gm *	12.35:1	40	48, 600	C=72.12	(Tran et al., 2012)
Dinoflagellate	Esterification - Sulfuric acid	1	30:100 (v:w)	65	2	C ≈100	(Chen et al., 2012)
	Transesterification - Potassium hydroxide	2	12:1	65	0.5, 100		
Chlorella pyrenoidosa	*Penicillium expansum* lipase (PEL)	20	3:1	50	48	Y=90.7	(Lai et al., 2012a)

*Lipase in activity in unit/gm

Hama and Kondo (2013) stated that the present price of enzyme catalyst is higher than its chemical counterpart. The enzymes are also prone to inactivation, which may limit its applicability to industrial scale production (Yan et al., 2012). To tackle these constraints researchers are looking for possible solutions. Use of immobilized enzymes provides advantage of its easy separation and reusability. Immobilization also provides stability to the enzyme. Lipases from different sources have varying specificity and stability. For ability to reuse the lipase catalysts, further improvements are required (Yan et al., 2012). In future, with advances in enzyme catalysis, it has potential to provide greener and sustainable conversion strategy for biodiesel production.

4. Enzymatic transesterification for biodiesel production

4.1 Lipases

Lipases are found in animals, plants and microorganisms and play a key role in the metabolism of oils and fats. Lipases take part in the deposition, transfer and metabolism of lipids (Villeneuve et al., 2000). Lipases are hydrolases (EC 3.1.1.3) that act on carboxyl ester bonds in triglycerides to yield fatty acids and glycerol. Lipase catalyzes this reaction at the lipid-water interface. The structure of lipase has a central L-sheet with an active site consisting of serine on a nucleophilic elbow placed in a groove. This groove is covered by a peptide lid; when lipase comes in contact with a lipid-water interface this peptide lid go through conformational changes making the active site accessible for the acyl moiety (Jegannathan et al., 2008, Villeneuve et al., 2000). Lipases have both hydrolytic as well as synthetic activity and, thus, can take part in

various industrially important reactions like esterification and transesterification (alcoholysis and acidolysis) (Figure 2.1) (Sharma et al., 2001, Shah et al., 2003).

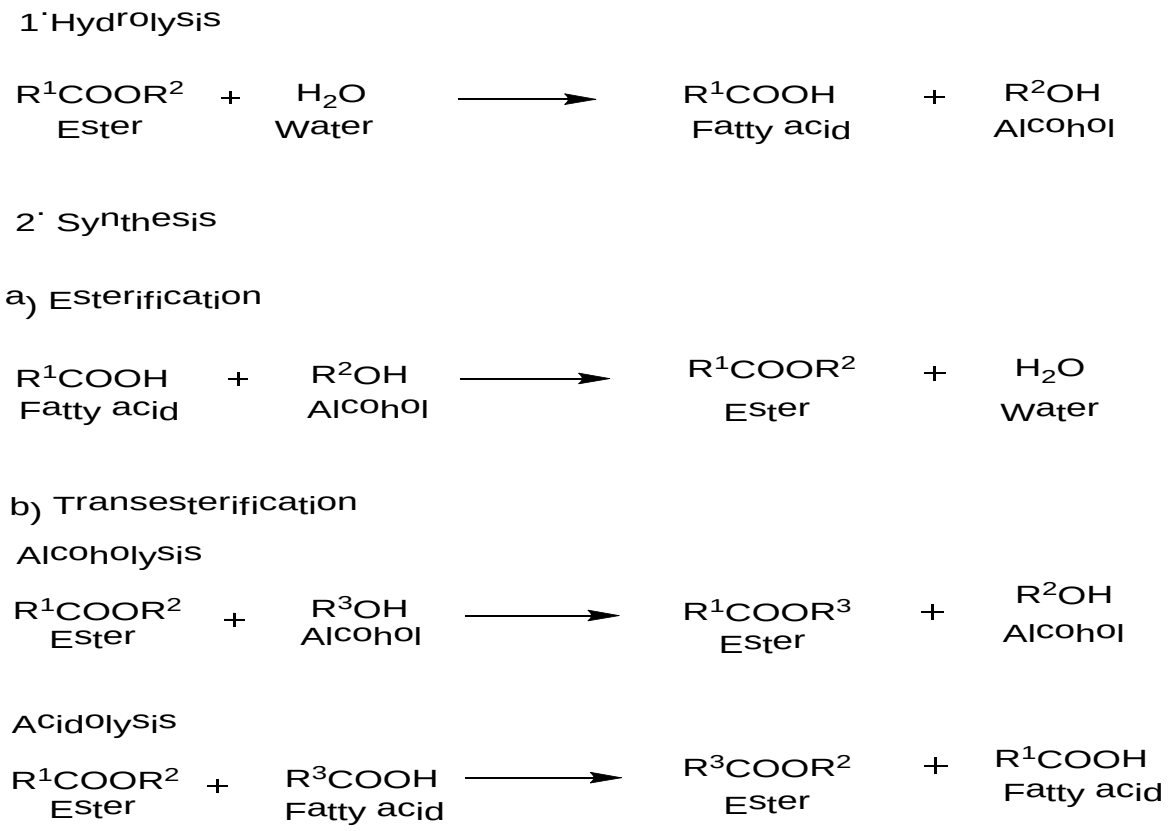


Figure 2.1: Hydrolytic and synthetic reactions catalyzed by lipase. R^1, R^2 and R^3 are different alkyl groups.

Lipases are used as catalysts in food processing, detergent, pharmaceutical, paper, cosmetics and chemical synthesis industries (Gupta et al., 2004, Shah et al., 2003, Sharma et al., 2001). Lipases from fungi and bacteria are easy to produce in bulk amounts because of their extracellular nature (Ghaly et al., 2010, Gupta et al., 2004). Lipases from different sources have different properties and catalytic efficiency (Bajaj et al., 2010). Lipases can effectively convert triglycerides to FAAE, thus attracting interest in the biodiesel field. Researchers have proposed

lipases as a greener alternative to conventional chemical catalysts in the synthesis of biodiesel. Feedstock oil and short chain alcohols acting as acyl acceptors react in the presence of lipases. Lipases effectively convert triglycerides as well as FFA to FAAE. Lipase-catalyzed transesterification can be carried out in the range of 30-40ºC, though certain lipases from thermophilic sources can work at even higher temperatures (Bajaj et al., 2010). Regiospecific lipases specifically act on certain ester bonds in triglyceride molecule. The majority of regiospecific lipases act specifically on ester bonds at positions one and three of the triglyceride molecule. Activity of regiospecific lipases was found to be maintained and high conversion was observed because of acyl migration (Li et al., 2010a). Favourable conditions such as immobilization of lipase on polar supports and silica gel in a reaction mixture helps to maintain conversion ability of regiospecific lipases (Robles-Medina et al., 2009, Szczęsna Antczak et al., 2009). Non-specific lipases from *Candida antarctica, Candida rugosa, Candida cylindracea, Pseudomonas fluorescens* and *Pseudomonas cepacia* and 1,3-specific lipases from *Rhizopus oryzae, Aspergillus niger, Rhizopus delemar, Rhizomucor miehei* and *Thermomyces lanuginosus* are commonly used as catalyst for biodiesel production (Ghaly et al., 2010).

Biodiesel yield depends upon various factors like source of lipase, application technique of lipase, acyl acceptor, temperature, presence of solvent, and alcohol to oil molar ratio. Lipase catalysis can be done using both extracellular and intracellular lipases. The extracellular process employs free or immobilized lipase extracted from the cells while the intracellular process employs free or immobilized whole cells as the catalysts (Fukuda et al., 2001, Gog et al., 2012, Ranganathan et al., 2008). Both continuous and batch process can be used for conversion of oil to biodiesel by lipase-catalyzed transesterification. The literature available on application of lipase catalyst for biodiesel production from microalgae is scant. Thus, literature available with

other feedstocks is taken into consideration to review various aspects of lipase-catalyzed biodiesel synthesis.

4.2 Mechanism of lipase-catalyzed transesterification for biodiesel production

Transesterification of triglyceride oil by lipase catalysis using methanol is a sequence of three reversible consecutive reactions similar to the acid and base catalyzed transesterification. In the first step, triglycerides are converted to diglycerides. In the second step, diglycerides are converted to monoglyceride and lastly, monoglycerides are converted to glycerol molecules. Each conversion step yields one fatty acid methyl ester (FAME) molecule, yielding a total of three FAME molecules per triglyceride molecule (Ghaly et al., 2010, Shah et al., 2003, Turkan and Kalay, 2006). Lipase catalysis follows the ping-pong bi-bi mechanism where two substrates react to yield two products through formation of enzyme-substrate intermediates. In the transesterification of each ester bond, the reaction mechanism involves the hydrolysis of an ester bond to yield a fatty acid, followed by esterification of fatty acid and alcohol to produce a new alkyl ester (Bousquet-Dubouch et al., 2001, Cheirsilp et al., 2008). The lipase active site has an Asp-His-Ser amino acid triad that plays an important role in catalytic activity by donating and accepting protons. Two groups which play an important role in the catalytic mechanism are a hydroxyl group of serine that acts as nucleophile and a nitrogen atom on histidine that takes part in proton mobilization. A carboxyl group of aspartic acid stabilizes the serine nucleophile as well as protonated histidine. The substrate (triglyceride) carbonyl carbon is attacked by a nucleophilic serine oxygen to form a tetrahedral lipase-substrate intermediate. Protons accepted by histidine are transferred to alkyl oxygen atoms of substrates to form diglycerides. Lipase-acyl complexes react with oxygen atoms of alcohols, which are added to the carbonyl bonds of lipase-acyl

complexes to form the second intermediate. Serine oxygen atoms accept a proton from histidine, which releases a FAAE (Al-Zuhair et al., 2007, Jegannathan et al., 2008).

Studies conducted on the kinetics of lipase-catalyzed transesterification shows that it follows Michaelis–Menten kinetics (Bousquet-Dubouch et al., 2001, Krishna and Karanth, 2001, Leca et al., 2010). In Michaelis-Menten kinetics, K_m determines affinity of enzyme for particular substrate and V_{max} determines the maximum rate of reaction. The affinity of an enzyme for the substrate is high if the K_m value is low. Most of the kinetic models proposed for lipase-catalyzed transesterification apply the ping-pong bi-bi mechanism when alcohol inhibition was taken in consideration. Knowledge of reaction kinetics aids in determining optimum reaction parameters, scaling-up of the process and reactor designing. Kinetics studies of lipase-catalyzed conversions involving other parameters like lipase type, lipase immobilization, the solvent used, effect of temperature, reactant concentrations and mass transfer limitations has been scarcely studied and needs further investigation (Al-Zuhair et al., 2007, Gog et al., 2012).

4.3 Immobilization

The major obstacle in enzymatic catalysis for biodiesel production is the high cost of the enzymes. A possible solution to reduce the process costs incurred is the use of immobilized lipases. Immobilization of lipase helps to reduce the cost of catalyst as it can be easily separated and reused. Immobilization also confers stability to lipase towards temperature, chemicals (alcohol and glycerol) and denaturation (Bajaj et al., 2010). Immobilized lipases have shown faster and higher catalytic activity compared to free lipases (Iso et al., 2001). Iso *et. al*. (2001) carried out transesterification of triolein using free *Pseudomonas fluorescens* lipase, as well as immobilized lipase on porous kaolinite particles. Propanol and butanol were used as acyl

acceptors and results showed that with propanol as acyl acceptor the reaction was completed in 10 h with immobilized lipase, while, to achieve a 90% conversion, the free lipase took 25 h. When free lipase were used as catalyst, formation of aggregates were reported which rendered less active sites available for reaction, while immobilization expressed every lipase molecule on the surface, making all the active sites available for reaction.

Adsorption, entrapment, encapsulation and cross-linking are the basic techniques used for immobilization of lipase. Among these, adsorption is the most widely used technique for immobilization as it is easy and cost effective process (Tan et al., 2010). Toyonite, cellulose polypropylene, silica gel, polypropylene, hydrotalcite, sephadex, siliconized glass, sepharose, accurel, diatomaceous earth, spherosil, celite, textile membrane, acrylic resin, and anion resin are commonly used carriers in the adsorption technique (Ghaly et al., 2010, Jegannathan et al., 2008). Adsorption is also favourable because it faces less mass transfer limitations compared to entrapment and cross linking. However, as lipases are attached to support materials by weak forces, which causes enzyme leaching and thus exhibits reduced activity when used repeatedly (Jegannathan et al., 2008). Biodiesel production from soybean oil was carried out by Liu et al. (2011) using *Burkholderia cenocepacia* lipase adsorbed onto macroporous resin. With optimum reaction conditions, 98% biodiesel yield was obtained. A low cost immobilization carrier, cotton membrane was studied by Nie et al. (2006) for immobilization of lipase from *Candida* sp. This lipase was studied in both batch and continuous processes and was found suitable for industrial application. In a batch process, transesterification of salad oil gave 96% yield of biodiesel. In the continuous process with salad oil and waste oil, biodiesel yields reached 93 and 92%, respectively (Nie et al., 2006).

In cross-linking technique for the immobilization of lipase, enzyme molecules are chemically linked to form matrix-free aggregates. Gluteraldehyde is a commonly used reagent for cross-linking of enzymes while other substances are bisdiazobenxidine and hexamethylene diisocyanate (Ghaly et al., 2010). Cross-linked enzyme aggregates (CLEA) show a high transesterification rate because of high concentration of lipase per unit volume and more robust structure which is tolerant to denaturation compared to the free lipases (Jegannathan et al., 2008, Kumari et al., 2007). CLEA have very small particle size, less than 10 µm, which makes its separation from the product difficult (Jegannathan et al., 2008). Another drawback of CLEA is that because of aggregate structure all the active sites would not be on the surface, which makes the process mass transfer limiting (Kumari et al., 2007).

Entrapment of lipase in gels offers stability and a better activity. Alginate is commonly used as gel for entrapment of lipase. Other polymers and gels used are methylenebisacrylamide, calcium alginate, kappa-carrageenan and phyllosilicate sol-gel matrix (Ghaly et al., 2010, Jegannathan et al., 2008, Jegannathan et al., 2009). The entrapment process is easy but costlier than adsorption. The conversion efficiency of immobilized lipase by entrapment is low because of poor diffusion and mass transfer limitations. Lipase was entrapped in sol-gel supported on diatomaceous earth and studied by Meunier and Legge (2012) for its catalytic activity with triolein and methanol as substrate. The methyl oleate yield obtained was 80% at 40°C in 6 h. Immobilized lipase with this technique showed stability even after storage for 1.5 years. The only disadvantage is that water adsorbed on diatomaceous earth inhibits lipase activity. Although, a drying step in immobilization procedure improves the catalytic activity, it adds to the cost of enzyme (Meunier and Legge, 2012). In the encapsulation of lipase, enzyme molecules are enclosed within a porous material like beads and capsules. Encapsulation prevents lipase

leaking and also improves mass transfer. Rate of conversion of substrate to product with encapsulated lipase was low because of limited permeability and pore clogging (Ghaly et al., 2010, Jegannathan et al., 2008). Jegannathan et al. (2010) studied transesterification of palm oil with methanol catalyzed by *Burkholderia cepacia* lipase (lipase PS) encapsulated within biopolymer kappa-carrageenan. The reaction carried out at optimum conditions yielded 100% conversion of methyl esters. When repeatedly used for five cycles, the lipase showed 82% conversion relative to the first cycle.

Cubes of polyurethane foam are the most popular biomass support particles (BSP) for immobilization of lipase-producing whole-cells (Ban et al., 2002). BSP enable use of intracellular lipase in both batch and continuous process possible. BSP provide stability to intracellular lipase as well as make their separation and reuse easy (Ban et al., 2002, Hama et al., 2011).

In order to reduce the cost of lipase catalysis, its reusability plays a key role in the economics in production of biodiesel. With a simple separation process, immobilized lipases can be reused without loss of its activity (Iso et al., 2001). Shimada et al. (1999) reported that immobilized *Candida antarctica* lipase can be used repeatedly for 50 cycles. Novozyme 435 lipase (extracted from *Candida antarctica* and immobilized on acrylic resin), when used with methyl acetate as acyl acceptor, was found to be reusable for 100 cycles (Du et al., 2004). The activity of immobilized *Enterobacter aerogenes* lipase was reported to reduce to 50% after 20 cycles. This loss of activity was attributed to loss of enzyme during the separation process after each cycle (Kumari et al., 2009). Li et al. (2006) stated that Novozyme 435 and Lipozyme TL IM (*Thermomyces lanuginosus* lipase) could be reused for 200 cycles when methanolysis is

carried out in tert-butanol as solvent. *Rhizopus oryzae* cells immobilized with BSP when treated with glutaraldehyde can be repeatedly used without loss of activity for six cycles. Glutaraldehyde treatment helps enzyme molecules to aggregate by cross-linking. Cross-linking prevents enzyme leakage, provides extra stability and thus prevents the activity of BSP (Ban et al., 2002).

4.4 Extracellular lipase application

Extracellular lipases have been frequently used for biodiesel production (Table 2.4). Complex process involved in its production, separation and purification steps make these enzymes expensive (Ghaly et al., 2010). Although in extracellular application, lipases can be used either in its free or immobilized form, the latter is preferred for a high conversion of biodiesel and its reusability. Recently, free lipases have not been studied extensively due to their comparatively lower conversion rate and higher process cost. The molar ratio of alcohol to oil, enzyme concentration, temperature, water content, presence of solvent play key role to determine conversion efficiency of lipase catalyzed transesterification. Vegetable oil transesterification catalyzed by *Candida antarctica* lipase was studied by Shimada et al. (1999) with step-wise addition of methanol. The reaction conditions optimized were: temperature of 30°C using 4% immobilized lipase by weight of oil and three moles of methanol added in three steps. After 48 h of reaction, the methyl ester content in biodiesel reported was 98.4%.

Table 2.4: Various extracellular lipases used for biodiesel production

Lipase	Feedstock (Oil)	Acyl acceptor (molar ratio)	Solvent	Enzyme conc (% wt/oil wt)	Water content (% wt/ oil wt)	Reaction conditions [Temp (°C), Time (h), Stirring rate (rpm)]	Biodiesel yield (Y)/ conversion (C) %	Ref.
Candida sp 99-125	Glycerol trioleate	Methanol, (3:1)	n-hexane	20	20	40, 12, 180	Y = 80.6	(Lu et al., 2009)
Candida antarctica Novozyme 435	Sunflower	Ethyl acetate, (11:1)	-	10	-	50, 12, 150	Y = 92.7	(Modi et al., 2007)
Candida antarctica Novozyme 435	Palm	Isobutanol, (6:1)	-	3	-	40, 40, 300	C = 100	(Naranjo et al., 2010)
Candida rugosa	Soybean	Methanol, (4:1)	n-hexane	60	-	35, 30	C = 87	(Xie and Wang, 2012)
Thermomyces lanuginosus Lipozyme TL IM	Crude palm	Methanol,	tert-butanol	7.5	-	40, 4 , 150	Y = 95.15	(Sim et al., 2010)
Pseudomonas cepacia (free lipase)	Soybean	Methanol, (3:1)	-	-	-	35, 90 , 150	Y > 80	(Kaieda et al., 2001)
Pseudomonas cepacia	Soybean	Methanol, (6.6:1)	-	-	-	30, 24	C = 90	(Li et al., 2011a)
Burkholderia cepacia Amano lipase PS	Palm	Methanol, (7:1)	-	52.5	10	30, 72, -	C = 100	(Jegannathan et al., 2010)
Jatropha curcas	Jatropha	Ethanol, (4:1)	-	-	-	40, , 200	C = 87.4	(Nahak et al., 2010)

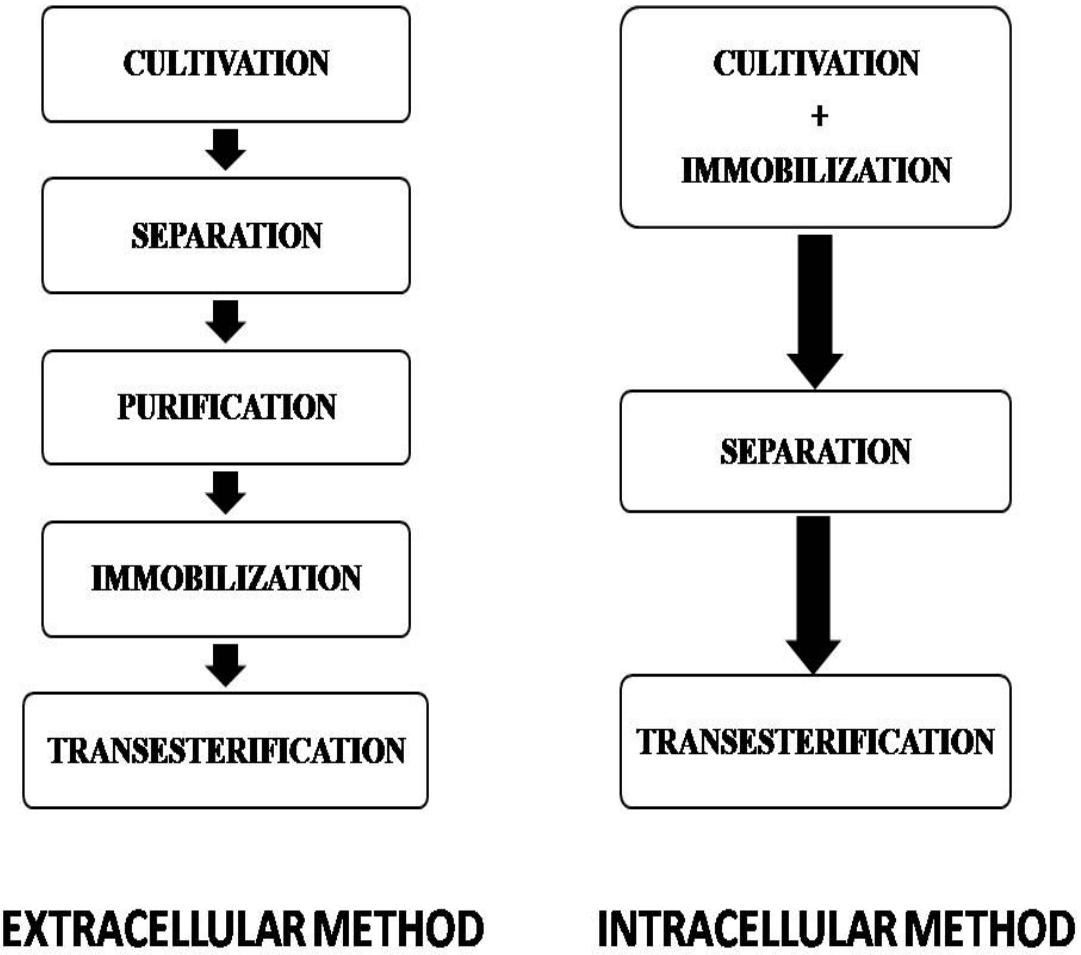
Extracellular lipase-catalyzed transesterification can be carried out in either a batch process or a continuous process. Batch processes are widely-accepted at the laboratory as well as industrial scale. Stirred tank reactors (STR) are the most-used reactors for transesterification reactions (Fjerbaek et al., 2009). Easy handling and control are the advantages with batch reactors. On the other hand, shear stress due to stirring can reduce the stability of the enzyme in stirred tank reactors. Researchers have investigated continuous process as an alternative to reduce the production cost of biodiesel (Fjerbaek et al., 2009, Helwani et al., 2009). In the continuous process, packed bed reactors (PBR) are used in which immobilized enzyme is packed in a jacketed column and the reaction mixture is pumped through this column in an upward direction. To reduce the viscosity of the reaction mixture, use of solvents is preferred in a continuing process. Enzyme concentration and flow rate are important variables determining PBR performance (Robles-Medina et al., 2009). Chen et al. (2011b) used Novozyme 435 in a packed-bed reactor for conversion of soybean oil to biodiesel. In the presence of tert-butanol as solvent and with optimized reaction conditions, 83.31% conversion was reported to have been achieved.

4.5 Whole cell (intracellular) lipase application

As the cost of extracellular lipase is the limiting factor for their successful industrial application, intracellular lipases are gaining interest among researchers (Fukuda et al., 2008, Sun et al., 2011). In the intracellular process, lipase-producing bacteria, yeast and filamentous fungi can be effectively used as whole cell catalysts (Table 2.5). The ease of immobilization and lipase display on the cell surface are key selection criteria for an organism to be used as a whole cell catalyst (Fukuda et al., 2008). Intracellular application of lipase reduces the cost of catalysis as

extraction of the enzyme from the organism and purification steps are eliminated (Robles-Medina et al., 2009) (Figure 2.2).

Figure 2.2: Comparison of steps involved in extracellular and intracellular (whole cell) lipase applications.



Methanolysis of soybean oil carried out using *Rhizopus oryzae* cells immobilized within BSP in tert-butanol as solvent yielded 72% biodiesel under optimum conditions. Stability of the

whole cell catalyst was maintained by the use of tert-butanol which reduces the methanol inhibition (Li et al., 2007). Transesterification of soybean oil was carried out in a solvent-free system with five commercial lipases and whole cell lipase of *Rhizopus chinensis.* Extracellular lipases from *Candida antarctica, Pseudomonas cepacia, Pseudomonas cepacia* immobilized on ceramic, *Candida rugosa* and porcine pancreas gave biodiesel yield of 30, 27, 27.4, 5 and 24.1% respectively, whereas, whole cell *Rhizopus chinensis* lipase showed a 28.4% biodiesel yield. He et al. (2008) emphasized the potential of whole cell lipase catalysis at industrial scale production owing to their low cost and comparable yield to that of commercial lipases. Ban et al. (2002) studied the effect of cross-linking treatment by glutaraldehyde on *Rhizopus oryzae* cells immobilized within BSP. In their study, transesterification of soybean oil was carried out by adding methanol in a step-wise fashion and the conversion was monitored for six batch cycles of 72 h each. Glutaraldehyde-treated whole cells showed conversion of 70–83% in six batch cycles, while the conversion efficiency of untreated cells dropped to 50% after the sixth batch. The cells grown on specific oil as carbon source showed high catalytic activity in converting that same oil, when it was used as feedstock for biodiesel production. This trend is attributed to action of oil carbon source as lipase inducers (Zeng et al., 2006). Intracellular lipase can be used in continuous production of biodiesel which can be scaled up to industrial level. Whole cell lipase catalysts immobilized on BSP can be packed in a PBR and the reaction mixture containing oil and alcohol passed through the column to obtain FAAE (Yoshida et al., 2012).

Table 2.5: Various whole cell (intracellular) lipases used for biodiesel production

Whole cell lipase	Feedstock (Oil)	Acyl acceptor (molar ratio)	Solvent	Enzyme conc (% wt/ oil wt)	Water content (% wt/ oil wt)	Reaction conditions [Temp (°C), Time (h), Stirring rate (rpm)]	Biodiesel yield (Y) /conversion (C) %	Ref.
Rhizopus oryzae	Soybean	Methanol (5.2:1)	tert-butanol	12	3.1	35, 24, 130	Y = 72	(Li et al., 2007)
Rhizopus oryzae	*Jatropha curcas*	Methanol, (3:1)	-	4	5	30, 60, 150	C = 80	(Tamalampudi et al., 2008)
Rhizopus chinensis (lyophilized mycelia)	Soybean	Methanol, (3:1)	-	8	2	30, 72, 150	Y = 86	(He et al., 2008)
Recombinant *E.coli* expressing *Serratia marcescens* YXJ-1002 lipase (lyophilized cells)	Waste grease	Methanol, (4:1)	-	8	5	30,72, 500	Y = 97.2	(Li et al., 2012)
Mold-fungus JN7	Marine microalgae DY54	Methanol	n-hexane	-	-	40, 72, 150	Y = 68.2	(Xiao et al., 2010)
Mold-fungus JN7	*Chlorella* sp.	Methanol	n-hexane	-	-	40, 72, 150	Y = 50.3	(Xiao et al., 2010)

5. Factors affecting the enzymatic transesterification for biodiesel production

5.1 Water content

Water is essential to maintain lipase conformation and it also increases the interfacial area between aqueous and organic phase where lipases act (Tan et al., 2010). Water content in reaction mixture can be determined by either water activity (a_w) or as weight percentage of feedstock oil (Chowdary and Prapulla, 2002, Lu et al., 2009). Water activity (a_w) is the ratio of vapour pressure of a given system to that of pure water (Szczęsna Antczak et al., 2009). Excess water takes part in transesterification reactions and leads to hydrolysis and thus can reduce the yield of alkyl esters. Optimum water content for the transesterification reaction is therefore very important. The optimum water content in the reaction depends upon the lipase and feedstock used, immobilization technique employed and type of solvent (Lu et al., 2009). Considering the scaling up and process design because of its convenient application water content measured in weight percentage could be a suitable choice for the optimization compared to water activity. Nie et al (2006) studied the effect of water content (0-40% by weight of salad oil) on conversion of salad oil to methyl esters by using lipase from *Candida* sp. Methyl ester yield increased with increasing water content and maximum yield was obtained in the range of 10-15%. Further increase in water content decreased the yield of methyl esters. This behaviour was attributed to high water content favouring hydrolysis and low water content favouring esterification. Methanolysis and hydrolysis of glycerol trioleate were studied by Lu et al. (2009) with lipase from *Candida* sp. and results showed that 20% water content by weight of trioleate produced the highest yield. Different water substitutes like tert-butanol and surfactants when added in reaction, could not match the yield from the water-added reaction. Water took part in subsequent

hydrolysis and esterification. Water also diluted the methanol which had an inhibitory effect on the lipase (Lu et al., 2009). Lipases from different sources showed different responses towards water content. Methanolysis of vegetable oil was studied with *Rhizomucor miehei, Fusarium heterosporum, Candida antarctica, Rhizopus delemar* and *Aspergillus niger. Candida antarctica* lipase showed that initial water content decreased the reaction rate but does not affect its catalytic ability (Shimada et al., 1999).

5.2 Temperature

Lipases from different source show varying optimum temperature in the range of 20 to 70°C for their activity. Moderate temperature requirements by lipase-catalyzed transesterification make this process less energy-intensive. An increase in temperature increases the enzyme activity up to optimum temperature, beyond which denaturation of enzyme occurs thereby decreasing its activity. The deciding factors for optimum temperature of the lipase-catalyzed reaction include immobilization, stability of lipase, alcohol to oil molar ratio and type of solvent (Ghaly et al., 2010, Szczęsna Antczak et al., 2009). Transesterification of crude palm oil with methanol by using commercially available Lipozyme TL IM showed 96.15% FAME yield at 40°C, compared to a yield of 85.86% at 30°C. Although, denaturation of enzyme at 40°C is more than that at 30°C, the yield was higher at 40°C because of lower viscosity of the reaction mixture that facilitated rapid mass transfer rate (Sim et al., 2010). Immobilization conferred thermal stability to lipase. When both free and immobilized *Pseudomonas fluorescens* lipase was studied with triolein and propanol as substrate for biodiesel production, results showed that the decrease in production of propyl oleate at 70°C was more, in case of free lipase, compared to immobilized lipase. Immobilized lipase at 70°C showed a conversion rate of more than 30% propyl oleate,

while free lipase showed a propyl oleate conversion of less than 12% at the same temperature (Iso et al., 2001).

5.3 Inhibition by alcohol

Alcohol is a popular acyl acceptor for the transesterification reaction for biodiesel production. Methanol is the most widely-used alcohol for transesterification because of its low chain length which results in a high biodiesel yield, and also it's being least expensive among the alcohols (Gog et al., 2012). Nelson et al. (1996) studied primary and secondary short chain alcohols in transesterification catalyzed by lipase and found that in solvent-free systems, short chain alcohols like methanol and ethanol produced lower yields compared to higher alcohols. For complete conversion of oil to methyl esters, three moles of methanol are required. The lipase activity was inhibited if more than 1.5 molar equivalents of methanol are present in the reaction mixture (Shimada et al., 1999). This inhibition was attributed to the insoluble alcohol droplets in oil which caused denaturation of the enzyme. To overcome this inhibition, various alternatives have been suggested by researchers including step-wise addition of methanol, use of other acyl acceptors, use of solvent and use of methanol-tolerant lipase. In the production of methyl esters from vegetable oil using 4% (by weight of oil) immobilized *Candida antarctica* lipase and one mole equivalent of methanol added in three steps, a high conversion of 98.4% was obtained at 30ºC (Shimada et al., 1999). Watanabe et al. (2000) developed a two-step methanol addition batch process and three-step methanol addition continuous process for methyl ester production from vegetable oil by using immobilized *Candida antarctic*a lipase. In the batch process more than 95% conversion was achieved while that in continuous process was 93%.

Acyl acceptors other than methanol have also been studied for their effect on lipase inhibition. Methyl acetate was used by Du et al. (2004) as an acyl acceptor for biodiesel production from soybean oil catalyzed by Novozyme 435 and 92% yield of methyl esters was reported. Modi et al. (2007) used ethyl acetate as an acyl acceptor for Novozyme 435-catalyzed biodiesel production and reported a yield of >90%. Methyl acetate and ethyl acetate are however, expensive and require more molar equivalents for complete conversion of oil.

Use of a solvent in the reaction mixture increases the alcohol solubility and, thus, minimizes alcohol inhibition (Ghaly et al., 2010). Iso et al. (2001) studied transesterification catalyzed by immobilized *Pseudomonas fluorescens* lipase with different short chain alcohols. It was found that a solvent (e.g. 1,4-dioxane) is required when methanol and ethanol are used as acyl acceptors, while propanol and butanol can perform in a solvent-free reaction mixture. Lipases from different sources have varying catalytic activity towards different alcohols (Nelson et al., 1996). Lipase from *Mucor miehei* was found to be efficient with primary alcohols, while lipase from *Candida antarctica* showed better efficiency with secondary alcohols (Nelson et al., 1996). *Photobacterium lipolyticum* (M37) lipase was found to be more methanol-tolerant compared to *Candida antarctica* lipase B (Novozyme 435), in a one-step methanol addition reaction for biodiesel production (Yang et al., 2009). A novel approach of adding silica gel in the reaction mixture was studied in biodiesel production of canola oil using Novozyme 435 and Lipozyme RM IM as catalyst. Silica gel has been reported to absorb methanol and controlled its availability in the reaction mixture, thus reducing the methanol inhibition of enzyme activity (Lee et al., 2011). Lee et al. (2013) used dimethyl carbonate (DMC) as acyl acceptor as well as reaction solvent in conversion of triglyceride from *Chlorella* sp. KR-1 using lipase (Novozyme

435) as a catalyst and the FAME conversion obtained was over 90% even when the catalyst was repeatedly used for more than 10 times.

5.4 Inhibition by glycerol

Glycerol also has an inhibitory effect on lipase activity. Glycerol, being one of the products of lipase-catalyzed transesterification reaction, drives reaction equilibrium in the reverse direction. Also, glycerol molecules form a hydrophilic environment around the immobilized lipase molecule, thus preventing the hydrophobic substrate to come into contact with the enzyme (Szczęsna Antczak et al., 2009). The continuous removal of glycerol from the reaction mixture and use of solvents are the solutions to minimize glycerol inhibition (Fjerbaek et al., 2009). Polar solvents like tert-butanol and novel solvents like ionic liquids dissolve glycerol and thus minimize its negative effect. Lipases show good stability and improved yield in such solvent systems (Gog et al., 2012, Kumari et al., 2009). In lipase-catalyzed transesterification, acyl acceptors other than short chain alcohols which do not lead to glycerol formation have recently gathered interest. When methyl acetate was used as an acyl acceptor, triacetylglycerol was formed as a byproduct which did not show any negative effect on biodiesel yield (Xu et al., 2003). Zhang et al. (2010) reported transesterification of palm oil and dimethyl carbonate (DMC) as acyl acceptor catalyzed by Novozyme 435 in a solvent-free system. A FAME yield of 90.5% was obtained at optimum reaction conditions of 10:1 DMC to oil ratio, 20% lipase (based on oil weight), 55°C reaction temperature and 24 h duration. In the reaction, lipase activity was not inhibited because instead of glycerol, glycerol dicarbonate was formed as a byproduct. The activity of Novozyme 435 remained unaltered even after eight reaction cycles, without any significant loss in FAME yield.

5.5 Effect of solvent

Lipase-catalyzed transesterification can be carried out in the presence or absence of a solvent. The presence of a solvent reduces viscosity and helps in the proper mass transfer. A solvent increases the solubility of oils and alcohol, thus providing a better interaction of substrates with the active sites of the enzymes. Thus, the presence of a solvent renders a high yield and reduces the enzyme inhibition by alcohol (Gog et al., 2012, Robles-Medina et al., 2009). Commonly-used solvents are n-hexane, n-heptane, petroleum ether and tert-butanol (Fjerbaek et al., 2009, Ghaly et al., 2010). Hydrophobic organic solvents were preferred for enzymatic transesterification because these solvents allowed water molecules to aggregate near the enzyme, which was necessary for its enhanced activity (Fjerbaek et al., 2009). Polar solvents altered the water microlayer around the enzyme molecule, leading to its denaturation. When the transesterification of microalgal lipids was done with intracellular lipase in the presence of both n-hexane and tert-butanol, a higher yield of FAME was obtained in non-polar n-hexane as compared to the polar tert-butanol (Xiao et al., 2010). The addition of solvent however increased the overall production cost and resulted in an additional step of solvent separation from the products. Solvents, due to their toxicity and flammability, have a deteriorating effect on the environment and hence their application must be minimal.

6. Enzymatic transesterification for microalgal lipids

Enzymatic transesterification of microalgal oil is realistic and environmental friendly alternative to the conventional chemical catalysis. In this approach both feedstock and the catalyst contributes to make the overall biodiesel production process greener. Sustainable

feedstock like microalgae and environmental friendly biocatalyst renders overall biodiesel process with several benefits like minimal land and water footprint, mild reaction conditions, less wastewater generation, pure products etc. Lipase enzyme from various bacterial and fungal sources has been used by several researches for conversion of various edible and non-edible feedstock oils (Fjerbaek et al., 2009, Fukuda et al., 2001, Raita et al., 2011, Zheng et al., 2012). Application of lipase catalysis for microalgal oil still in its early stages, as very few published articles are there investigating this area. Xiong et al. (2008) used enzyme as a feasible alternative to the conventional chemical catalysts for conversion of microalgal lipids. In their study, they carried out conversion of *Chlorella protothecoides* lipids facilitated by immobilized *Candida* sp. lipase at 38ºC with 3:1 methanol to oil ratio and a conversion efficiency of 98% was obtained. Screening of lipases from various sources, immobilization of lipases, and inhibition of lipase activity by short chain alcohols, process optimization, co-solvent application and reducing enzyme catalysis cost during their application for conversion of microalgal lipids are key areas which needs attention from researchers. Tran et al. (2012) immobilized lipase from *Burkholderia* sp. on magnetic particles (alkyl-grafted Fe_3O_4–SiO_2) and used to catalyze both direct transesterification of wet microalgal (*Chlorella vulgaris* ESP-31) biomass as well as lipids extracted from *Chlorella vulgaris* ESP-31. Direct transesterification gave 97.3% biodiesel conversion while transesterification of extracted oil gave 72.1% biodiesel conversion (Tran et al., 2012). Lai et al. (2012a) investigated the conversion of *Chlorella pyrenoidosa* lipids catalyzed by *Penicillium expansum* lipase (PEL) and *Candida antarctica* lipase B (Novozyme 435) in ionic liquid [BMIm][PF_6]) and tert-butanol as reaction solvent. They found under optimum reaction conditions PEL showed 90.2% yield in ionic liquid solvent and 48.6% yield in tert-butanol while, Novozyme 435 showed 86.2% yield in ionic liquid solvent and 44.4% yield in tert-

butanol. Their results showed that PEL can be employed as efficient catalyst for conversion of microalgal lipids to biodiesel in ionic liquid as reaction medium.

Whole cell lipase application can help to reduce the cost of enzymatic transesterification. Whole cell catalysis for microalgal lipids conversion to biodiesel needs to be investigated for selection of suitable organism as lipase catalyst and its process optimization. Xiao et al. (2010) carried out transesterification of microalgal lipids by using locally isolated lipase-producing fungus JN7 as a whole cell catalyst. Transesterification of microalgal lipids was done using intracellular lipase in the presence of both n-hexane and tert-butanol, highest yield of FAME was obtained in non-polar n-hexane compared to polar tert-butanol. The reaction carried out in the presence of n-hexane at 40ºC after 72 hours yielded 68.2% FAME. Enzymatic approach of microalgal lipid conversion holds immense potential towards the sustainable production of biodiesel. This technology is still in its early stages and needs attention from researchers. Biocatalytic approach for conversion of microalgal lipids needs more investigation to select suitable lipase and optimize the process parameters as well as to overcome the challenges of high cost and enzyme inhibition.

7. Knowledge gaps

The knowledge gaps in the context of the research are presented as follows

1. Compared to upstream, literature on downstream processing (drying of biomass, extraction and conversion of microalgal lipids to biodiesel etc.) is scant.

2. Very few reports are available on application of enzyme catalyst for conversion of microalgal lipids.

8. Aims and Objectives of study

Algal biodiesel coupled with enzymatic conversion has potential as an environmentally friendly green fuel source. A comprehensive study of lipase catalysis on microalgae-derived oil for biodiesel production is necessary, in order to form a basis for further scaling-up of the process.

Aim: To produce biodiesel from microalgae-derived oil using extracellular and intracellular lipases as catalysts for the transesterification reaction.

Specific objectives

- To investigate the transesterification process using extracellular lipase from selected bacterial and fungal sources.
- To investigate the transesterification process by intracellular (whole cell) catalysis using lipase-producing microorganisms.
- To compare immobilized whole cell versus extracellular lipase catalyzed transesterification of microalgal oil.
- To characterize the fuel properties of the final product (biodiesel) for the purposes of compliance.
- To conduct a techno-economic feasibility study of the optimized process.

CHAPTER THREE: CULTIVATION, DRYING AND EXTRACTION OF LIPIDS FROM MICROALGAL BIOMASS

1. Introduction

Microalgae have emerged as a promising feedstock for the biodiesel production. Microalgal biodiesel has several advantages over the crop-based biodiesel, such as high potential for biomass production with minimal land and freshwater requirement; no arable land requirement, no food security concerns; a higher carbondioxide sequestration potential; wastewater utilization during cultivation; and the production of value-added products from de-oiled microalgal biomass. The production of a sustainable and economically-viable biodiesel from microalgae is however still a challenge (Chisti, 2007, Huang et al., 2010, Rawat et al., 2013, Scott et al., 2010).

Biodiesel production from the microalgae includes steps like cultivation, harvesting and drying of microalgal biomass, extraction of lipids from biomass and conversion of lipids to biodiesel. Photoautotrophic cultivation of microalgae is an economically viable method for large scale biomass production. Microalgae essentially require light, carbon dioxide, inorganic nutrients, and water for their growth (Chen et al., 2011a). Several microalgal species have been studied for their biodiesel production potential. Indigenous oil-producing microalgal strains are considered suitable for the large scale cultivation. South Africa has vast coastal areas as well as inland water bodies. Microalgal strains like *Chlorella* sp., *Scenedesmus* sp., *Nannochloropsis* sp. are prominently found in water bodies in the South Africa (Griffiths et al., 2010, Griffiths et al., 2011). *Scenedesmus* sp. has shown potential in terms of lipid yield as well as suitable fatty acid profile, to be used as biodiesel feedstock (Abd El Baky et al., 2012, Li et al., 2011b). Open pond

cultivation systems are less cost intensive and easier to operate compared to closed photobioreactors. However, low productivity and contamination are the limitations associated with this system (Rawat et al., 2013).

Harvesting and drying of microalgal biomass are crucial steps in the commercial production of microalgae. The harvesting method is dictated largely by the microalgal strain and the cultivation procedure. Harvesting is followed by drying of the wet biomass. Drying of harvested biomass is necessary to increase the efficiency of lipid extraction from biomass. Drying methods may include natural sun drying or using advanced techniques like freeze drying, drum drying, oven-drying, spray-drying and fluidized bed-drying (Chen et al., 2009a). Despite sun drying being amongst the slower methods, it is cost and energy effective compared to the other techniques. Freeze drying is widely used for microalgal biomass. Freeze drying is a gentle process in which all the cell constituents are preserved without rupturing the cell wall (Brennan and Owende, 2010, Chen et al., 2009a, Halim et al., 2012). Extraction of lipids from microalgae is carried out by using either physical (mechanical expeller) or chemical methods (solvent extraction) or by using a combination of these methods. Cell disruption followed by solvent extraction is a widely-used strategy for oil extraction. Disruption techniques like autoclaving, bead-beating, sonication, microwave irradiation and osmotic shock are usually coupled with solvent extraction for improved lipid yields. Conventional soxhlet extraction is a time consuming process taking several hours for complete extraction of lipids, on the other hand techniques like microwave and sonication are rapid and efficient (Halim et al., 2012, Kumari et al., 2011). The extracted lipids are then converted to biodiesel via transesterification (Brennan and Owende, 2010, Cravotto et al., 2008, Halim et al., 2011, Sharma et al., 2011c). Due to the vast diversity of microalgae, investigating suitable harvesting, drying, cell disruption, and extraction techniques

for a particular microalga becomes necessary. Development of effective and economical drying, cell disruption and extraction techniques are required to address the challenge of scaling up of the biodiesel production process from pilot to industrial scale.

In this study, *Scenedesmus obliquus* was chosen as the microalgal feedstock for biodiesel synthesis. Open circular ponds were used for cultivation of microalgae for biomass production. This chapter includes comparison of drying and cell disruption techniques for effective extraction of lipids from fresh water *S. obliquus* grown in an open circular pond. The effect of drying and cell disruption techniques on lipid quality was also assessed for its suitability for biodiesel production.

2. Materials and methods

2.1 Chemicals and reagents

A mixed FAME standard comprising of 37 components was obtained from Sigma-Aldrich. All organic solvents (chloroform, ethanol, methanol, iso-propanol, dichloromethane, hexane and toluene) and other chemicals purchased from Sigma-Aldrich (USA) were of analytical grade.

2.2 Cultivation of microalgae

A microalgal strain *Scenedesmus obliquus* isolated from the Durban region, Kwa-Zulu Natal, South Africa was used for the present study. *S. obliquus* was grown under natural light (400 – 1200 $\mu mol.m^{-2}.s^{-1}$) and water temperatures ranging from 18 – 27°C in an open circular pond of 8000 L capacity located at Kingsburgh Wastewater Treatment Works facility (-30.075068, 30.856904) for biomass production. BG11 medium was used as a nutrient medium

to culture the microalgae (Ehimen et al., 2010, Lee et al., 2010). Mixing and aeration of the suspended algal biomass was accomplished by submersible pumps having a flow rate of 110 $L.min^{-1}$. Lipid accumulation in *S. obliquus* cells was monitored using Nile Red staining (Axiolab HBO 50 epi fluorescent microscope Carl Zeiss, Germany) (Moazami et al., 2011, Mutanda et al., 2011b). The biomass yield of *S. obliquus* was monitored by gravimetric analysis (Mutanda et al., 2011a). Dewatering was necessary to obtain a thick biomass slurry. Harvesting of biomass was initially done on day 21 by gravitational settling to remove the bulk amount of water followed by centrifugation to obtain a thick slurry. All the biomass required for study was collected from same batch of open pond cultivation and was stored in -84°C.

2.3 Drying of biomass

The harvested wet microalgal biomass was dried using three different drying techniques (sun drying, oven drying, and freeze drying). The thick slurry of wet biomass obtained from 20 L microalgal culture after gravitational settling followed by centrifugation was used for the different drying techniques. Microalgal biomass was sun dried on a drying bed lined with white plastic of 1500 µm thickness at ambient temperature (25 – 30°C) for 72 h (Rwehumbiza et al., 2012). For freeze drying, wet biomass was frozen overnight at −84°C and lyophilized using a freeze dryer (Mini lyotrap, LTE scientific Ltd. United Kingdome). Oven drying was carried out using a hot air oven for 12 h at 60°C (Cheirsilp and Torpee, 2012). The dried microalgal biomass was crushed in a mortar and pestle and the dry microalgal powder was stored in desiccator to avoid moisture absorption. The harvesting and drying techniques were repeated to accumulate substantial dried biomass for further experiments.

2.4 Cell-disruption and extraction of lipids

Cell disruption and extraction of *S. obliquus* biomass was carried out by slight modification in methods used by Lee et al. (2010) for microwave extraction and Kumari et al. (2011) for sonication extraction. The dried biomass (2 g) was added to a 40 ml mixture of chloroform and ethanol (1:1, v/v) and subjected to cell disruption by microwave (Milestone S.R.L., Italy, output power 1200 W) at 100°C for 10 min at 1000 W. The mixture was centrifuged to separate solvent mixture from the cell debris. The mixture was vacuum filtered followed by distillation of solvent in a rotary evaporator at 70°C. The amount of crude lipid was quantified gravimetrically and the lipid yield (%) was calculated. For sonication-assisted extraction, microalgal biomass (2 g) was mixed in solvent mixture (20 ml) and the oil was extracted using a sonicator (Misonix XL-2000-010, USA, output power 100 W, output frequency 22.5 KHz) in 50 ml tubes at 15 KHz for 2 min. The solvent mixture was centrifuged to separate solvent mixture with lipids and residual biomass in the form of a pellet. Residual biomass was again mixed with solvent mixture (20 ml) and subjected to sonication followed by centrifugation. Solvent mixture was pooled together and filtered through a vacuum filter. The solvent was removed in a rotary evaporator at 70°C to obtain the microalgal oil. The crude microalgal lipid was measured gravimetrically and the lipid yield (%) was quantified. The lipid yields obtained from biomass dried by the three different drying processes using the two disruption techniques viz. microwave and sonication were compared.

2.5 Lipid characterization

The lipid quality was assessed by determination of saponification value and acid value using ASTM methods D5558-95, Reapproved 2011; and D664-07 respectively (Kafuku et al., 2010, Noshadi et al., 2012). The potentiometric titration for determination of

acid and saponification value was carried out by using an automatic titrator (TIM 855 Titration manager, Radiometer Analytical, Titralab, France). For saponification value, pre-weighed lipid samples were boiled with alcoholic KOH in a round bottom flask with a condenser attached to it. After complete saponification, the contents of the flask were titrated with HCl (0.5 M). Saponification value was determined using following formula:

$$\text{Saponification value} = \frac{28.05(\text{A}-\text{B})}{\text{Weight of lipid sample}} \quad (1)$$

Where, A- End point of blank

B- End point of lipid sample

For acid value, pre-weighed lipid samples were dissolved in toluene and iso-propanol (1:1 v/v) and titrated with alcoholic KOH. Acid value was determined using the following formula:

$$\text{Acid value} = \frac{(\text{A}-\text{B})\times \text{M} \times 56.1}{\text{Weight of lipid sample}} \quad (2)$$

Where, A- End point of lipid sample

B- End point of blank

M- Concentration of alcoholic KOH

Molecular weight of lipid was calculated according to formula given by Xiong et al. (2008):

$$\text{Molecular weight of lipid} = \frac{168300}{(\text{SV}-\text{AV})} \quad (3)$$

Where, SV- saponification value of microalgal lipids

AV- acid value of microalgal lipids

2.6 Fatty acid profile

For fatty acid profile analysis, lipids were converted to fatty acid methyl esters (FAMEs) and analyzed by gas chromatography. The extracted lipids were subjected to simultaneous esterification and transesterification using sulfuric acid as catalyst and methanol as an acyl acceptor for its conversion to FAMEs. The reaction conditions were 30:1 methanol to oil molar ratio; temperature: 60°C; catalyst concentration: 10% w/w of oil; time: 4 h and 1ml hexane (D'Oca et al., 2011). The rate of stirring was kept constant at 200 rpm in an orbital shaker incubator (Model TU-454, mrc Ltd. Israel). The FAMEs were then analyzed by gas chromatography (Shimadzu GC-2014, Japan) equipped with a flame ionization detector and a capillary column (SP2380, Supelco Analytical, USA). The oven temperature was programmed to start at 60°C and kept at hold for 2 min, then initially increased to 160°C at a ramp rate of 10°C.min^{-1} and then to 240°C at a ramp rate of 7°C.min^{-1} and again kept at hold for 1 min. The injector and detector temperature was 250°C and nitrogen was used as the carrier gas.

2.7 Statistical analysis

The data was expressed as mean value ± SE (Standard Error). The results were tested for significance at the 0.05 level by comparing mean values obtained from experiments for different drying and cell disruption techniques, employing one-way analysis of variance.

3. Results and discussion

3.1 Biomass production

The cultivation of microalgae in open ponds has potential for large scale commercial production of microalgal biomass (Figure 3.1). The microscopic observation showed dominance

of *S. obliquus* (>95 %) in the culture grown in the open pond. Photoautotrophic growth of *S. obliquus* under natural sunlight supplemented with BG11 as nutrient medium produced substantial amount of biomass. Lipid accumulation in *S. obliquus* was assessed by Nile Red staining; neutral lipid bodies in the cytosol are stained yellow in colour (Figure 3.2). The biomass yield of *S. obliquus* reached 1.16 $g.L^{-1}$ on the 21st day of cultivation.

Figure 3.1: Cultivation of *S. obliquus* in open ponds

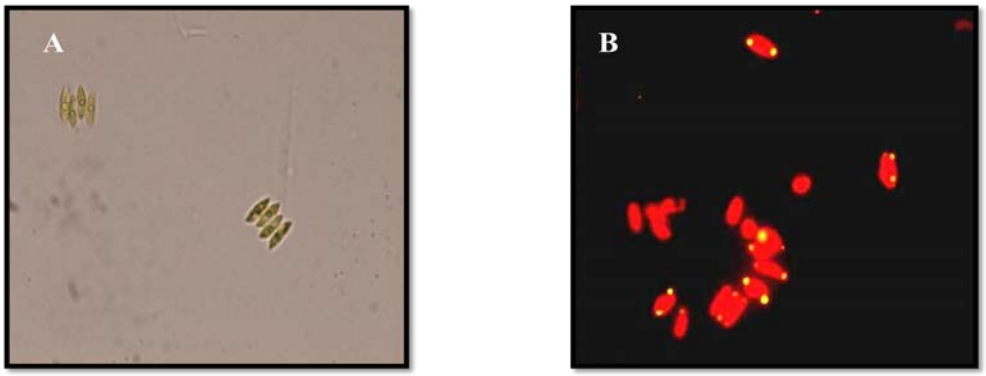


Figure 3.2: Microscopy images of *Scenedesmus obliquus* (A) without Nile Red staining (B) With Nile Red staining.

3.2 Effect of drying technique

Water has to be removed from the microalgal biomass slurry to increase the efficiency of lipid extraction. In the microwave-assisted solvent extraction, the lipid yields of *S. obliquus* biomass dried by freeze drying, oven drying and sun drying were found to be 29.65 ± 1.05%, 28.63 ± 0.42%, and 28.33 ± 1.37% lipid.g^{-1} DCW (dry cell weight) respectively (Figure 3.3). Whereas, in case of sonication-assisted solvent extraction, lipid yields by freeze drying, oven drying and sun drying were found to be 19.85 ± 0.35%, 18.8 ± 0.1% and 18.9 ± 0.5% lipid.g^{-1} DCW respectively (Figure 3.3). Thus, no significant difference in the lipid yield was observed among the three drying techniques ($p > 0.05$) for the latter extraction method.

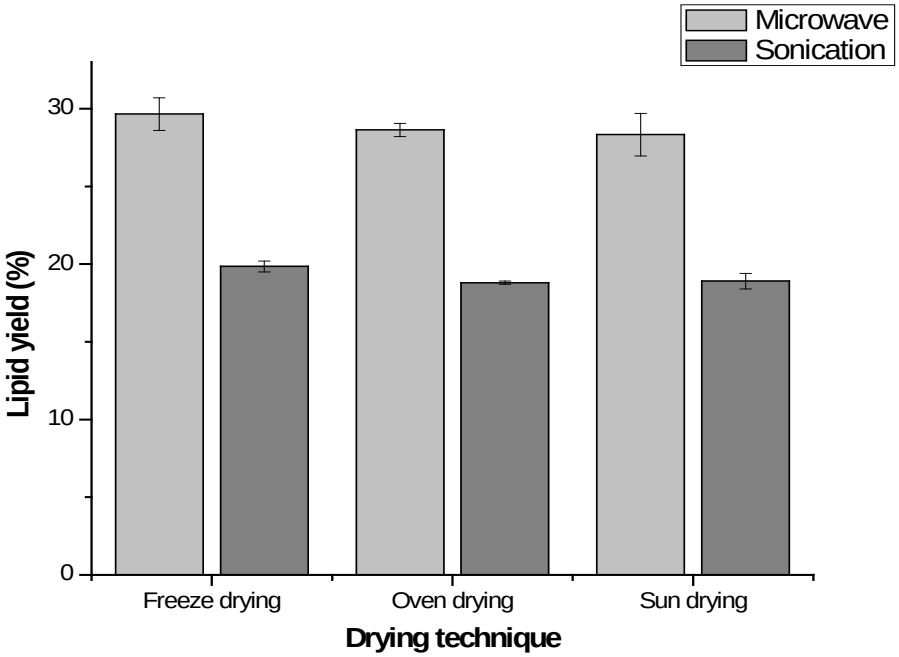


Figure 3.3: Effect of drying and cell disruption techniques on lipid yield (%) from algal biomass.

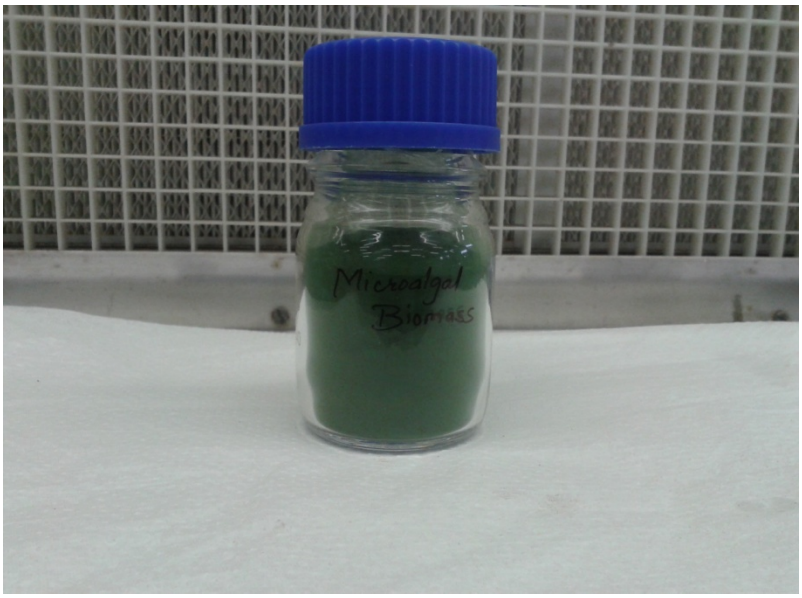


Figure 3.4: Freeze dried microalgal (*S. obliquus*) biomass

Our findings confer with research by Balasubramanian et al. (2013) who reported biomass of *Nannochloropsi*s sp. was dried using oven drying, freeze drying and sun drying and there was no significant effect of drying technique on the total lipid yield after extraction. Among the three techniques, freeze drying and oven drying are energy-intensive methods. The time taken for drying by the three techniques varied. Sun drying took substantially longer (72 h) for drying microalgal biomass compared to oven drying (12 h) and freeze drying (24 h). The choice of drying technique is also influenced by the scale of microalgae production. The economical feasibility offered by sun drying makes it a preferred choice for a large scale production of dry biomass from the wet microalgae. However, for sun-drying, requirement of large land area may be a constraint. The Freeze drying technique is suitable for the lab scale studies as it operates at gentle conditions and keeps microalgal cellular components preserved.

3.3 Effect of cell disruption techniques and lipid extraction

The commonly utilized extraction methods viz. Bligh and Dyer and Folch method employ a mixture of chloroform and methanol as solvent (Kumari et al., 2011). However, methanol is known to be highly toxic and hence was substituted with ethanol. A mixture of chloroform and ethanol was used as solvent for the extraction of lipids (Ramluckan et al., 2014). The extraction of lipid from microalgae by solvent, assisted with a cell disruption technique (microwave or sonication) results in a better yield as these techniques enhances the contact between cellular lipids and solvents. Both microwave and sonication techniques of cell disruption avoids use of the toxic chemicals used for cell disruption and also greatly reduce the time of lipid extraction when compared to conventional chemical cell disruption techniques and soxhlet extraction. In the sonication technique, sound waves forms cavitation bubbles in liquid medium which after collapsing generates chemical and mechanical energy that disrupts the cell

wall of microalgae (Vyas et al., 2010). Sonication enhances lipid extraction from microalgal cells by facilitating solvent access through cell disruption and proper mass transfer. In the microwave technique, the microwave energy facilitates rotation of molecular dipoles, thus disrupting weak hydrogen bonds. This effect causes movement of dissolved ions which increase the solvent penetration into microalgal biomass and improves lipid extraction (Cravotto et al., 2008). In this study, the effect of microwave and sonication on the lipid yield has been studied where a comparatively higher yield of lipid was observed with microwave (Figure 3.3). The microwave mode of lipid extraction gave a high yield of 29.65 ± 1.05% lipid.g^{-1} DCW, compared to sonication where the yield observed was 19.85 ± 0.35% lipid.g^{-1} DCW in case of freeze dried microalgal biomass. The microwave technique showed a similar trend of higher lipid yields in oven dried (28.63 ± 0.43%) and sun dried (28.33 ± 1.37%) biomass compared to oven dried (18.8 ± 0.1%) and sun dried (18.9 ± 0.5%) biomass extracted by the sonication technique. Microwave-assisted lipid extraction gave 49.37 to 52.29% better yield compared to sonication-assisted lipid extraction of biomass dried by selected drying techniques. Higher yields by microwave technique can be attributed to several factors. In the microwave technique, electromagnetic waves penetrate the cell matrix and interact at molecular level, which makes heating more efficient (Manco et al., 2012). In conventional solvent extraction, direction of mass transfer is from inside to outside while heat transfer direction is from outside to inside. However, with microwaves both mass and heat transfer occurs from inside the cell matrix to outside solvents. This causes the efficient cell disruption as well as lipid extraction (Iqbal and Theegala, 2013). Results from the present study are in compliance with a previous study by Lee et al. (2010) where the microwave method has been shown to extract higher amounts of lipids from *Scenedesmus* sp. as compared to sonication. In a previous study by Iqbal

and Theegala (2013), microwave-assisted lipid extraction and soxhlet extraction was studied for lipid extraction of *Nannochloropsis* sp. using chloroform and ethanol as solvent, soxhlet extraction yielded 49% lipid.g^{-1} DCW while microwave-assisted extraction gave slightly higher lipid yield of 53% lipid.g^{-1} DCW. The microwave technique has an easy mode of operation and thus can be scaled up for an industrial scale lipid extraction.

3.4 Characterization of lipid

Lipid characteristics viz. saponification value, acid value and fatty acid profile has an influence on the choice of conversion technique to be adopted for the production of biodiesel. It also subsequently influences the fuel properties of biodiesel. A high saponification value of lipids extracted from *S. obliquus* (172.52 ± 1.16 to 177.2 ± 0.82 mg KOH.g^{-1}) showed that it can be used as efficient feedstock for biodiesel production (Table 3.1). A high acid value (21.01 ± 0.52 to 29.75 ± 0.54 mg KOH.g^{-1}) indicated the presence of a high amount of free fatty acids (FFA). Acid value results showed that drying method has significant effect on amount of FFA content in total lipids ($p < 0.05$) (Table 3.1). Highest acid value was determined in lipids extracted from biomass dried using sun drying (29.75 ± 0.54 mg KOH.g^{-1}) followed by oven drying (26.17 ± 0.84 mg KOH.g^{-1}) and freeze drying (21.01 ± 0.52 mg KOH.g^{-1}). This high acid value of lipids extracted from sun-dried microalgal biomass could be because of lipid oxidation caused by long exposure to light and UV (ultraviolet) irradiation and enzyme degradation (Balasubramanian et al., 2013).

Table 3.1: Characterization of *Scenedesmus obliquus* lipids

Property (unit)	Freeze Dried	Oven dried	Sun dried
Saponification value (mg KOH.g^{-1})	177.2 ± 0.82	172.52 ± 1.16	175.45 ± 1.62
Acid value (mg KOH.g^{-1})	21.01 ± 0.52	26.17 ± 0.84	29.75 ± 0.54
Molecular weight (g.mole^{-1})	1077.53	1149.98	1155.11

Results achieved in the present study are in compliance with a study by Balasubramanian et al. (2013), who found variation in FFA content of lipids extracted from *Nannochloropsis* sp. dried using sun drying, oven drying and freeze drying. High FFA content in *S. obliquus* lipid also necessitates the use of either an acid catalyst or a biocatalyst which can convert feedstocks with high FFA efficiently.

3.5 Fatty acid profiles of *S. obliquus*

Palmitic acid (C16:0) was found to be the most dominant fatty acid in the lipids extracted from *S. obliquus*, despite using different drying and cell disruption techniques, contributing from 31.67 ± 0.32 to 55.18 ± 2.07% to the total lipid constituents. The other prominent fatty acids identified in the lipid extracts were C16:1 (4.40 ± 0.09 to 8.86 ± 0.32%), C18:1 (6.87 ± 0.48 to 16.66 ± 0.85%), C18:2 (6.88 ± 0.17 to 12.72 ± 0.08%), C18:3 (8.84 ± 0.12 to 15.30 ± 0.13%) and C20:1 (1.41 ± 0.04 to 8.85 ± 2.48%) (Figures 3.4 and 3.5). Fatty acid profile results showed that there is a variation in percentage composition of saturated fatty acids, monounsaturated fatty acid and polyunsaturated fatty acids, depending upon the drying as well as the cell disruption technique (Figures 3.5 and 3.6). Oven dried and freeze dried biomass showed higher percentage

of saturated fatty acid (46.43 to 58.44%) than sun dried biomass (44.33 to 44.98%). Microalgal lipid components, specifically polyunsaturated fatty acids, show swift response to various factors like temperature, pH, light, nutrient depletion etc (Borges et al., 2011, Gao et al., 2013). Lipids extracted by the microwave technique showed 39.61 to 54.1% of unsaturated fatty acids while lipids extracted by the sonication technique showed 47.65 to 52.85% of unsaturated fatty acids. The high temperatures used in the microwave technique as well as free radical formation during sonication technique could cause the oxidative cleavage of unsaturated bonds (Gerde et al., 2012, Viswanathan et al., 2012,).

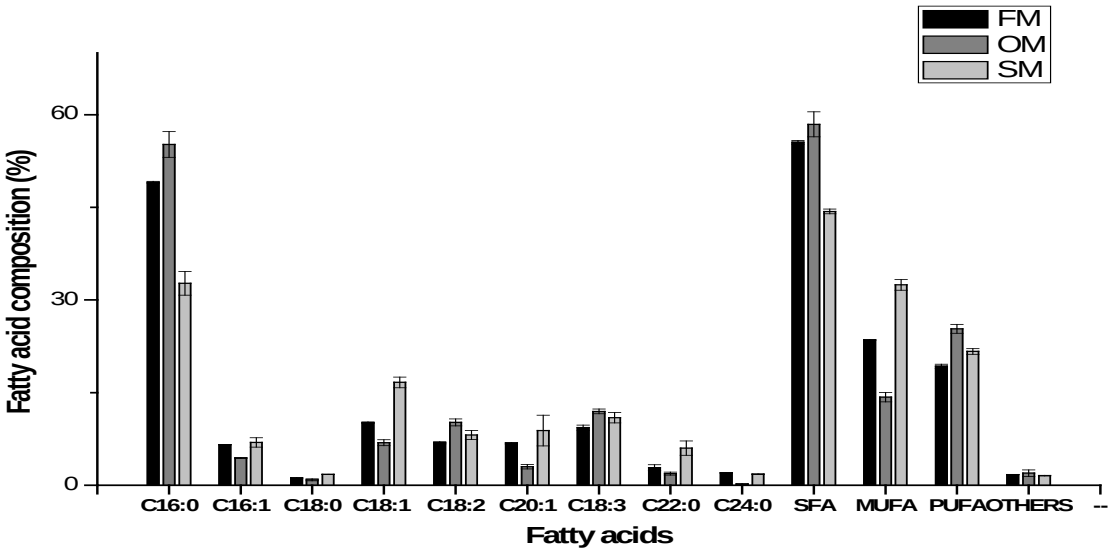


Figure 3.5: Lipid profile obtained by the microwave technique (FM- freeze dried biomass, OM- oven dried biomass and SM- sun dried biomass).

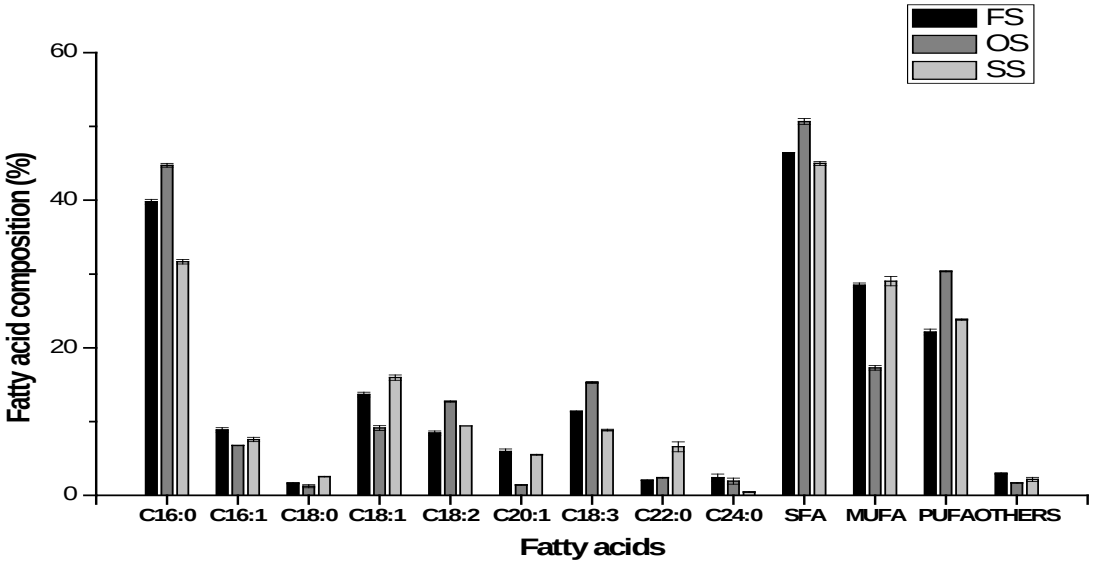


Figure 3.6: Lipid profile obtained by the sonication technique (FS- freeze dried biomass, OS- oven dried biomass and SS- sun dried biomass).

Fatty acid composition has a profound effect on the fuel property of biodiesel. There is an inverse relation between the oxidation stability and cold flow property of biodiesel (Sharma et al., 2008). A high content of saturated fatty acid is desirable for a better oxidation stability of biodiesel. This will be beneficial for the industry as biodiesel could be stored for longer periods. On the other hand, a high content of unsaturated fatty acid is beneficial for cold flow properties (cloud point, cold filter plugging point, and pour point) of biodiesel. This would lead to the possible usage of the fuel even in cold countries and in cold months in the tropical countries. However, the saturated fatty acids could be removed from the lipid by winterization and antioxidants could be added to retard the oxidation of the fuel. These processes (winterization and addition of antioxidants) will contribute to the overall cost of the fuel. Thus, it is desirable that there is a mixture of both saturated and unsaturated fatty acids in the lipid to achieve agreeable oxidation stability and cold flow properties. The concentration of linolenic acid in lipid should not exceed 12% (Gouveia et al., 2009). In the present study, the

concentration of linolenic acid (C18:3) varied from 8.84 ± 0.12 to 15.30 ± 0.13%. A high concentration of linolenic acid may lower the oxidation stability of the fuel and lead to its rancidity, if the fuel is not supplemented with antioxidants. In the present work, the concentration of linolenic acid was sometimes greater (ranging from 8.84 to 15.3%) than the specified limit (12%) in lipid extracted through sonication. Whereas, in the microwave assisted lipid extraction, the content of linolenic acid (ranging from 9.32 to 11.96%) was within the specified limit of 12%. Hence, it may be concluded that the lipid extracted by microwaves is superior to that from sonication for its suitability for biodiesel feedstock.

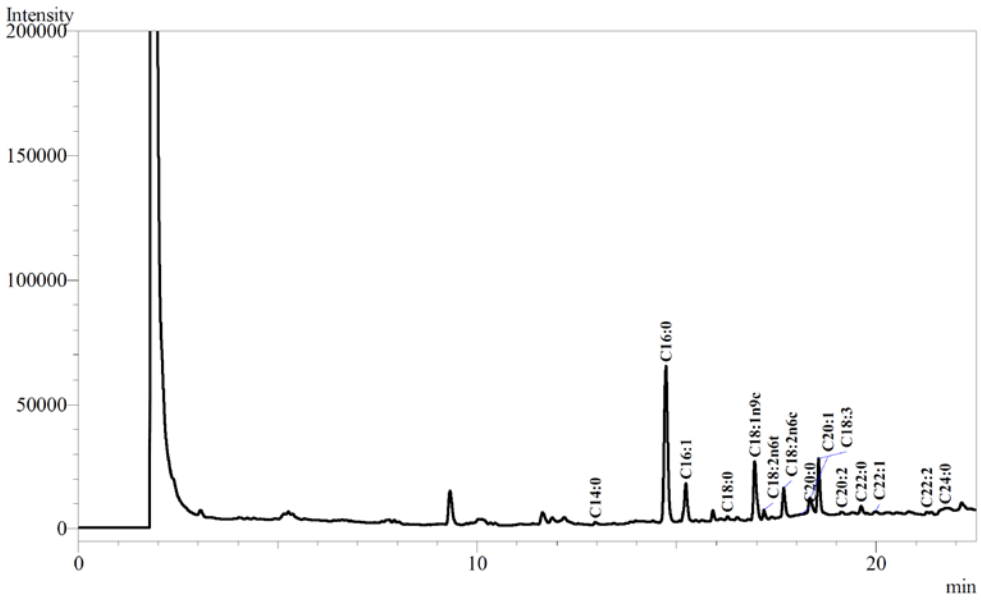


Figure 3.7: Chromatogram depicting fatty acid profile of lipids extracted by microwave technique from freeze dried *Scenedesmus obliquus* biomass.

Chromatogram of lipids extracted by microwave technique from freeze dried *S. obliquus* biomass showed suitable composition of fatty acids as well as linolenic acid content within EN14214 specifications (Figure 3.7). Thus for further study of conversion of microalgal lipids to

biodiesel using an enzyme catalyst, freeze drying and the microwave extraction techniques were chosen.

4. Conclusions

Drying methods and cell disruption techniques which are crucial for effective downstream processing in microalgal biodiesel synthesis were studied. The selected drying methods did not show a significant difference in the extraction of lipid. Microwave cell disruption resulted in a higher lipid yield compared to sonication. Lipids extracted from *S. obliquus* showed desirable saponification value, acid value, and lipid profile as a feedstock for biodiesel synthesis. Drying and cell disruption techniques have significant influence on lipid profile. Lipids extracted from freeze dried biomass using microwave extraction technique showed superior quality as biodiesel feedstock. Thus, freeze drying with gentle operating conditions and an efficient microwave cell disruption technique were used to process *S. obliquus* biomass and extract lipids for further studies. However, an easy and economical sun drying method with an efficient microwave cell disruption technique could be possible processing steps in further scaling up of biodiesel synthesis from *S. obliquus*.

CHAPTER FOUR: EXTRACELLULAR LIPASE APPLICATION

1. Introduction

Microalgal biodiesel has shown promising potential of alternate fuel in the light of dwindling petroleum fuel sources and food security concerns. Biodiesel is a mixture of monoalkyl esters of fatty acids derived from the natural resources like vegetable oils, animal fats and microalgae. The most widely accepted method for synthesis of biodiesel is transesterification of oils using an alkali catalyst and methanol as the acyl acceptor (Chisti, 2007, Fukuda et al., 2008). Microalgae, the third generation feedstock have shown immense potential for biodiesel production compared to the previous feedstocks (food and non-food crops). Microalgae can accumulate substantial amounts of lipids and can be grown in marginal lands without competing with food crops. Use of wastewater as the nutrient medium, CO_2 sequestration and other value-added products adds to the benefits provided by microalgae (Amaro et al., 2011, Rawat et al., 2013).

Although the chemical conversion method has been widely used for biodiesel synthesis, it has some challenges: additional neutralization and product purification steps; high energy requirements; and environmental concerns due to wastewater generation during downstream processing. On the other hand, the biocatalytic method offers the advantages of a high quality product which reduces downstream processing steps, less wastewater generation and energy consumption (Bajaj et al., 2010, Fjerbaek et al., 2009, Tan et al., 2010). Lipase-catalyzed conversion of feedstock oils to biodiesel is greener and a sustainable approach because of its environmentally-benign nature and low energy requirements. High specificity and activity result in high quality pure products which need fewer purification steps for further processing. Lipases have been employed by many researchers as a catalyst for transesterification of various

feedstocks for biodiesel synthesis (Chen et al., 2009b, Jegannathan et al., 2010, Lai et al., 2012a). Unlike alkali catalyst, lipases can be used for conversion of feedstock oils with high free fatty acids (FFA) (Atadashi et al., 2012, Kumari et al., 2007). Microalgal lipids have high FFA content, thus using lipase can provide significant advantages over the conventional conversion process using acid or alkali catalysts. Challenges faced when using lipases are its high cost, inhibition by short chain alcohols and slow reaction rate compared to chemical catalysts (Bisen et al., 2010, Fjerbaek et al., 2009). Enzyme-catalyzed biodiesel production is still under assessment stage for development of efficient process to overcome cost and inhibition constraints. There is comparatively sparse literature available about lipases being employed for conversion of microalgal lipids.

Conversion yields can be improved by screening suitable lipases from various sources and optimization of process parameters for selected lipases. Immobilization of lipase is a possible solution to reduce the cost, as immobilized lipase can be easily separated and reused (Robles-Medina et al., 2009). Adding methanol to stoichiometric molar ratio or step-wise addition of methanol can overcome the methanol inhibition of lipases (Fukuda et al., 2001, Watanabe et al., 2000). Another approach is to carry out reactions in suitable solvents which reduce the inhibitory effect by methanol (Gog et al., 2012). For the optimization studies of lipase-catalyzed biodiesel synthesis, response surface methodology (RSM) has been successfully applied (Ognjanovic et al., 2009, Shieh et al., 2003). RSM studies not only predict optimized levels of selected parameters but also provide information about the extent of parameter's influence on the process.

This chapter explores the application of extracellular lipase for the conversion of microalgal lipids to biodiesel. *Scenedesmus obliquus* grown in open circular ponds was selected as the

microalgal feedstock. Lipases from selected sources were screened for their catalytic performance. The selected lipases were chosen for optimization of reaction parameters using response surface methodology. A three level four factor Box-Behnken experiment design was employed for response surface methodology optimization. Effect of various reaction parameters on the lipase-catalyzed biodiesel conversion was studied. Step-wise addition of methanol was investigated to observe further improvement in the biodiesel conversion. Reusability of the selected lipases was also evaluated. The resulting biodiesel product was characterized for its fuel properties and compared with standard specifications.

2. Material and methods

2.1 Chemicals and reagents

The free and immobilized lipases were obtained from Sigma-Aldrich. *Pseudomonas fluorescens* and *Candida rugosa* lipases immobilized on immobead 150 (Sigma-Aldrich, Netherlands) were used as immobilized lipases. Free lipases used were Amano lipase from *Pseudomonas fluorescens* (Amano, Sigma-Aldrich, Japan), lipase from *Candida antarctica* (Sigma-Aldrich, Germany), lipases from porcine pancreas and wheat germ (Sigma-Aldrich, USA). A mixed fatty acid methyl ester (FAME) standard (37 components) and methyl heptadeconoate were obtained from Sigma-Aldrich (USA). All organic solvents and other chemicals purchased from Sigma-Aldrich (USA) were of analytical grade. *S. obliquus* lipids extracted using the microwave technique as described in Chapter three was used as microalgal biodiesel feedstock.

2.2 Screening of lipases

Selected free and immobilized lipases (free lipases from *P. fluorescens, C. antarctica*, porcine pancreas and wheat germ and immobilized lipases from *P. fluorescens* and *C. rugosa*) were used for transesterification of *S. obliquus* lipids. Reaction conditions were: temperature, 40°C; methanol to oil molar ratio, 4:1; water quantity, 5% of oil weight; enzyme concentration, 10% by oil weight with immobilized lipase and 1% by oil weight with free lipase based on lipase activity. For all the conversion experiments 0.1g of *S. obliquus* lipids was used as feedstock. Solvent n-hexane was added to the reaction mixture to provide proper mass transfer. Samples were taken out at regular interval from the reaction mixture and analyzed using gas chromatography to determine percentage fatty acid methyl ester (FAME) conversion. Gas chromatography conditions were described in section 2.6 of Chapter three. Methyl heptadeconoate was used as an internal standard and the 37 component FAME mix was used for peak identification. FAME conversion was calculated using formula by Lee et al. (2011).

$$\text{FAME Conversion (\%)} = \frac{\sum \text{TA} - \text{IA}}{\text{IA}} \times \frac{C_I \times V_I}{m} \times 100 \qquad \text{(Eq. 1)}$$

Where, ∑TA: total area of peaks from C14 to C24, IA: peak area of internal standard, C_I: concentration of internal standard (mg/L), V_I: volume of internal standard (ml) and m: mass of biodiesel sample (mg). Based on FAME conversion and time required, a lipase was selected for further optimization studies.

2.3 Optimization of process parameters

A three level four factorial Box-Behnken response surface methodology experimental design using Minitab Statistical Software was employed in this study, comprising of 27

experiments with three replicates at centre point. Optimization of reaction conditions for lipase-catalyzed transesterification of *S. obliquus* lipids was done taking FAME conversion (%) as response. The experimental design consisted of four factors: catalyst amount (wt% with respect to oil weight), reaction temperature (°C), water amount (wt% with respect to oil weight), and methanol to oil molar ratio. A range of three level factors was selected based on the previous findings reported by researchers. The temperature and methanol to oil molar ratio optimized in previous studies using lipase as catalyst for biodiesel synthesis were in the range of 30-55°C and 3:1-6:1 respectively (Kaieda et al., 2001, Kumari et al., 2009, Lu et al., 2009, Tongboriboon et al., 2010, Xie and Wang, 2012). Optimized water content and enzyme amount in previous studies were in the range 2-5% (Rodrigues and Ayub, 2011, Tongboriboon et al., 2010) and 5-20 % (Modi et al., 2007, Sim et al., 2010, Tongboriboon et al., 2010) with respect to oil weight respectively. Adding enzyme as weight percentage with respect to oil gives advantage of its easy application at large scale. Table 4.1 depicts the three levels of variables for the selected four factors i.e. catalyst amount (5, 10 and 15 wt% with respect to oil), reaction temperature (35, 45 and 55°C), water amount (1, 2.5 and 5 wt% with respect to oil) and methanol to oil molar ratio (3:1, 4:1 and 5:1) in lipase-catalyzed transesterification. The reaction time of 12 h and agitation speed of 200 rpm were kept constant for transesterification. The effect of independent factors on the dependent factors was analyzed by a quadratic equation:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4 + e \quad \text{(Eq. 2)}$$

Where, Y is the response (biodiesel conversion, %), a_0 is offset term; a_1, a_2, a_3 and a_4 are linear coefficients; a_{11}, a_{22}, $a_{33}$and a_{44} are the squared term coefficients; a_{12}, a_{13}, a_{14}, a_{23}, a_{24} and a_{34} are the interaction coefficients; and e is the error. X_1, X_2, X_3 and X_4 are catalyst amount, reaction temperature, water amount and methanol to oil molar ratio respectively.

Table 4.1: Factors and levels for experimental design using extracellular lipase

Factors	**Enzyme amount (wt% with respect to oil weight)**	**Temperature (°C)**	**Water content (wt% with respect to oil weight)**	**MeOH: Oil Molar ratio**
Level 1	-1 (5)	-1 (35)	-1 (1)	-1 (3:1)
Level 2	0 (10)	0 (45)	0 (2.5)	0 (4:1)
Level 3	1 (20)	1 (55)	1 (5)	1 (5:1)

MeOH- Methanol

2.4 Step-wise addition of methanol and reusability study

Step-wise addition of methanol was applied to study the improvement in conversion yields with optimized reaction parameters. Methanol was added using three different schemes. In scheme one, out of three moles, one mole of methanol was added at the beginning of reaction followed by one mole after every 2 h. In scheme two, one mole of methanol was added at the start of the reaction and one mole added after every 3 h. In scheme three, two moles of methanol was added at the beginning, while the remaining one mole was added after 4 h. For the reusability study, immobilized lipase was separated from the reaction mixture by filtering and then subsequently washing with n-hexane and distilled water. Recovered lipase was used with optimized parameters and selected scheme of step-wise methanol addition. A similar procedure was followed for subsequent batches of reaction. Reactions were carried out for 12 h and samples were analyzed by GC for percentage FAME conversion.

2.5 Biodiesel characterization

The FAME product obtained from *S. obliquus* via extracellular lipase-catalyzed conversion was tested for fuel characteristics using standard methods. A FAME profile was detected by gas chromatography analysis. Properties like density, ester content, linolenic acid content, acid value (ASTM D664-07), was determined by the standard methods and elemental analysis was done using an elemental analyzer (Vario EL, Elementar Germany). Calorific value was determined by using calorimeter (Drycal modular calorimeter, Energy instrumentation). Properties like cetane number, iodine value and oxidative stability were determined using correlation equations (Wang et al. 2012) based on the fatty acid methyl ester composition in biodiesel product determined by gas chromatography.

The equations to calculate cetane number, iodine value and oxidative stability were as follows.

$$\text{Cetane number} = -0.1209 \times \text{DU} + 65.0958 \quad \text{(Eq 3)}$$

$$\text{Iodine value} = 0.6683 \times \text{DU} + 25.0364 \quad \text{(Eq 4)}$$

$$\text{Oxidative stability} = -0.0384 \times \text{DU} + 7.77 \quad \text{(Eq 5)}$$

Where, DU is degree of unsaturation. The DU was calculated from the FAME profile using the equation

$$\text{DU} = (\%\ \text{composition of MUFA}) + 2 \times (\%\ \text{composition of PUFA } C_n{:}\,2) \times (\%\ \text{composition of PUFA } C_n{:}\,3) + 4 \times (\%\ \text{composition of PUFA C\_n: 4}) \quad \text{(Eq 6)}$$

Where, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Cold filter plugging point (CFPP) was calculated by the formula given by Ramos et al. (2009)

$$\text{CFPP} = 3.1417 \times \text{LCSF} - 16.477 \quad \text{(Eq 7)}$$

Where, LCSF is long chain saturation factor. The LCSF was calculated from the FAME profile using equation

$$\text{LCSF} = 0.1 \times \%\text{composition of C16:0} + 0.5 \times \%\text{composition of C18:0} + 1 \times \%\text{composition of C20:0} + 1.5 \times \%\text{composition of C22:0} + 2 \times \%\text{composition of C24:0} \quad \text{(Eq 8)}$$

Properties of FAME product were compared with the ASTM and EN standards for its suitability as biodiesel.

3. Results and discussion

3.1 Selection of a suitable lipase

Lipases from different sources showed the different specificities and activities. Various bacterial and fungal lipases have been successfully employed in industrial applications. For the screening study, free lipases from fungal (*C. antarctica*), bacterial (*P. fluorescens*), animal (porcine pancreas) and plant (wheat germ) source along with immobilized lipases from fungal (*C. rugosa*) and bacterial (*P. fluorescens*) source were chosen. Immobilized *P. fluorescens* lipase has shown highest FAME conversion (33.87 ± 1.5%) followed by immobilized *C. rugosa* lipase (20.76 ± 0.49%) in 24 h reactions (Figure 4.1). The high FAME conversion by immobilized *P. fluorescens* lipase could be possible because of its high resistance towards methanol inhibition as compared to *C. rugosa* lipase (Kaieda et al., 2001). Soumanou and Bornscheuer (2003), in their study on lipase catalyzed synthesis of FAMEs from sunflower oil, found that *P. fluorescens* lipase performed better in the organic solvent n-hexane and has also shown higher resistance towards methanol tolerance compared to other selected lipases. Thus, *P. fluorescens* lipase was

selected as the biocatalyst for further optimization studies. Lowest FAME conversion was observed using wheat germ lipase. While using immobilized lipases as catalyst, the increase in percentage FAME conversion using 12 h and 24 h reaction times was not significant. All the optimization experiments were thus carried out for 12 h.

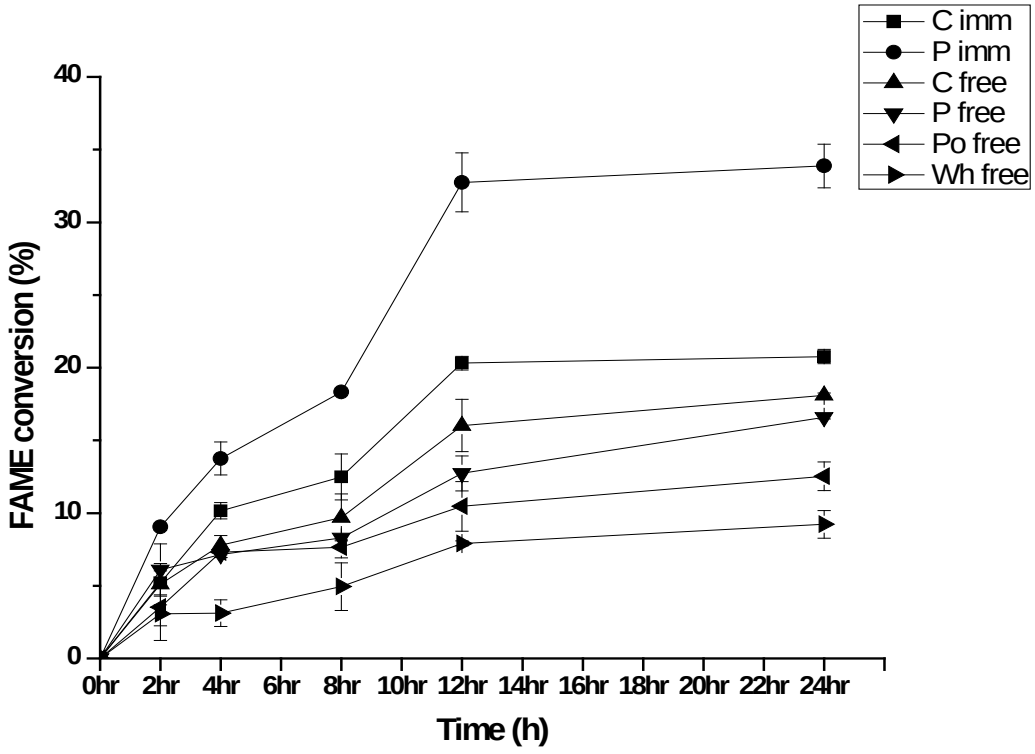


Figure 4.1: FAME conversion obtained by using immobilized and free extracellular lipases from different sources. (C imm: immobilized *C. rugosa* lipase, P imm: immobilized *P. fluorescens* lipase, C free: free *C. antarctica* lipase, P free: free *P. fluorescens* lipase, Po free: free porcine pancreas lipase and Wh free: free wheat germ lipase)

3.2 Effect of process parameters on FAME conversion

The optimization of reaction parameters for lipase-catalyzed transesterification of *S. obliquus* lipids was done using the Box-Behnken design of response surface methodology. All 27 experiments with response i.e. percentage FAME conversion are depicted in Table 4.2. Factors selected for optimization studies were catalyst amount (wt% with respect to oil weight), reaction temperature, water amount (wt% with respect to oil weight) and methanol to oil molar ratio. The factors and levels are described in section 2.3. Enzyme is added as a weight percentage with respect to oil, so that level optimized can be easily adopted for scaled-up conversion process at pilot or industrial scale. The coefficients were detected for full model using regression analysis. Based on the Box-Behnken design and results obtained from the experiments, the quadratic polynomial equation (Eq. 3) was obtained using multiple regression analysis. The equation with actual variables was:

R1 = 12.34 + 7.24 catalyst amount (A) – 10.49 temperature (B) – 2.61 water amount (C) – 8.74 methanol to oil molar ratio (D) + 2.95 catalyst amount^2 (A^2) + 8.03 temperature ^{2} (B^2) – 0.17 water amount ^{2} (C^2) + 4.49 methanol to oil molar ratio^2 (D^2) – 4.5 catalyst amount × temperature (AB) – 1.09 catalyst amount × water amount (AC) – 3.41 catalyst amount × methanol to oil molar ratio (AD) + 5.71 temperature × water amount (BC) + 14.53 temperature × methanol to oil molar ratio (BD) + 3.88 water amount × methanol to oil molar ratio (CD) (Eq. 3)

R1 was the observed response (percentage FAME conversion) of lipase catalyzed transesterification of *S. obliquus* lipids. A, B, C and D are the coded values of independent factors catalyst amount (wt% with respect to oil weight), reaction temperature, water amount (wt % with respect to oil weight) and methanol to oil molar ratio, respectively.

Table 4.2: Box-Behnken model results for reaction condition optimization.

Exp. No.	Coded values of reaction variables				FAME Conversion (%)
	Enzyme concentration (A)	Temperature (B)	Water content (C)	MeOH: Oil Molar ratio (D)	
1	0	1	0	-1	7.62
2	1	-1	0	0	53.23
3	0	-1	0	1	15.51
4	-1	0	0	-1	12.06
5	0	0	-1	1	8.74
6	-1	0	0	1	8.61
7	1	0	1	0	18.03
8	0	1	-1	0	10.27
9	1	1	0	0	13.75
10	-1	0	-1	0	12.48
11	1	0	0	1	15.27
12	1	0	0	-1	32.39
13	0	1	1	0	22.17
14	0	1	0	1	14.70
15	0	0	0	0	12.21
16	0	0	1	-1	19.79
17	0	0	1	1	7.39
18	0	-1	1	0	13.31
19	1	0	-1	0	27.26
20	-1	0	1	0	7.63
21	-1	1	0	0	5.37
22	0	-1	0	-1	66.55
23	0	0	-1	-1	36.65
24	0	0	0	0	12.46
25	0	-1	-1	0	24.26
26	0	0	0	0	12.35
27	-1	-1	0	0	26.87

MeOH- Methanol

Table 4.3: Analysis of variance (ANOVA) for response surface quadratic model.

	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	4549.92	4549.92	324.99	5.27	0.003
Linear	4	2947.38	2947.38	736.85	11.95	<0.001
Enzyme amount (A)	1	629.52	629.52	629.52	10.21	0.008
Temperature (B)	1	1319.87	1319.87	1319.87	21.41	0.001
Water content (C)	1	81.89	81.89	81.89	1.33	0.272
MeOH: Oil Molar ratio (D)	1	916.09	916.09	916.09	14.86	0.002
Square	4	435.22	435.22	108.8	1.76	0.201
Interaction	6	1167.32	1167.32	194.55	3.16	0.043
Residual Error	12	739.81	739.81	61.65		
Lack-of-Fit	10	739.78	739.78	739.78	4774.89	<0.001
Pure Error	2	0.03	0.03	0.02		
Total	26	5289.73				

R-Sq = 86.01% (DF – degree of freedom; Seq. SS – sequential sum of squares; Adj. SS – adjusted sum of squares; Adj. MS – adjusted mean of squares; F – probability distribution; p - probability)

The statistical significance of the designs was determined by the F-test for analysis of variance (ANOVA) which is depicted in Table 4.3. The ANOVA analysis indicates that the quadratic polynomial equation (Eq 3) was significant and represents a definite relationship between the response and variables with p value (0.003) less than 0.05 and agreeable coefficient of determination (R-Sq = 86.01%). The model terms are significant when the “Prob > f” values are less than 0.05. Thus, from the Table 4.3, it is evident that the most significant factor was temperature (0.001), followed by methanol to oil molar ratio (0.002) and catalyst amount (0.008). The water content in the reaction mixture was not found to significantly influence the

biodiesel conversion (0.272). Among the interactions of parameters, only temperature and methanol to oil molar ratio interaction (BD) showed significant influence (0.003).

The FAME conversion of experimental runs (Exp. No. 1-27) with various levels of selected factors is given in Table 4.2. Highest biodiesel conversion (66.55%) was achieved from *S. obliquus* lipids at 35°C, methanol to oil ratio of 3:1 with 10% enzyme amount and 2.5% water content based on oil weight (Exp. No. 22). Enzyme catalysts are known to be specific and their activity is greatly influenced by the reaction parameters like temperature and inhibitory reactants. In the experimental runs by RSM, response depends on the collective influence of selected parameters. Despite this, highest FAME conversion achieved at 35°C was 66.55%, which is greater than that achieved at 45°C (36.65%) and 55°C (22.17%). This clearly indicated that beyond the optimum temperature of 35°C the increase in temperature led to decrease in *P. fluorescens* lipase activity. Bacterial lipases are known to perform better in the temperature range 30-60°C (Gupta et al., 2004). With increase in temperature, activity of lipase also increases, but after a certain point due to enzyme denaturation, the activity declines. The optimum reaction temperature was determined to be 35°C, which is moderate and makes the conversion process less energy intensive. Similarly, with the increase in methanol to oil molar ratio, the percentage of FAME conversion declined. Highest FAME conversions achieved at selected molar ratios were 66.55% at 3:1 and 53.23% at 4:1, which sharply declined to 15.51% at 5:1. Stoichiometrically, for transesterification of oils, three moles of methanol are required. Presence of excessive methanol in reaction mixture severely affects the enzyme conformation causing loss of activity. The amount of catalyst is directly proportional to product conversion. However, it can greatly influence the economics of process. With the 5% lipase in reaction mixture based on oil weight, highest FAME conversion achieved was 26.87%, while at 10% and 20% lipase

amount this increased to 66.55% and 53.23%, respectively. Thus, 10% lipase was chosen as the optimum. From the ANOVA results, it was observed that water amount did not influence the biodiesel conversion significantly. Hence 2.5% water content based on lipid weight, which yielded the highest FAME conversion, was chosen as optimum.

The lowest FAME conversion achieved was 5.37% (Exp. No. 21) at 55°C (methanol to oil molar ratio of 4:1 with 5% enzyme amount and 2.5% water content based on oil weight). In this experiment (Exp. No. 21), the most influencing parameters like temperature, methanol to oil molar ratio and enzyme amount deviated from the optimum levels. RSM experiments and results allowed determination of optimum levels of selected parameters for *P. fluorescens* lipase catalyzed biodiesel conversion of *S. obliquus* lipids. Statistical analysis also highlighted the most influencing parameters, which can form the basis for a scaled-up process scheme.

3.3 Effect of step-wise methanol addition

Stoichiometrically, three moles of methanol are required for complete conversion of feedstock lipids to FAMEs. Methanol is known to inhibit lipase activity if present in excessive amounts. Thus, step-wise addition of methanol was applied in this study to improve the conversion efficiency. Three schemes as described in section 2.6 were investigated for FAME conversion. In scheme one (S1), one mole of methanol was added at 0, 2 and 4 h of the reaction, while in scheme two (S2), one mole of methanol was added at 0, 3 and 6 h of reaction. In scheme three (S3), two moles of methanol were added at 0 h followed by one mole at 4 h. In the control experiment, methanol was not added in step-wise manner. Highest FAME conversion of 90.81 ± 1.61% was achieved in reactions which followed scheme two of methanol addition (Figure 4.2). The FAME conversion achieved in scheme one was 80.74 ± 0.89% and in scheme

three was 75.25 ± 0.61% (Figure 4.2). The FAME conversion varied depending upon the dose and time of methanol addition. Lower FAME conversion in scheme one and three could be because of presence of excess methanol present in the beginning of reaction. In scheme one (S1), two mole of methanol were added within 2 h of reaction since its commencement. While, in scheme three (S3), two mole of methanol were present at the onset of reaction. Shimada et al. (1999) studied the conversion of vegetable oil catalyzed by *Candida antarctica* lipase using step-wise methanol addition. In their study, with a three step methanol addition scheme 98.4% FAME conversion was achieved. Step-wise methanol addition controls the amount of methanol in the reaction mixture. In this way, inhibition caused by excessive insoluble methanol can be controlled. Step-wise addition of methanol ensures operational stability to the lipase and thus results in a higher conversion.

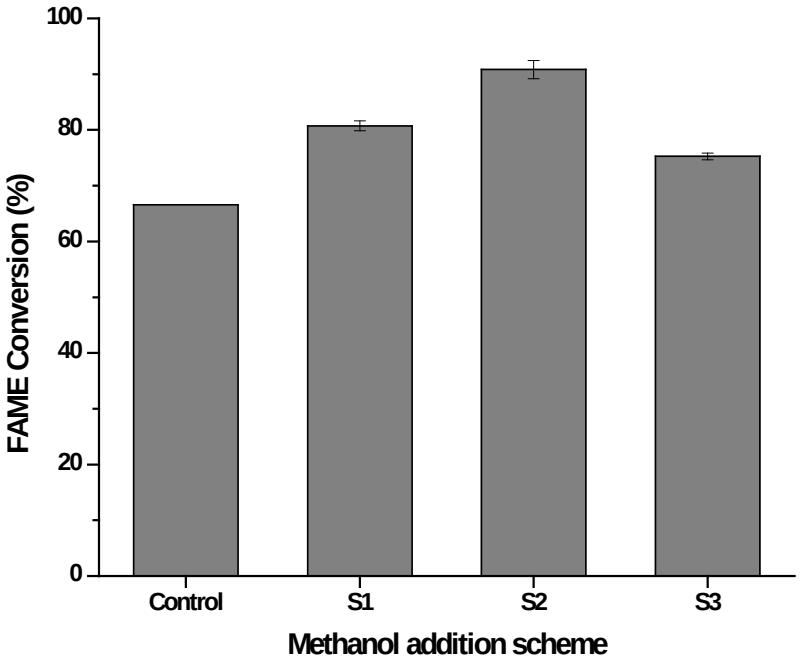


Figure 4.2: FAME conversion obtained after applying step-wise methanol addition schemes.

3.4 Reusability of lipase

Immobilized *P. fluorescens* lipase was separated from the reaction mixture and reused with optimized parameters and optimal step-wise methanol addition scheme, in repeated batches. FAME conversion of 86.75% was achieved for the fourth batch of reaction (Figure 4.3).

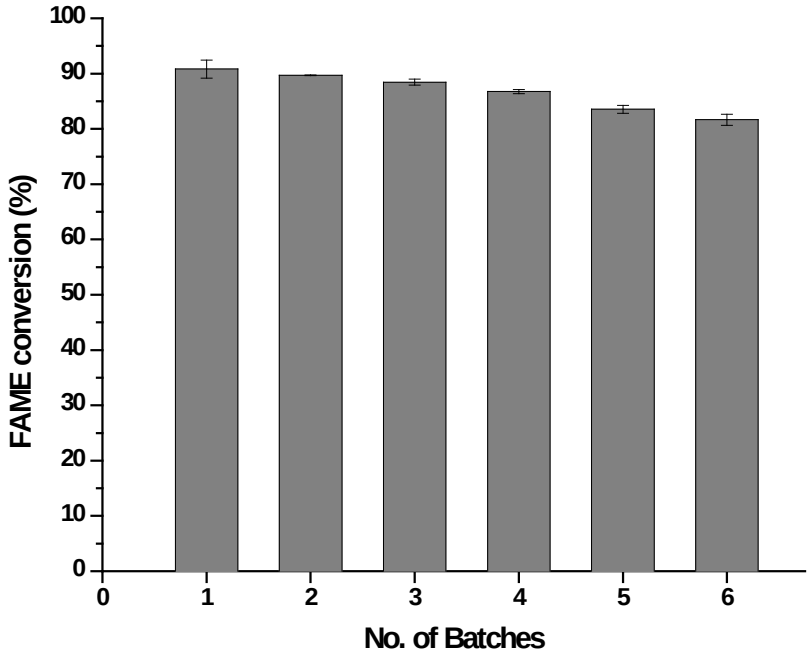


Figure 4.3: FAME conversion obtained after reusing *P. fluorescens* lipase.

After batch number five and six, FAME conversions obtained were 83.55 and 81.65% respectively. The FAME conversion (86.75%) achieved in fourth batch of reaction was over 95% relative to conversion achieved in first batch (90.81%), while FAME conversions of batch number five and six were less than 95% relative to conversion achieved in first batch. Thus, from the reusability experiments it was concluded that immobilized lipase has shown stability and relatively high FAME conversion upto four repeated uses. However, lipase can be used

repeatedly for more batches of reaction but at the cost of compromised product quality. Lipase activity is known to decrease after repeated use. This is due to a number of reasons like long exposure to methanol and hexane, conformational changes in lipase and leaking of enzyme from the support material. Reuse of the lipase catalyst can greatly reduce the cost of biodiesel conversion towards a more economically feasible process.

3.5 Fuel properties of biodiesel

Biodiesel is considered as suitable for use in compression ignition engines, only if it complies with the specifications set by agencies like ASTM (American Society for Testing and Materials) and EN (European norms, European Committee for Standardization) (Atadashi et al., 2012). Fuel properties of biodiesel are influenced by number of factors such as feedstock quality, fatty acid composition of feedstock, conversion technique and post production steps. Fatty acid composition of feedstock influences properties like oxidative stability, cetane number and cold flow properties. High saturation in fatty acids favours oxidative stability and desirable cetane number, while it is considered unsuitable for cold flow properties. High unsaturation in fatty acids is considered good for cold flow properties; on the other hand, it is not suitable for oxidative stability. The fatty acid methyl ester composition of *S. obliquus* biodiesel synthesized by *P. fluorescens* lipase catalysis is shown in Table 4.4. *S. obliquus* biodiesel contained 34.9% saturated fatty acids and 64.06% unsaturated fatty acids, while degree of unsaturation and long chain saturated factor were 110.19 and 6.81, respectively.

Table 4.4: Fatty acid methyl ester composition of *S. obliquus* biodiesel

FAME	**Composition (%)**
Myristic acid methyl ester (C14:0)	0.79
Palmitic acid methyl ester (C16:0)	30.44
Palmitoleic acid methyl ester (C16:1)	6.89
Stearic acid methyl ester (C18:0)	1.78
Oleic acid methyl ester (C18:1)	13.34
Linoleic acid methyl ester (C18:2)	17.60
Linolenic acid methyl ester (C18:3)	11.17
Arachidic acid methyl ester (C20:0)	0.67
Eicosenoic acid methyl ester (C20:1)	4.86
Eicosadienoic acid methyl ester (C20:2)	3.77
Behenic acid methyl ester (C22:0)	0.46
Erucic acid methyl ester (C22:1)	3.41
Docosadienoic methyl ester (C22:2)	3.01
Lignoceric acid methyl ester (C24:0)	0.76
Total saturated fatty acids methyl esters	34.90
Total unsaturated fatty acid methyl esters	64.06

The properties of *S. obliquus* biodiesel synthesized by *P. fluorescens* lipase catalysis observed are shown in Table 4.5, along with American biodiesel standard (ASTM 6751) and European biodiesel standard (EN14214). *S. obliquus* biodiesel showed a cetane number value of 51.77, which is higher than the specified standards. High cetane number suggests that the biodiesel has efficient combustion properties (Sinha et al., 2008). Calorific value is important fuel property which determines the energy content and forms basis for comparison with petroleum fuel. Calorific value of *S. obliquus* biodiesel was found to be 37.67 $MJ.kg^{-1}$, which is comparable to petroleum diesel (44.8 $MJ.kg^{-1}$) (Sinha et al., 2008).

Table 4.5: Properties of *S. obliquus* biodiesel via extracellular enzyme catalysis

Biodiesel characteristics	**Units**	***S. obliquus* Biodiesel**	**ASTM 6751**	**EN 14214**
Cetane number	-	51.77	min 47	min 51
Calorific value	$MJ.kg^{-1}$	37.67	-	-
Density	$kg.m^{-3}$	877	860-900	860-900
Methyl ester content	%	90.81	-	min 96.5
Linolenic acid methyl ester content	%	11.17	-	max 12
Acid value	$mgKOH.g^{-1}$	0.42	max 0.8	max 0.5
Iodine number	$g100.g^{-1}$	98.68	-	max 120
Cold filter plugging point (CFPP)	°C	4.9	-	-
oxidative stability	h	3.54	min 3	min 6
Carbon	wt%	78.11	-	-
Hydrogen	wt%	13.53	-	-
Sulfur	wt%	<0.001	max 0.05	-

Acid value observed was 0.42 $mgKOH.g^{-1}$ which is well within the specified limits of ASTM (0.8) and EN (0.5) standards. This suggests that despite the high FFA content in microalgal lipids, enzyme catalysts are efficient enough to convert these FFAs to biodiesel. Linolenic acid methyl ester content as specified by EN14214 standards should be lower than the 12%. Linolenic acid (C18:3) is highly unsaturated fatty acid with 3 double bonds, which if present in higher amounts can lead to biodiesel rancidity. *S. obliquus* biodiesel showed linolenic methyl ester content of 11.17% which is well within the specifications. Methyl ester content in *S. obliquus* biodiesel is 90.81% which is lower than the specified limits (96.5%) by EN14214 standards, which needs further improvement through efficient conversion. Sulfur content is important for biodiesel characterization as well as environmental concerns (Li et al., 2011c). *S. obliquus* biodiesel has not shown detectable sulfur content. Low sulfur content ensures reduced SOx emission from engines. At low temperatures, biodiesel can cause plugging of filters and lines.

EN14214 does not specify limits for cold filter plugging point, but each country can specify limits depending on time of year and climate (Ramos et al., 2009). The biodiesel prepared in this study showed a cold filter plugging point of 4.9 °C, which is due to the higher content of saturated fatty acids. Oxidation stability ensures stability of biodiesel during prolonged storage, which otherwise leads to its deterioration. Oxidation stability of *S. obliquus* biodiesel obtained via extracellular enzyme catalysis is 3.54 h, which is higher than the ASTM specifications (min 3 h). Chen et al. (2012) in their study to characterize *Scenedesmus* sp. biodiesel found an acid value of 0.52 mgKOH.g^{-1}, sulfur content 0.02%, methyl ester content 91% and oxidative stability 5.42 h. Most of the fuel properties of biodiesel obtained from *S. obliquus* via extracellular enzyme catalysis comply with the specified ASTM standards and EN standards.

4. Conclusions

Immobilized *P. fluorescens* lipase showed better catalytic efficiency among the selected lipases from various source. Biodiesel was synthesized using *S. obliquus* lipids catalyzed by immobilized *P. fluorescens* lipase. Microalgal feedstock and lipase as biocatalyst makes biodiesel synthesis sustainable and greener. Response surface methodology was undertaken to efficiently optimize reaction parameters for immobilized *P. fluorescens* lipase-catalyzed biodiesel synthesis. Improved biodiesel conversion of 90.81% was obtained at 35°C, with 10% enzyme amount, 2.5% water content based on oil weight and methanol to oil ratio of 3:1, where methanol is added in three steps. Immobilized *P. fluorescens* lipase can be reused for the four repeated batches without much loss in relative FAME conversion (> 95%) of the initial batch. Most of the fuel properties of biodiesel comply with the ASTM 6751 and EN 14214 specifications for standard biodiesel.

CHAPTER FIVE: WHOLE CELL LIPASE APPLICATION

1. Introduction

Lipase-catalyzed microalgal biodiesel production has been associated with several advantages such as low energy requirements, pure quality products and minimal wastewater generation. Lipases are capable of catalyzing both esterification of free fatty acids and transesterification of triglycerides (Fjerbaek et al., 2009). This gives lipase catalysts an advantage over conventional chemical catalysts while using high free fatty acid containing feedstock lipids from microalgae, avoiding environmental and corrosion concerns (Fjerbaek et al., 2009, Robles-Medina et al., 2009). Several researches have investigated application of extracellular lipases for biodiesel production. Extracellular lipases from *Candida* sp., *Pseudomonas* sp., *Thermomyces lanuginosus* and *Rhizomucor miehei* have been employed in the biodiesel synthesis (Kaieda et al., 2001, Torres et al., 2002, Watanabe et al., 2000, Xu et al., 2003).

However, industrial application of commercial immobilized lipases is still limited, due to its high cost (Helwani et al., 2009, Robles-Medina et al., 2009). Whole cell lipase application, where microbial cells producing lipases are directly applied as a catalyst, is gaining interest. In whole cell application, the extraction and purification steps involved in commercial lipase production are avoided. Thus, enzyme catalysts costs are greatly reduced in this approach. Moreover, simple cultivation and immobilization of whole cell catalysts could be implemented at large scale production plants, and whole cell catalysts can be easily prepared in large amounts. Several lipase-producing microorganisms such as bacteria, fungi and yeasts have been applied as

whole cell catalysts for conversion of vegetable oils (Ban et al., 2002, Sun et al., 2011, Zeng et al., 2006). Whole cell lipases can be easily immobilized into biomass support particles (BSP). *Rhizopus oryzae* whole cell lipase immobilized into polyurethane foam as BSP was studied by several researchers for conversion of vegetable oils to biodiesel (Li et al., 2007, Zeng et al., 2006). Cells of *Rhizopus* sp, *Aspergillus* sp., *Rhizomucor* sp., and *Candida* sp. are robust and have been successfully used for lipase production (Ellaiah et al., 2004, Kunigo et al., 2013, Liu et al., 2014).

Immobilization of whole cells provides stability as well as improves reusability. Packaging material like polystyrene can be used as the biomass support particle because of its chemical stability and mechanical strength (Gautam et al., 2002, Li et al., 2010b). Lipase activity is inhibited by the presence of alcohols in the reaction mixture. Methanol added as an acyl acceptor causes lipase activity inhibition. Therefore, step-wise addition of methanol and use of solvent as the reaction medium has been suggested by researchers to overcome this constraint. There are very few studies where whole cell lipases have been applied for conversion of microalgal lipids to biodiesel. Xiao et al., (2010) studied conversion of lipids from marine and fresh water microalgae using locally-isolated fungus as the whole cell catalyst. They found higher biodiesel yield from marine microalgae (68.2%) compared to fresh water microalgae (50.3%) when whole cell catalysts were applied. They also observed that the whole cell fungus showed higher yields when n-hexane was used as reaction solvent compared to tert-butanol. Recently, Surendhiran et al., (2014) investigated the conversion of *Chlorella salina* lipids employing *Rhodotorula mucilaginosa* MTCC8737 yeast as whole cell catalyst immobilized on sugarcane bagasse chips. In their study, with methyl acetate as acyl acceptor, they observed

85.29% biodiesel yield at optimum conditions. Applying whole cell lipase catalyst for conversion of microalgal lipids is a novel approach which needs more investigation.

In this chapter, the application of whole cell lipase from *Aspergillus* sp. DUT01 and *Candida* sp. DUT04 was studied for conversion of microalgal lipids to biodiesel. *Scenedesmus obliquus* grown in open circular ponds was selected as the microalgal feedstock. Immobilized and free whole cells from selected sources were screened for their catalytic performance. Selected fungal strain was identified using sequence based identification method using ribosomal internal transcribed spacer region (Balajee et al., 2009). Selected whole cell lipase was chosen for optimization of reaction parameters using response surface methodology (RSM). A three level four factor Box-Behnken experiment design was employed for response surface methodology optimization. The effect of various reaction parameters on the whole cell lipase-catalyzed biodiesel conversion was also studied. Step-wise addition of methanol was investigated to observe further improvement in the biodiesel conversion. Reusability of selected whole cell lipase was also evaluated. The eventual biodiesel product was characterized for fuel properties and compared to standard specifications.

2. Material and methods

2.1 Chemicals and reagents

Aspergillus sp. DUT01 and *Candida* sp. DUT04 were obtained from the Department of Biotechnology and Food Technology, Durban University of Technology. A mixed fatty acid methyl ester (FAME) standard (37 components) and methyl heptadeconoate were obtained from Sigma-Aldrich, USA. All organic solvents and other chemicals purchased from Sigma-Aldrich were of analytical grade. Cubes of polystyrene packaging material (5mm×5mm×5mm cuboids)

were used as biomass support particles (BSP). *Scenedesmus obliquus* lipids extracted using the microwave technique, as described in Chapter three, was used as microalgal feedstock lipids.

2.2 Cultivation of lipase-producing organisms and immobilization

Aspergillus sp. DUT01 and *Candida* sp. DUT04 were grown in basal medium described by Li et al., (2007). Composition of basal medium was 30 $g.L^{-1}$ vegetable oil, 70 $g.L^{-1}$ peptone, 1.2 $g.L^{-1}$ $NaNO_3$, 1.2 $g.L^{-1}$ KH_2PO_4 and 0.5 $g.L^{-1}$ $MgSO_4.7H_2O$. Cultures were grown in 500 ml flasks with culture volumes of 100 ml. Cultures were incubated at 35°C in reciprocal shaker with 100 rpm for 72 h with 100 pre-sterilized BSPs. For free whole cell preparation, cultures were incubated at the same conditions without addition of BSPs. After a 72 h cultivation period, BSPs were separated by filtration followed by washing with distilled water. For free whole cell lipase, mycelium was separated and washed with distilled water. Mycelium and BSPs were frozen in -84°C and dried in freeze drier for 8 h.

Weight of cell mass immobilized within BSPs was determined gravimetrically by measuring BSP weights prior to and after immobilization. Lipase activity of free and immobilized whole cell lipases was determined by the olive oil hydrolysis method (Pinsirodom and Parkin, 2001). Olive oil emulsion was prepared by mixing 10 g olive oil and 10 g gum arabic in 200 ml of 50 mM sodium phosphate buffer (pH 8). The reaction mixture consisted of 5 ml olive oil gum arabic emulsion 10 mg lyophilized free whole cell lipase or 5 BSPs in case of immobilized whole cell lipase. The hydrolysis was carried out at 30°C, 250 rpm for 20 min and the reaction was stopped by adding 95% ethanol. The reaction mixture without addition of enzyme was used as a blank. Hydrolyzed fatty acids were determined by titration with 0.1 N

NaOH, using phenolphthalein as the indicator. One unit of activity was expressed as the release of 1 µmol fatty acid per min in the reaction mixture.

2.3 Screening of whole cell lipases from different sources

Free and immobilized whole cell lipases (*Aspergillus* sp. DUT01 and *Candida* sp. DUT04) were used for transesterification of *S. obliquus* lipids. Reaction conditions were: temperature, 40°C; methanol to oil molar ratio, 5:1; water quantity, 5% of oil weight; number of BSPs, 5 for immobilized whole cell lipase and free whole cell lipase, 20% by oil weight (keeping the dry cell weight constant in immobilized and free form). For all conversion experiments 0.1g of *S. obliquus* lipids was used as feedstock. To provide proper mass transfer, the n-hexane was added to the reaction mixture. Samples were removed at regular interval from the reaction mixture and analyzed using gas chromatography to determine percentage fatty acid methyl ester (FAME) conversion as described in section 2.2 of Chapter four. Based on FAME conversion and time required, a suitable whole cell lipase was selected for further optimization studies.

2.4 Identification and phylogenetic characterization

The fungal isolate chosen for whole cell lipase production was identified by molecular techniques. DNA extraction was done by using the ZR fungal/bacterial DNA kit (Zymo Research) according to the manufacturer's instructions. Amplification of genomic DNA was done by using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (primers synthesized by Inqaba Biotechnical Industries (Pty) Ltd South Africa) (Balajee et al., 2009, Magnani et al., 2005, White et al., 1990). PCR products were sequenced by Inqaba. Assessment for similar sequences was carried out

using the BLAST (Altschul et al., 1990). The nucleotide sequences were aligned in CLUSTAL X (www.clustal.org) and edited using BioEdit (version 7.1) and exported to MEGA5.10 for evolutionary analysis. A phylogenetic tree was constructed from the alignment and bootstrap analysis was performed using 1000 replicates by the neighbour-joining method (Saitou and Nei, 1987). The nucleotide sequence was submitted to GenBank (NCBI).

2.5 Optimization of process parameters

Optimization was carried out employing a three-level, four-factorial Box-Behnken response surface methodology experimental design, using Minitab Statistical Software. Experimental design comprised of 27 experiments with 3 replicates at centre point. Optimization of reaction conditions for whole cell lipase-catalyzed transesterification of *S. obliquus* lipids was done taking FAME conversion (%) as the response. The experimental design consisted of four factors: reaction temperature, methanol to oil molar ratio, water amount (wt% with respect to oil weight) and number of BSPs. The range of three levels for factors was selected, based on the findings reported by Li et al (2007). Their optimization study for *Rhizopus oryzae* whole cell catalyzed biodiesel production from soybean oil took methanol to oil molar ration in the range 3:1 to 7:1 and water content in the range 1 to 5 wt% with respect to oil weight. The temperature optimized for whole cell lipase catalyst in previous studies was in the range of 30 to 50°C (He et al., 2008, Li et al., 2007, Xiao et al., 2010).

The three levels of variables for the selected four factors were: reaction temperature (35, 45 and 55°C), methanol to oil molar ratio (3:1, 5:1 and 7:1), water amount (1, 2.5 and 5 wt% with respect to oil) and number of BSPs (4, 5 and 6) in whole cell lipase catalyzed transesterification (Table 5.1). The reaction time of 36 h and agitation speed of 200 rpm were

kept constant for transesterification. The effect of independent factors on the dependent factors was analyzed by the following quadratic equation:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4 + e \quad \text{(Eq. 1)}$$

where, Y is the response (biodiesel conversion, %), a_0 is offset term; a_1, a_2, a_3 and a_4 are linear coefficients; a_{11}, a_{22}, $a_{33}$and a_{44} are the squared term coefficients; a_{12}, a_{13}, a_{14}, a_{23}, a_{24} and a_{34} are the interaction coefficients; and e is the error. X_1, X_2, X_3 and X_4 are reaction temperature, methanol to oil molar ratio, water amount and number of BSPs respectively.

Table 5.1: Factors and levels for experimental design

Factors	Temperature (°C)	MeOH: Oil Molar ratio	Water content (wt% with respect to oil weight)	Number of BSPs
Level 1	-1 (35)	-1 (3:1)	-1 (1)	-1 (4)
Level 2	0 (45)	0 (5:1)	0 (2.5)	0 (5)
Level 3	1 (55)	1 (7:1)	1 (5)	1 (6)

2.6 Step-wise addition of methanol and reusability study

To overcome methanol inhibition, step-wise addition of methanol was applied with optimized reaction parameters. Methanol was added using two different schemes. In scheme one, out of five moles, two moles of methanol were added at the beginning of reaction followed by 1.5 moles each after 12 h and 18 h. In scheme two, three moles of methanol was added at the start of the reaction and two moles after 12 h. For the reusability study, immobilized whole cell lipase was separated from the reaction mixture by filtering and then subsequently washing with n-hexane and distilled water. Recovered BSPs were used with optimized parameters and the

selected scheme of step-wise methanol addition. A similar procedure was followed for subsequent batches of reaction. Reactions were carried out for 36 h and samples were analyzed by GC for percentage FAME conversion.

2.7 Characterization of biodiesel

Biodiesel prepared from *S. obliquus* via whole cell lipase catalysis was characterized for its FAME profile and fuel properties. Properties like density, ester content, linolenic acid content and acid value (ASTM D664-07) was determined by standard methods and elemental analysis was done using elemental analyzer (Vario EL, Elementar Germany). Calorific value was determined by using calorimeter (Drycal modular calorimeter, Energy instrumentation). The FAME profile and percentage composition was used to detect other fuel properties. Properties like cetane number, iodine value and oxidative stability were determined using correlation equations given by Wang et al. (2012) and cold filter plugging point (CFPP) was calculated by the formula given by Ramos et al. (2009) as described in section 2.5 of Chapter four. Properties of FAME product were compared with the ASTM and EN standards for its suitability as biodiesel.

3. Results and discussion

3.1 Catalytic properties of whole cell lipase

Catalytic properties of free and immobilized whole cell lipase are listed in Table 5.2. Amount of cells immobilized on BSP was determined gravimetrically. It was found that for both *Aspergillus* sp. DUT01 and *Candida* sp. DUT04, weight of dry cells immobilized on BSP were almost similar i.e., 4 and 3.9 $mg.BSP^{-1}$, respectively. Hydrolytic activity was determined by the olive oil hydrolysis method. *Aspergillus* sp. DUT01, whether free or immobilized, has higher activity than *Candida* sp. DUT04.

Table 5.2: Catalytic properties of whole cell lipases

Whole cell lipase	Immobilized cell weight on BSP (mg/BSP)	Hydrolytic activity (IU/g dry cells)	Hydrolytic activity (IU/BSP)
Aspergillus sp. DUT01 immobilized	4	137.5	0.55
Candida sp. DUT04 immobilized	3.9	115.39	0.45
Aspergillus sp. DUT01 free	-	177.5	-
Candida sp. DUT04 free	-	132.5	-

3.2 Selection of suitable whole cell lipase

Free and immobilized whole cell lipases from *Aspergillus* sp. DUT01 and *Candida* sp. DUT04 were screened for their catalytic performance to synthesize biodiesel. Lyophilized mycelium from *Aspergillus* sp. DUT01 and *Candida* sp. DUT04 were used as free whole cell lipases. Biomass support particles (BSPs) with immobilized cells of *Aspergillus* sp. DUT01 and *Candida* sp. DUT04 were used as immobilized whole cells. BSPs with *Aspergillus* sp. DUT01 whole cell lipase has shown highest FAME conversion (29.05 ± 0.65%) followed by BSPs with *Candida* sp. DUT04 (27.43 ± 0.09%) in 48 h reactions (Figure 5.1). Both the free whole cell lipases have shown lower FAME conversion than the BSPs. Thus, immobilized *Aspergillus* sp. DUT01 whole cell was selected for optimization studies. While using immobilized whole cells as catalyst, the increase in percentage FAME conversion observed from 36 h to 48 h reaction time was not significant. All the optimization experiments were therefore carried out for 36 h. Molecular identification confirmed the selected fungal strain as *Aspergillus niger*. The BLAST (Basic local alignment search tool) results of the nucleotide sequence had shown 99% homology with *A. niger*. Nucleotide sequence of the strain was submitted to GenBank (Accession number: KJ949677). Taxonomic position of the strain was further confirmed by phylogenetic analysis. A

phylogenetic tree depicting close relationship of the strain to other *Aspergillus* strains was constructed. Rhizopus and corynobacterium were used as the out group. (Figure 5.2).

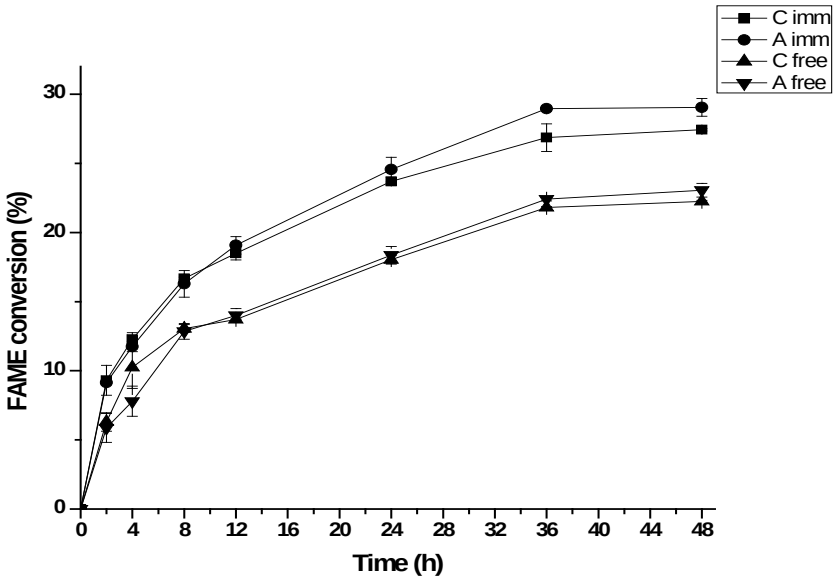


Figure 5.1: FAME conversion obtained after using immobilized and free whole cell lipases from different sources. (C imm: immobilized *Candida* sp. DUT04 whole cell lipase, A imm: immobilized *Aspergillus* sp. DUT01 whole cell lipase, C free: free *Candida* sp. DUT04 whole cell lipase and A free: free *Aspergillus* sp. DUT01 whole cell lipase)

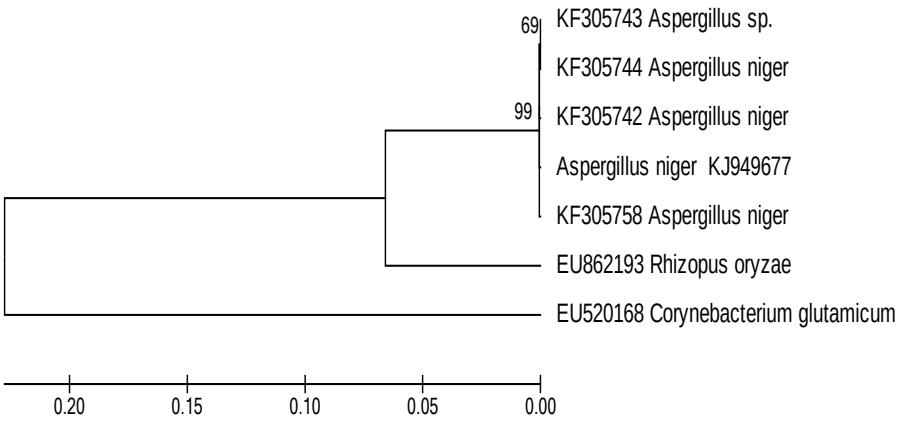


Figure 5.2: Phylogenetic tree confirm the close relationship of the *Aspergillus* strain used in this study to *Aspergillus niger** strain.

3.3 Effect of process parameters on FAME conversion

The optimization of reaction parameters for whole cell lipase-catalyzed transesterification of *S. obliquus* lipids was done using Box-Behnken design of response surface methodology. All 27 experiments with response i.e. percentage FAME conversion is listed in Table 5.3. Factors selected for optimization study were reaction temperature, methanol to oil molar ratio, water amount (wt% with respect to oil weight) and number of BSPs. The coefficients were detected for model using regression analysis. Based on the Box-Behnken design and results obtained from the experiments, the quadratic polynomial equation (Eq. 2) was obtained using multiple regression analysis. The equation with actual variables is:

R1 = 32.22 - 6.76 temperature (A) – 3.42 methanol to oil molar ratio (B) + 1.53 water amount (C) – 2.14 number of BSPs (D) + 0.49 temperature ^{2} (A^2) - 8.76 methanol to oil molar ratio^2 (B^2) +1.43 water amount ^{2} (C^2) + 3.11 Number of BSPs^2 (D^2) – 1.85 temperature x methanol to oil molar ratio (AB) – 2.03 temperature × water amount (AC) – 7.51 temperature × Number of BSPs (AD) + 1.20 methanol to oil molar ratio × water amount (BC) + 2.18 methanol to oil molar ratio × Number of BSPs (BD) + 7.11 water amount × Number of BSPs (CD) (Eq. 2)

where, R1 is the observed response (% FAME conversion) of whole cell lipase catalyzed transesterification of *S. obliquus* lipids. A, B, C and D are the coded values of independent factors i.e. reaction temperature, methanol to oil molar ratio, water amount (wt% with respect to oil weight) and number of BSPs respectively.

Table 5.3: Box-Behnken model results for reaction condition optimization.

Exp. No.	Coded values of reaction variables				FAME Conversion (%)
	Temperature (A)	MeOH: Oil Molar ratio (B)	Water content (C)	No. of BSPs (D)	
1	0	-1	1	0	30.64
2	0	-1	0	-1	28.49
3	-1	1	0	0	27.25
4	0	-1	-1	0	37.44
5	1	0	0	-1	38.41
6	-1	0	0	-1	39.41
7	1	0	1	0	25.46
8	1	1	0	0	14.58
9	0	0	-1	1	19.90
10	-1	-1	0	0	28.43
11	0	1	0	1	24.77
12	0	1	1	0	20.21
13	0	0	0	0	31.85
14	0	1	0	-1	21.28
15	0	1	-1	0	22.19
16	1	0	0	1	22.70
17	0	0	-1	-1	45.42
18	0	0	1	1	41.17
19	0	0	0	0	32.44
20	1	-1	0	0	23.19
21	0	0	1	-1	38.25
22	-1	0	1	0	45.15
23	-1	0	0	1	53.76
24	1	0	-1	0	22.97
25	0	-1	0	1	23.23
26	-1	0	-1	0	34.51
27	0	0	0	0	32.38

MeOH: Methanol

Table 5.4: Analysis of variance (ANOVA) for response surface quadratic model

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	1950.55	1950.55	139.325	4.87	0.005
Linear	4	774.22	774.22	193.554	6.76	0.004
Temperature (A)	1	549.53	549.53	549.53	19.19	0.001
MeOH: Oil Molar ratio (B)	1	141.1	141.1	141.1	4.93	0.046
Water content (C)	1	28.39	28.39	28.39	0.99	0.339
No. of BSPs (D)	1	55.2	55.2	55.2	1.93	0.19
Square	4	692.74	692.74	173.186	6.05	0.007
Interaction	6	483.59	483.59	80.599	2.81	0.06
Residual Error	12	343.65	343.65	28.637		
Lack-of-Fit	10	343.43	343.43	34.343	321.41	0.003
Pure Error	2	0.21	0.21	0.107		
Total	26	2294.2				

R-Sq = 85.02 % (DF – degree of freedom; Seq. SS – sequential sum of squares; Adj. SS – adjusted sum of squares; Adj. MS – adjusted mean of squares; F – probability distribution; p - probability)

The statistical significance of the designs was determined by F-test for analysis of variance (ANOVA) (Table 5.4). The ANOVA analysis specifies that the quadratic polynomial equation (Eq 3) was significant and represents definite relationships between the response and variables with p value (0.005) less than 0.05 and agreeable coefficient of determination (R-Sq = 85.02%). The model terms are significant when the “Prob > f” values are less than 0.05. Thus, from Table 5.4, it is evident that most significant factor was temperature (0.001), followed by methanol to oil molar ratio (0.046). While the water content (0.339) and number of BSPs (0.19) in reaction mixture were not found to significantly influence biodiesel conversion. Among other parameter interactions, only temperature and number of BSPs interaction (0.016) and water content and number of BSPs interaction (0.021) has shown significant influence.

The percentage FAME conversion of experimental runs (Exp. No. 1-27) with various levels of selected factors is shown in Table 5.3. Highest biodiesel conversion (53.76%) was achieved from *S. obliquus* lipids at 35°C, methanol to oil ratio of 5:1 and 2.5% water content based on oil weight with 6 BSPs (Exp. No. 23). Lipases activity is greatly influenced by reaction parameters like temperature and short chain alcohols. In the experimental runs for RSM, response depends on the collective influence of selected parameters. Despite this fact, highest FAME conversion achieved at 35°C was 53.76% which is greater than that achieved at 45°C (41.17%) and 55°C (38.41%). This clearly indicates that beyond the optimum temperature of 35°C, increase in temperature leads to decrease in the *A. niger* whole cell lipase activity. Temperature is an important parameter for lipase activity. High temperature can denature lipase conformation, while low temperature can slow down lipase activity. Talukder et al. (2013), in their study on *Aspergillus nomius* whole cell catalyst for palm oil biodiesel synthesis, found 40°C as the optimum temperature.

For transesterification of oils, stoichiometrically three moles of methanol are required. Presence of excessive methanol in reaction mixture severely affects the enzyme, resulting in loss of activity. Highest FAME conversions achieved at selected molar ratios were 30.64% at 3:1 and 53.76% at 5:1 which declined to 27.25% at 7:1. Thus, methanol to oil molar ratio of 5:1 was observed as the optimum. Talukder et al. (2013) in their study using *Aspergillus nomius* whole cell lipase, optimized methanol to oil molar ratio was also 5:1, where they stated that use of tert-butanol as reaction solvent minimizes methanol inhibition. In this study, n-hexane was used as reaction solvent, which could have aided in minimizing methanol inhibition of *A. niger* whole cell lipase.

From the ANOVA results it was observed that water amount and number of BSPs did not influence biodiesel conversion significantly. Hence, 2.5% water content based on lipid weight and 6 BSPs, which gave highest FAME conversion, was chosen as optimum reaction conditions. The lowest FAME conversion achieved was 14.58% (Exp. No. 8) at 55°C, methanol to oil molar ratio of 7:1 with 2.5% water content based on oil weight and 5 BSPs. In this experiment (Exp. No.8), most influencing parameters like temperature, methanol to oil molar ratio deviated from the optimum levels. RSM experiments and results yielded optimum levels of selected parameters for *A. niger* whole cell lipase catalyzed biodiesel conversion of *S. obliquus* lipids. Statistical analysis also highlighted the most influencing parameters which can form the basis for scaled-up process schemes.

3.4 Effect of step-wise methanol addition

Methanol is known to inhibit lipase activity if present in excessive amounts. Thus step-wise addition of methanol was applied in this study to improve the FAME conversion. Two schemes, as described in section 2.5, were investigated for FAME conversion. In scheme one (S1), two moles of methanol was added at the beginning and 1.5 moles each at 12 and 18 h of reaction, while in scheme two (S2), three moles of methanol was added at the beginning and two moles after 12 h of reaction. Figure 5.3 depicts the percentage FAME conversion achieved for the two different schemes of methanol addition as well as in the control where methanol was not added in a step-wise manner. Highest FAME conversion of 80.97 ± 0.51% was achieved in the reaction which follows scheme one of methanol addition. In scheme two, at the beginning of reaction three moles of methanol were present, thus could have resulted in the lower FAME conversion. Step-wise methanol addition controls the amount of methanol in the reaction mixture. In this way, inhibition caused by excessive insoluble methanol can be controlled. Step-

wise addition of methanol ensures operational stability to the whole cell lipase and thus results in higher conversions.

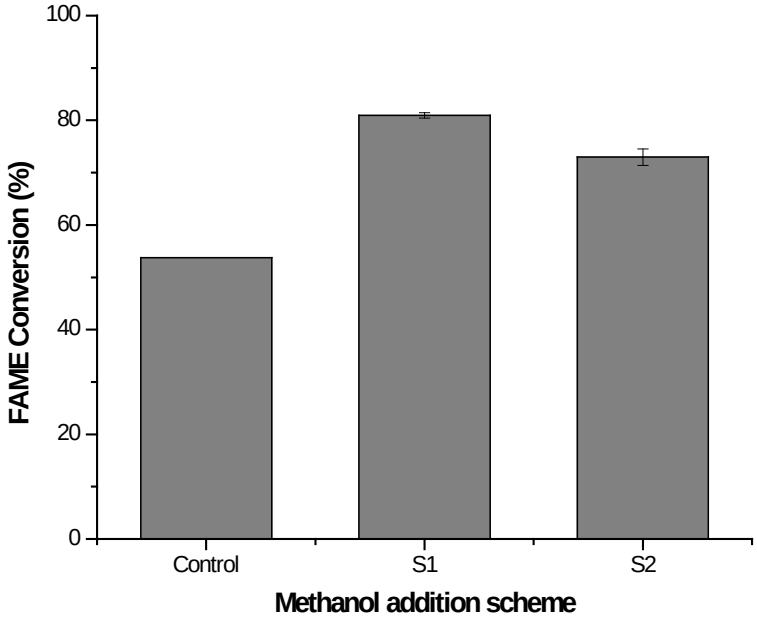


Figure 5.3: FAME conversion obtained after applying step-wise methanol addition schemes.

3.5 Reusability of whole cell lipase

Experiments were performed for investigating reusability and stability of *A. niger* whole cell lipase for FAME conversion. The BSPs were separated from the reaction mixture and reused with optimized parameters and the chosen step-wise methanol addition scheme, in repeated batches. The FAME conversion results for reuse of immobilized *A. niger* whole cell lipase for repeated batches are depicted in Figure 5.4. A FAME conversion of 76.76% was achieved for the second reaction batch. For the third batch, 68.83% FAME conversions was obtained. The FAME conversion obtained in second batch was 94.81% and in third batch was 85% relative to FAME

conversion achieved in first batch. Thus, from the reusability experiments it can be concluded that immobilized *A. niger* whole cell lipase has shown stability and relatively high FAME conversion for the second batch. Lipase activity is known to decrease after repeated use. This could be possible because of number of reasons like long exposure to methanol and hexane, conformational changes in lipase, and leaking of whole cell enzyme from the BSPs. Reuse of lipase catalyst can greatly influence the economics involved in biodiesel conversion towards a more economically feasible process.

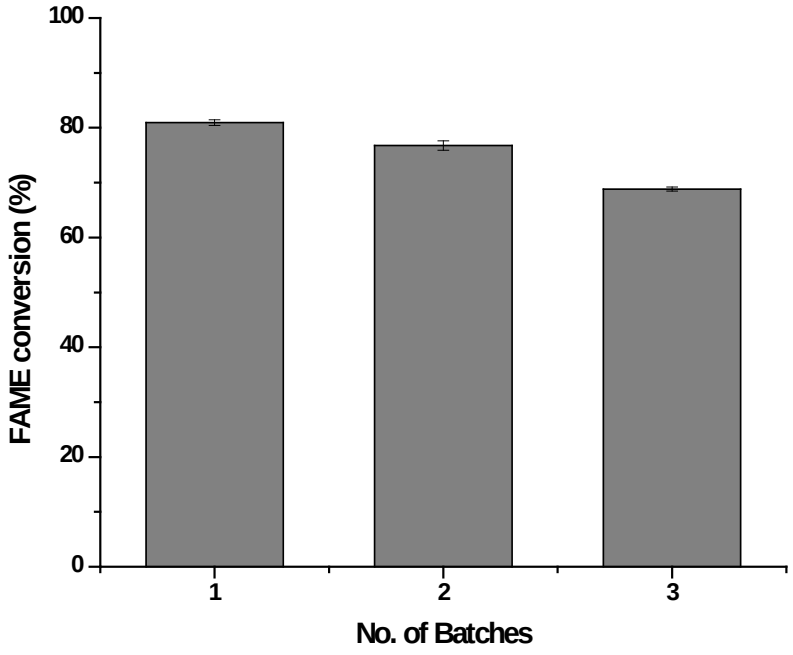


Figure 5.4: FAME conversion obtained after reusing *A. niger* whole cell lipase.

3.6 Fuel properties of biodiesel

Standard biodiesel specifications are set by agencies like ASTM (American Society for Testing and Materials) and EN (European norms, European Committee for Standardization) for its suitability to use in compression ignition engines (Atadashi et al., 2012). A number of factors such as feedstock quality, fatty acid composition of feedstock, conversion technique and post production steps influence the fuel properties of biodiesel. Degree of saturated and unsaturated fatty acids in fatty acid composition of feedstock influences properties like oxidative stability, cetane number and cold flow properties. High saturation in fatty acids is considered suitable for oxidative stability and desirable cetane number, while it is responsible for hampering cold flow properties. High unsaturation in fatty acids favours suitable cold flow properties. On the other hand, this is considered unsuitable for oxidative stability. The fatty acid methyl ester composition of *S. obliquus* biodiesel synthesized via whole cell catalysis, degree of unsaturation and long chain saturation factor are shown in Table 5.5. *S. obliquus* biodiesel synthesized via whole cell catalysis has 33.61% saturated fatty acids and 66.06% unsaturated fatty acids, while degree of unsaturation and long chain saturated factor were 110.46 and 6.35, respectively.

Table 5.5: Fatty acid methyl ester composition of *S. obliquus* biodiesel

FAME	**Composition (%)**
Myristic acid methyl ester (C14:0)	0.16
Palmitic acid methyl ester (C16:0)	30.79
Palmitoleic acid methyl ester (C16:1)	4.24
Stearic acid methyl ester (C18:0)	0.64
Oleic acid methyl ester (C18:1)	15.07
Linoleic acid methyl ester (C18:2)	14.95
Linolenic acid methyl ester (C18:3)	10.52
Arachidic acid methyl ester (C20:0)	0.86
Eicosenoic acid methyl ester (C20:1)	10.84
Eicosadienoic acid methyl ester (C20:2)	5.07
Behenic acid methyl ester (C22:0)	0.49
Erucic acid methyl ester (C22:1)	2.02
Docosadienoic methyl ester (C22:2)	3.35
Lignoceric acid methyl ester (C24:0)	0.68
Saturated fatty acids methyl esters	33.61
Unsaturated fatty acid methyl esters	66.06

The properties of the biodiesel produced are shown in Table 5.6, along with American biodiesel standard (ASTM 6751) and European biodiesel standard (EN14214). The cetane number value obtained was 51.74, which is higher than the specified standards. High cetane number is associated with efficient combustion properties of biodiesel (Sinha et al., 2008). Calorific value is important fuel property determining the energy content and suitability as transport fuel. Calorific value of *S. obliquus* biodiesel synthesized via whole cell catalysis was found to be 38.93 $MJ.kg^{-1}$, which is comparable to petroleum diesel (44.8 $MJ.kg^{-1}$) (Sinha et al., 2008).

Table 5.6: Properties of *S. obliquus* biodiesel via whole cell enzyme catalysis

Biodiesel characteristics	**Units**	***S. obliquus* biodiesel**	**ASTM 6751**	**EN 14214**
Cetane number	-	51.74	min 47	min 51
Calorific value	$MJ.kg^{-1}$	38.93	-	-
Density	$kg.m^{-3}$	869	860-900	860-900
Methyl ester content	%	80.97	-	min 96.5
Lenolenic acid methyl ester content	%	10.52	-	max 12
Acid value	$mgKOH.g^{-1}$	0.48	max 0.8	max 0.5
Iodine number	$g.100g^{-1}$	98.86	-	max 120
Cold filter plugging point (CFPP)	°C	3.5	-	-
oxidative stability	h	3.53	min 3	min 6
Carbon	wt%	65.36	-	-
Hydrogen	wt%	12.68	-	-
Sulfur	wt%	<0.001	max 0.05	-

Acid value obtained was 0.48 mg.KOH g^{-1}, which is well within the specified limits of ASTM (0.8) and EN (0.5) standards. *S. obliquus* lipids have high free fatty acid content. The acid value of biodiesel proved that whole cell lipase catalyst is efficient in converting these FFAs to biodiesel. Linolenic acid methyl ester content should be lower than the 12%, as specified by EN14214 standards. Higher amounts of linolenic acid (C18:3), which is a highly unsaturated fatty acid with 3 double bonds, can lead to biodiesel rancidity. *S. obliquus* biodiesel showed linolenic methyl ester content of 10.52%, which is well within the specifications. *S. obliquus* biodiesel has 80.97% methyl ester content, which is lower than the EN14214 specified limit (96.5%). Sulfur content in biodiesel is associated with environmental concerns (Li et al., 2011c). Low sulfur content ensures reduced SOx emission from the engines. *S. obliquus* biodiesel did not show detectable sulfur content. Low temperatures can cause plugging of filters and lines while using biodiesel. EN14214 does not specify limits for cold filter plugging point, but each country

can specify limits according to time of year and local climate (Ramos et al., 2009). The cold filter plugging point of biodiesel prepared in this study was found to be 3.5°C. Oxidation stability ensures stability of biodiesel which is important for long term storage, which otherwise leads to its deterioration. Oxidation stability observed for *S. obliquus* biodiesel obtained via whole cell lipase catalysis was 3.53 h, which is greater than the limit specified by ASTM (min 3 h) but lower than the EN14214 specifications (min 6 h). Chen et al. (2012) characterized *Scenedesmus* sp. biodiesel and found an acid value of 0.52 $mgKOH.g^{-1}$, sulfur content of 0.02%, methyl ester content of 91% and oxidative stability of 5.42 h. Fuel properties of biodiesel obtained from *S. obliquus* via whole cell lipase catalysis therefore comply with the specified ASTM standards and EN standards.

4. Conclusions

Immobilized *A. niger* as whole cell lipase showed higher catalytic efficiency compared to immobilized *Candida* sp. DUT04 whole cell lipase. Biodiesel was synthesized using *S. obliquus* lipids catalyzed by immobilized *A. niger* as whole cell lipase. Whole cell lipase application for conversion of microalgal lipids could alleviate cost concerns associated with commercial lipases. Response surface methodology was undertaken to efficiently optimize reaction parameters for immobilized *A. niger* whole cell lipase-catalyzed biodiesel synthesis. Improved biodiesel conversion of 80.97% was obtained at 35°C, methanol to oil ratio of 5:1 with 2.5% water content based on oil weight and 6 BSPs and where methanol is added in three steps. Immobilized *A. niger* whole cell lipase can be reused for two batches without much loss in relative FAME conversion (> 94%) of the initial batch. Most of the fuel properties of biodiesel comply with the ASTM 6751 and EN 14214 specifications for standard biodiesel.

CHAPTER SIX: COMPARISON AND TECHNO-ECONOMIC ANALYSIS OF ENZYMATIC AND CHEMICAL METHODS

1. Introduction

Biodiesel is considered as suitable renewable alternative for transport fuel. With the increase in fossil fuel prices and energy demand, biodiesel production is expected to grow higher (Chisti, 2007, Schenk et al., 2008). Microalgae has shown promising potential as a biodiesel feedstock. Transesterification of lipids to biodiesel in presence of methanol and suitable catalyst is the basic route for biodiesel production. Industrial scale biodiesel production plants are currently applying the conventional homogeneous chemical catalyst for conversion of feedstock lipids (Sotoft et al., 2010). Enzyme catalyst has shown several environmental and technological benefits, but its implementation at large scale is still not possible because of high cost and slow reaction rates. Several researchers are focusing on improving conversion as well as reducing cost of enzyme-catalyzed biodiesel production (Bajaj et al., 2010, Fjerbaek et al., 2009).

Lipase-catalyzed conversion of lipids operates at mild reaction conditions, yield high quality products and generates less wastewater (Bajaj et al., 2009). Immobilization of lipase can improve economics as it provides stability to enzyme as well as it can be reused. Extracellular commercial lipases are costly, thus application of lipase producing whole cells as catalyst is being investigated as a possible solution to reduce catalyst cost. Successful realization of lipases as a catalyst at industrial scale biodiesel production needs complete evaluation of performance, environmental impact and cost of lipase-catalyzed process (Fukuda et al., 2008, Ghaly et al., 2010).

In previous chapters, drying and extraction of microalgal lipids and conversion of microalgal lipids via extracellular and whole cell lipase catalysis has been studied. For drying, three techniques viz. freeze drying, oven drying and sun drying were studied. For lipid extraction, microwave and sonication techniques were investigated. For conversion of microalgal lipids using *Pseudomonas fluorescens* extracellular lipase and *Aspergillus niger* whole cell lipase, process parameters and step wise methanol addition were optimized.

In this chapter, extracellular and intracellular lipase-catalyzed conversions were compared with a conventional homogeneous chemical catalyst. Energy consumption and costs involved in these processes were also estimated. Similarly, drying and extraction processes studied were also compared in terms of energy consumption and costs involved. Challenges for the successful implementation of lipase-catalyzed conversion of microalgal lipids were also discussed.

2. Material and methods

2.1 Drying of microalgal biomass and extraction of lipids

Microalgae *S. obliquus* was cultivated in open circular pond under photoautotrophic mode of cultivation. Biomass was harvested by gravitational settling followed by centrifugation. Obtained wet microalgal biomass was dried by using three different drying techniques viz. freeze drying, oven drying and sun drying as described in section 2.3, Chapter three. Lipids were extracted from dried biomass by microwave and sonication assisted solvent extraction as described in section 2.4 Chapter three. Three drying and two extraction methods were compared based on energy consumption and process cost.

2.2 Conversion of microalgal lipids by enzymatic methods

S. obliquus lipids were converted to biodiesel via enzyme-catalyzed conversion. Enzyme catalysts were applied in extracellular as well as intracellular (whole cell) mode of application. In Chapter four, immobilized *P. fluorescens* lipase was found to be a suitable extracellular lipase for microalgal lipid conversion. Process parameters and a methanol step-wise addition scheme were optimized as described in Chapter four. In Chapter five, *A. niger* whole cells immobilized within BSPs were chosen as suitable whole cell lipase, process parameters and methanol step wise addition scheme was optimized. Conversion of *S. obliquus* lipids was carried out using extracellular lipase as described in Chapter four and for whole cell lipase as described in Chapter five. FAME conversion was determined by gas chromatography analysis, as described in Chapter four. After reaction completion one ml of n-hexane and one ml distilled water was added and the reaction mixture is allowed to separate into organic and aqueous layers. The organic n-hexane layer containing FAMEs was separated from the reaction mixture while the aqueous layer contained unreacted methanol and other debris. Immobilized lipases in both extracellular and intracellular application were allowed to settle, separated and washed with n-hexane and distilled water for their reuse. Solvents were removed from the organic layer, separated from the reaction mixture and FAMEs were measured gravimetrically. The FAME yield was calculated by using the following formula:

$$\text{FAME yield (\%)} = \frac{\text{Weight of FAME}}{\text{Weight of lipids}} \times 100 \qquad \text{(Eq 1)}$$

2.3 Conversion of microalgal lipids by the chemical method

Microalgal lipids are also subjected to conventional homogeneous acid catalyzed biodiesel conversion. Because of high free fatty acid content in microalgal lipids, sulphuric acid was chosen as the catalyst as it is able to catalyze simultaneous esterification and transesterification. The reaction conditions were: *S. obliquus* lipids: 0.1g, methanol to oil molar ratio of 30:1; temperature: 60°C; catalyst concentration: 10% w/w of oil; time: 4 h; n-hexane: 1ml (D'Oca et al., 2011). The rate of stirring was kept constant at 200 rpm in an orbital shaker incubator (Model TU-454, mrc Ltd.). The FAME conversion was determined as described in Chapter four and FAME yield as per Eq. 1, given in this chapter.

2.4 Energy input and cost estimation for various methods

Flow sheets were drawn for extracellular lipase, whole cell lipase and homogeneous acid catalyzed biodiesel synthesis from microalgal lipids. Various steps involved, instrumentation required and process parameters were considered, while conducting techno-economic analysis. Process flow sheets were used to calculate energy and cost of process as well as to compare the processes by technological aspects.

Energy input for drying, extraction and conversion techniques was calculated using the following equation**:**

$$E\ (kW) = P \times t / 1000 \qquad \text{(Eq. 2)}$$

where, P is the power (W) consumption of all the instruments used, t the operating time (h) of the instruments.

Cost analysis was carried out based on the raw material and energy usage for the processing of microalgal biomass (per kilogram). Costs estimated were used for comparative analysis between enzymatic and the chemical method. Costs for labour, capital expenditure and tax were not included in the process cost estimation. Raw material costs for analytical grade and industrial grade chemicals were provided by the suppliers. In case of enzyme catalysts, cost estimation was also calculated considering its reuse potential. For reuse consideration, enzyme cost was divided by number of batches for which it can be reused without much loss in activity. Energy consumption and costs were compared for the different types of processes.

3. Results and discussion

3.1 Comparison of enzymatic and chemical methods

Lipids were converted to biodiesel using extracellular lipase, whole cell lipase and homogeneous acid catalyst. Among the enzymatic methods, extracellular lipase from *P. fluorescens* was shown to have better conversion (90.81 ± 1.61%) and yield (96.28 ± 0.46%), compared to whole cell lipase from *A. niger* (80.97 ± 0.52 and 90.82 ± 1.43%). Homogeneous acid showed 95.73 ± 0.56% biodiesel conversion and 97.08 ± 0.24% biodiesel yield. Enzymatic methods have shown lower biodiesel conversion, as well as yields as compared to the homogeneous acid method (Figure 6.1).

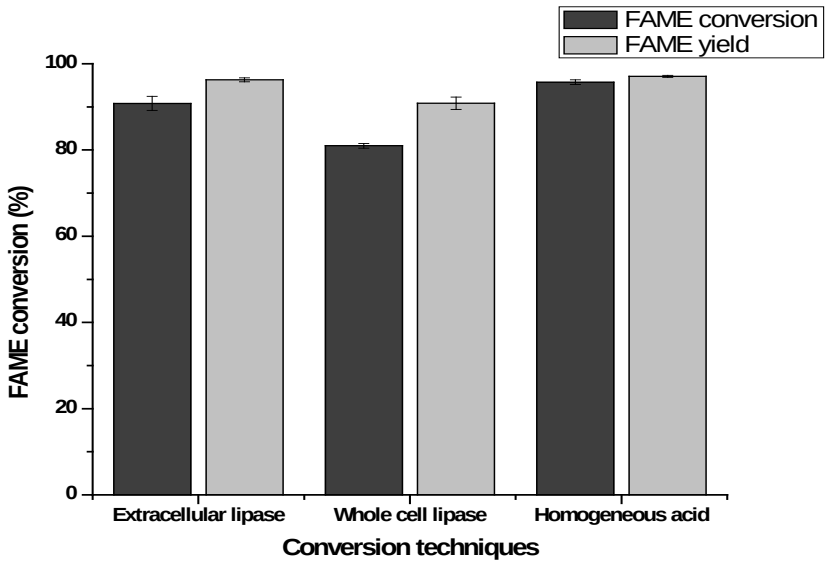


Figure 6.1: Comparison of enzymatic and chemical catalytic methods.

In case of time consumption, extracellular enzyme performed better than whole cell lipase. Extracellular lipase conversion had an optimum time of 12 h, compared to 36 h for whole cell lipase. Homogeneous acid catalyzed conversion time was 4 h, which was less than both the enzymatic methods (Table 6.1). Optimized temperature for immobilized lipase whether in extracellular for or intracellular form (whole cell) was found to be 35°C. Homogeneous acid catalyst operates at relatively higher temperature of 60°C (D'Oca et al., 2011). Lower temperature requirements for enzyme catalysts, makes the conversion process less energy intensive than chemical catalysts.

Table 6.1: Reaction parameters and conversion efficiency of enzymatic and chemical methods

	Extracellular lipase	**Whole cell lipase**	**Homogeneous acid**
Catalyst	Immobilized *P. fluorescens* lipase	Immobilized whole cell *A. niger* lipase	Sulphuric acid
Temperature	35°C	35°C	60°C
Time	12 h	36 h	4 h
MeOH:Oil molar ratio	3:1	5:1	30:1
Water content	2.5%	2.5%	-
Catalyst concentration	10%	6 BSPs	10%
Solvent amount	1ml	1ml	1ml
FAME conversion	90.81±1.61%	80.97±0.52%	95.73±0.56
FAME yield	96.28±0.46%	90.82±1.43%	97.08±0.24%
Reuse	4 batches	2 batches	-

Immobilized *A. niger* whole cell lipase was found to be more tolerant towards methanol (Methanol: Oil ratio 5:1) compared to immobilized *P. fluorescens* extracellular lipase (Methanol: Oil ratio 3:1). While, in case of sulphuric acid catalyst methanol to oil molar ratio was 30:1. Enzyme catalysis thus needs less amount of toxic methanol, which makes the process safer and environmentally friendly. Immobilized lipase catalysts can be reused for conversion of microalgal lipids, while homogeneous acid catalyst cannot be separated and leads to acidic wastewater generation. Immobilized *A. niger* whole cell lipase was found to be less stable compared to immobilized *P. fluorescens* extracellular lipase. Results from the study suggested that, the immobilized *P. fluorescens* extracellular lipase can be reused for four batches and the immobilized *A. niger* whole cell lipase can be reused for two batches. Reuse of immobilized lipase aids in reducing the catalyst cost involved in conversion process. Thus considering the conversion and reuse, *P. fluorescens* extracellular lipase can be selected as suitable enzyme catalyst for microalgal biodiesel production.

3.2 Techno-economic analysis of drying and extraction methods

Techno-economic evaluation of selected drying methods revealed that the freeze drying method was the most energy consuming method, compared to oven and sun drying. Energy input for freeze drying was 21.96 kWh, and for oven drying, 6 kWh (Table 6.2). High energy input in freeze drying is attributed to the sophisticated instrumentation needed to freeze and then vacuum-dry the microalgal biomass. However, freeze drying produced the best results for lipid extraction, as well as for lipid quality compared to oven and sun drying. Oven drying requires high temperature (≈60°C) which adds to energy input, as well as influences the lipid quality in terms of acid value and fatty acid composition. Oven drying was the least time-consuming among the selected drying techniques. Sun drying had negligible energy input as no sophisticated instrumentation was required and drying was accomplished using natural sunlight. However, the time taken by sun drying was highest (72 h). Also, sun drying requires land area for spreading biomass which could be a concern for its large scale application. Microwave and sonication cell disruption techniques were assessed, where the microwave technique produced higher lipid yields than the sonication technique (Chapter three). Energy input for extraction of 1 kg microalgal biomass by the microwave technique was 23.6 kWh, while that of sonication technique was 28.6 kWh (Table 6.3). Extraction process cost for 1 kg microalgal biomass by microwave extraction was estimated to be R3657 using analytical grade solvents and R164.86 using industrial grade solvents. Extraction process cost for 1 kg microalgal biomass by sonication extraction was estimated to be R3662.9 using analytical grade solvents and R170.75 using industrial grade solvents. Microwave techniques can be easily implemented at large scale production facilities.

Table 6.2: Energy consumption and process cost for drying techniques.

	Price (R)	**Oven drying**		**Freeze drying**		**Sun drying**	
		Qnt	Cost	Qnt	Cost	Qnt	Cost
Energy Input	1.18 $R.kWh^{-1}$	6.00	7.07	21.96	25.91	-	-
Total			7.07		25.91	-	-

R-South African Rand, Qnt-Quantity

Table 6.3: Energy consumption and process cost for extraction techniques.

			Microwave			**Sonication**		
	Price ($)		Qnt	Cost		Qnt	Cost	
	AN grade	IN grade		AN grade	IN grade		AN grade	IN grade
Energy Input	1.18 $R.kWh^{-1}$	1.18 $\$.kWh^{-1}$	23.60	27.83	27.83	28.60	33.72	33.72
Chloroform	120.86 $R.L^{-1}$	9.42 $R.L^{-1}$	10 L	1208.7	94.21	10 L	1208.7	94.21
Ethanol	242.05 $R.L^{-1}$	4.28 $R.L^{-1}$	10 L	2420.5	42.82	10 L	2420.5	42.82
Total				3657	164.86		3662.9	170.75

AN- Analytical grade, IN-Industrial grade, R-South African Rand, Qnt-Quantity

3.3 Techno-economic analysis of conversion methods

Conversion of microalgal lipids to biodiesel is a crucial step. Processing cost for 1 kg of microalgal biomass conversion was calculated based on raw material and energy input required for conversion of lipids to biodiesel. Both analytical grade and industrial grade chemical prices were considered while drawing cost estimates (Table 6.4).

Table 6.4: Energy consumption and process cost for enzymatic and chemical catalytic methods.

	Price AN grade	Homogeneous acid		Extracellular lipase		Whole cell lipase	
		Qnt	Cost	Qnt	Cost	Qnt	Cost
Sulphuric acid	170.75 $R.kg^{-1}$	30 gm	5.14				
P. fluorescens lipase	118338.04 $R.kg^{-1}$			30 gm	3550.03 887.48*		
A. niger whole cell lipase	69.69 $R.1000BSP^{-1}$					18000 BSP	1254.36 627.23*
Sodium hydroxide	555.93 $R.kg^{-1}$	25 gm	13.92				
n-hexane	288.83 $R.L^{-1}$	6l	1733	6l	1733	6l	1733
Methanol	57.81 $R.L^{-1}$	329.73 ml	19.06	32.97 ml	1.93	54.9 6ml	3.21
Energy input	1.18 $R.kWh^{-1}$	3.08	3.75	3.05	3.64	5.06	6.21
Total			1774.86		5288.71 2616.15*		2996.78 2369.65*
	Price IN grade	**Homogeneous acid**		**Extracellular lipase**		**Whole cell lipase**	
		Qnt	**Cost**	**Qnt**	**Cost**	**Qnt**	**Cost**
Sulphuric acid	5.89 $R.kg^{-1}$	30 gm	0.21				
P. fluorescens lipase	12846.54 $R.kg^{-1}$			30 gm	385.4 96.35*		
A. niger whole cell lipase	9.21 $R.1000BSP^{-1}$					18000 BSP	165.72 82.86*
Sodium hydroxide	4.28 $R.kg^{-1}$	25 gm	0.11				
n-hexane	8.46 $R.L^{-1}$	6l	50.74	6l	50.74	6l	50.74
Methanol	3.43 $R.L^{-1}$	329.73 ml	1.18	32.97 ml	0.11	54.96 ml	0.21
Energy input	1.18 $R.kWh^{-1}$	3.08	3.75	3.05	3.64	5.06	6.21
Total			55.78		439.89 150.84*		222.57 139.71*

AN- Analytical grade, IN-Industrial grade, R-South African Rand and Qnt-Quantity
*Considering reuse potential

The homogeneous acid-catalyzed conversion process cost was calculated to be 1774.86 $R.kg^{-1}$ biomass using analytical grade chemicals and 55.78 $R.kg^{-1}$ using Industrial grade chemicals (Table 6.4). Extracellular lipase catalyzed conversion process cost was calculated to be 5288.71 $R.kg^{-1}$ biomass using analytical grade chemicals and 439.89 $R.kg^{-1}$ using industrial grade chemicals. In the case of whole cell lipase-catalyzed conversion process, cost was calculated to be 2996.78 $R.kg^{-1}$ biomass using analytical grade chemicals and 222.57 $R.kg^{-1}$ using industrial grade chemicals (Table 6.4). Jegannathan et al., (2011) found that manufacturing cost for immobilized enzyme conversion was 206.96% higher than the chemical conversion method. For comparative analysis to give better idea at commercial scale production, process costs calculated using industrial grade chemical prices were considered. Process cost for extracellular lipase was approximately 8 times and for whole cell lipase 4 times that of homogeneous acid-catalyzed process. When reuse potential of enzyme catalysts was considered process cost for homogeneous acid, extracellular lipase and whole cell lipase were 55.78, 150.84 and 139.71 $R.kg^{-1}$ (Table 6.4). This clearly indicates that the reuse potential of enzyme catalysts aids in reducing the catalyst cost. In this study, reuse potential for extracellular lipase was four batches and for whole cell lipase, two batches. Reusability of enzyme depends upon its stability, immobilization and enzyme leaking. High cost of enzyme catalyst is the biggest challenge for successful realization of this process at commercial scale. Enhancing stability to improve reusability of lipases could reduce the enzymatic conversion process cost.

Homogeneous acid-catalyzed conversion takes place at 60°C, while both enzymatic conversions take place at 35°C. Energy input for extracellular lipase catalyzed conversion is lowest (3.05 kWh) followed by homogeneous acid (3.08 kWh) and whole cell lipase (5.06 kWh) conversion, respectively. High energy input in whole cell lipase process is due to the slow

reaction rate which takes 36 h. Process flow sheets gives an idea about processing steps involved in biodiesel production process using chemical and biocatalysts as well as forms basis for comparison. Process flow of homogeneous catalyst for biodiesel production indicates that neutralization and extra purification steps are necessary for maintaining product quality (Figure 6.2). When enzymatic catalysts were applied for biodiesel synthesis, neutralization and extra water washing steps are completely avoided (Figures 6.3 and 6.4). Less purification steps and no addition of chemicals keep wastewater output to minimal levels in case of enzyme catalysis. For homogeneous acid-catalyzed conversion, the product obtained needs tedious purification steps. Neutralization and water washing steps in homogeneous acid catalyzed conversion leads to generation of acidic and alkaline wastewater. Wastewater generated needs treatment before its safe discharge, which adds to the overall process cost of homogeneous acid-catalyzed conversion. Reaction solvent n-hexane is the second-most contributor to the raw material cost after catalyst, in case of enzymatic conversion. Solvent provides proper mass transfer of reactants as well as reduces the inhibition of enzyme caused by methanol. All three process flow sheets showed that after first purification step n-hexane can be recovered and reused. Reuse of solvent could aid in reducing the process cost. Microalgae can be grown in varied climatic conditions by applying suitable cultivation strategy. Commercial enzymes are readily available; also for whole cell application lipase producing organisms can be produced in bulk amounts. Thus, biodiesel production using microalgae as feedstock and lipase as catalyst ensures the local energy security. Large scale facility can also create job opportunities. Sustainable production of biodiesel from microalgae using environmentally friendly biocatalyst has social, economical and environmental significance.

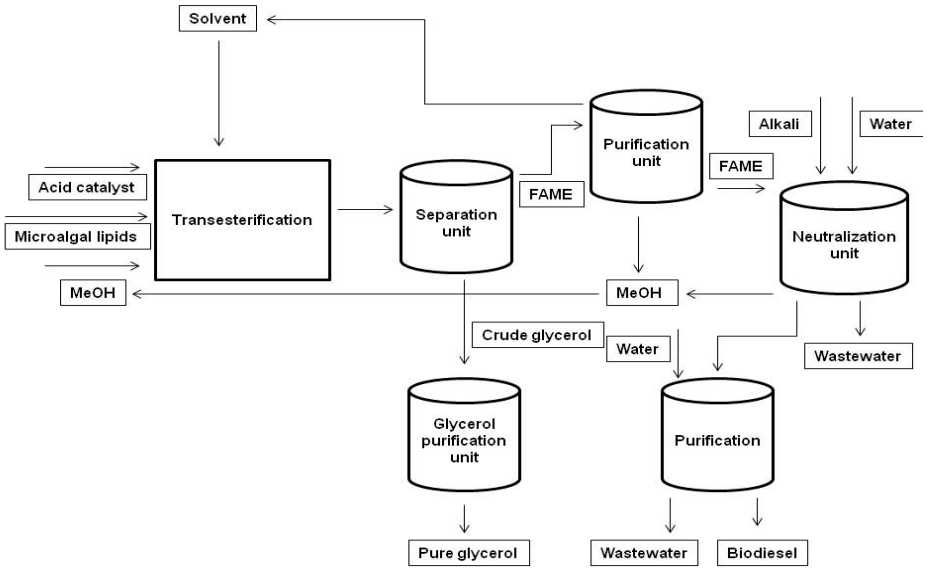


Figure 6.2: Process flow sheet for homogeneous acid-catalyzed conversion.

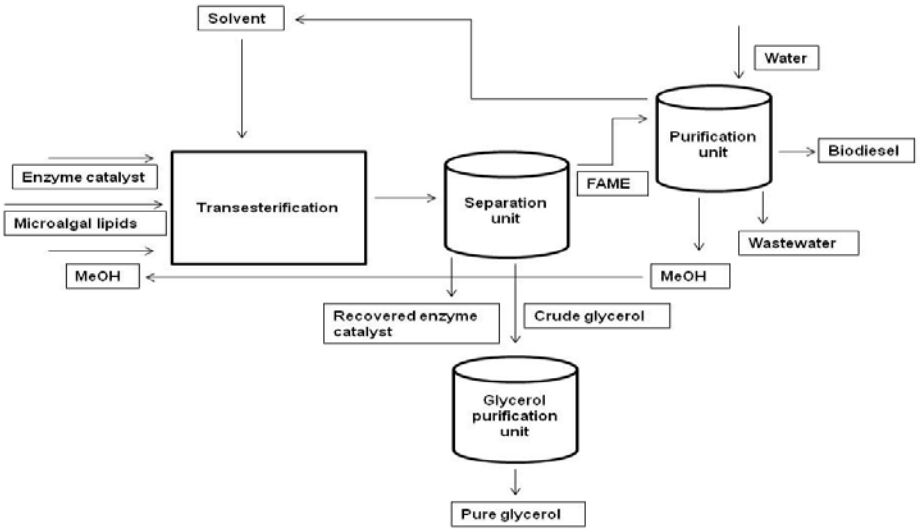


Figure 6.3: Process flow sheet for extracellular lipase-catalyzed conversion.

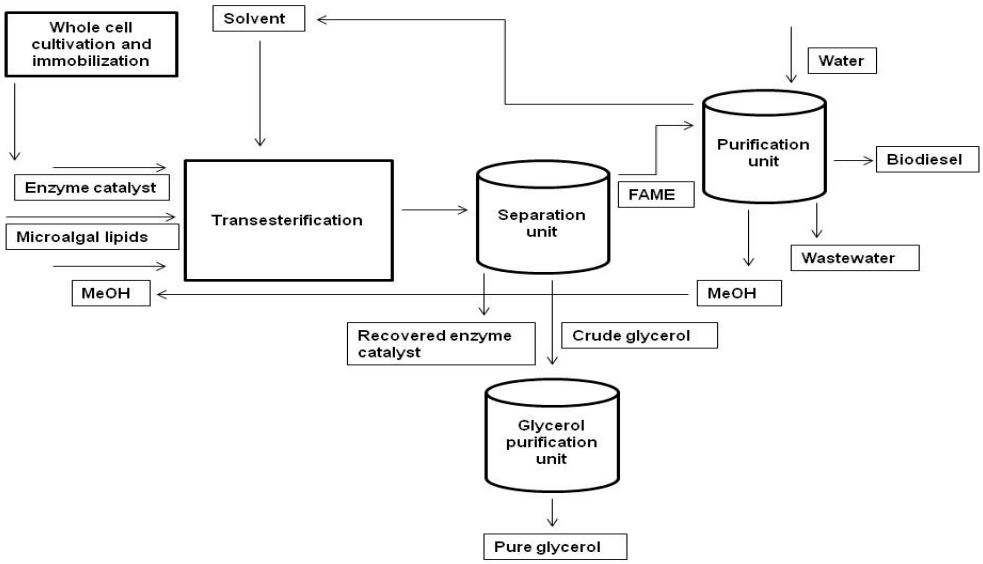


Figure 6.4: Process flow sheet for whole cell lipase-catalyzed conversion.

3.4 Challenges and future prospective for enzymatic conversion method

Enzymatic catalysis has shown promising potential to replace the conventional chemical catalysts with comparable biodiesel conversion and yields. High cost is the major bottleneck for commercial scale application. However, with the biotechnological advances, cost of commercial enzymes could be reduced in the near future. Immobilization and reusability of biocatalysts has shown potential to reduce the overall cost (Jegannathan et al., 2011). Extracellular lipase from *P. fluorescens* can be used for four batches and immobilized whole cell *A. niger* lipase can be used for two batches. Reusability potential of *P. fluorescens* and whole cell *A. niger* lipase was determined based on the relative FAME conversion to first batch conversion. Both biocatalysts can be reused for even more batches, but the product quality is compromised. Reusability of enzyme depends upon its stability on the support material as well as extent of exposure to the methanol and reaction solvent. Problems associated with stability of enzyme can be alleviated

with improved immobilization and solvent engineering strategies. Continuous mode of operation could also be applied to the enzymatic conversion strategy. Methanol inhibits the activity of enzyme, which reduces the conversion efficiency as well as biodiesel yield. Step-wise addition of methanol has shown that the biodiesel conversion efficiency can be increased by controlling the amount of methanol in the reaction mixture. Overall it can be concluded that enzyme catalyst holds significant prospects to be used at commercial scale biodiesel production, however to make it economically viable cost of enzyme catalysis needs to be reduced.

4. Conclusions

Immobilized extracellular lipase and homogeneous acid catalyst has shown comparable biodiesel conversion and yield. Whole cell lipase showed low biodiesel conversion and yield. Techno-economic analysis revealed that the process cost of both the enzymatic methods was higher than the chemical method. However, it can be reduced by improving the reusability of immobilized enzymes. Mild reaction conditions, low methanol requirement, less purification steps and wastewater output are the other technological benefits provided by enzymatic conversion methods. With biotechnological advances, enzyme cost is expected to be reduced in the near future. Thus, it can be concluded that enzyme catalyst could be utilized at large scale biodiesel production which makes the overall process greener and sustainable.

GENERAL CONCLUSIONS

Microalgal biodiesel has offered new prospects in the field of renewable energy. The main concern with regard to microalgal biodiesel production process is its efficiency and economical viability. Much of the research in this area focused on screening and developing microalgal cultivation systems. Downstream processing of microalgal biodiesel has still some bottlenecks that need to be addressed. Harvesting, drying of biomass, extraction of lipids and its conversion to biodiesel are still in its early stages. This work is focused primarily on the conversion of microalgal lipids to biodiesel using biocatalysts, which is a novel aspect. This study also deals with the preceding steps such as biomass drying and lipid extraction. Please find below a summary of significant aspects of the research findings:

Drying and cell disruption:

Results from this study showed that, drying and cell disruption techniques had a significant influence on the lipid characteristics such as degree of unsaturation and acid value. Freeze dried biomass yielded the best quality lipids with minimal free fatty acid content from *S. obliquus*. Sun drying technique showed minimum energy requirements compared to freeze and oven drying. Microwave assisted lipid extraction showed better results in terms of percentage lipid recovery as well as lipid quality, compared to sonication. The energy consumption of the microwave assisted extraction was less than that required for sonication. Freeze drying of biomass and microwave cell disruption technique are feasible for laboratory scale application. Freeze drying could be replaced with the more economical sun drying process at larger scale.

Enzymatic catalysis of biodiesel conversion

Immobilized lipase showed better catalytic performance compared to free lipases. The extracellular application of immobilized *P. fluorescens* lipase showed superior biodiesel conversion in screening studies. Immobilized *A. niger* whole cell lipase showed higher conversion efficiency compared to immobilized *Candida* sp. DUT04 whole cell lipase. Extracellular lipase-catalyzed conversion showed higher biodiesel conversion (90.81 ± 1.61%) and yield (96.28 ± 0.46%) compared to whole cell lipase-catalyzed process (C= 80.97 ± 0.52% and Y= 90.82 ± 1.43%). Whole cell lipase has shown more tolerance towards methanol. Immobilized lipases can be reused for biodiesel synthesis using microalgae, where, extracellular lipase showed better reuse potential than the immobilized whole cell lipase.

Process parameter optimization and step-wise methanol addition improved the biodiesel conversion in both cases. Temperature, methanol to oil molar ratio and catalyst amount were found to be the only factors to significantly influence the immobilized extracellular *P. fluorescens* lipase-catalyzed conversion. In case of immobilized *A. niger* whole cell lipase temperature and methanol to oil molar ratio were found to be significantly influencing biodiesel conversion. Biodiesel conversion and yield from lipase-catalyzed processes were lower compared to homogeneous acid-catalyzed conversion (C= 95.73 ± 0.56% and Y= 97.08 ± 0.24%) of *S. obliquus* lipids. However enzymatic conversion processes were accomplished at lower temperature and methanol to oil ratio, compared to conventional chemical catalyst conversion.

Fuel properties of *S. obliquus* biodiesel:

Biodiesel has to meet specifications set by international agencies for standards for its commercial use to be applicable. Biodiesel synthesized by enzymatic conversion of microalgal

lipid investigated in this study showed compliance in terms of cetane number, density, linolenic acid methyl ester content, acid value, iodine number, sulphur content with ASTM 6751 and EN 14214 standards. Calorific values of *S. obliquus* biodiesel synthesized by lipase catalyst were comparable to diesel fuel. However, the methyl ester contents for enzymatic conversions were lower than the EN 14214 specified limits of 96.5%. Oxidative stability of *S. obliquus* biodiesel synthesized by enzyme catalyst met the ASTM 6751 specifications, but was lower than the EN 14214 specifications. Cold filter plugging point of synthesized biodiesel needs further refinement. Overall it can be concluded that most of the fuel properties of *S. obliquus* biodiesel synthesized by extracellular and whole cell lipases were in compliance with international standard specifications.

Techno-economic analysis:

However, findings showed that conventional chemical conversion is more cost effective than the enzymatic conversion due to a significantly higher enzyme cost. Immobilization and reuse of lipase catalysts could improve the economics and make the conversion process comparable to conventional chemical catalysts. A further environmental benefit of using lipase catalyst is a reduced impact with minimal wastewater generation, compared to chemical catalysts. In addition, enzyme catalysts are advantageous in that purification steps are minimized and they have a lower energy requirement.

The commercialization of microalgal biodiesel relies heavily on successful downstream processing. Biomass drying and lipid extraction and conversion are key processes in this segment of biodiesel production. The use of lipase-catalyzed conversion of microalgal lipids to biodiesel is a fairly recent approach which shows great promise in the search for a sustainable, renewable

and eco-friendly liquid fuel. The findings presented in this thesis have academic as well as industrial significance, which could form a basis for environmentally friendly microalgal biodiesel production at an industrial scale. This study supports the potential of using microalgal biomass as a feedstock and lipase catalyzed conversion of lipids to biodiesel. The latter is certainly a greener and sustainable approach to cater for increasing renewable energy demands.

RECOMMENDATIONS

Based on the findings from this work, recommendations for studies on lipase-catalyzed biodiesel synthesis from microalgae are as follows:

- Improvement of lipase-catalyzed conversion via application of lipase mixtures, pretreatments and solvent engineering.
- Elucidation of lipase catalyzed reaction kinetics.
- Development of cost effective enzyme immobilization strategies for extracellular as well as whole cell lipases.
- Improvement of immobilized lipase stability to increase its potential for reuse.
- Life cycle analysis of biodiesel production using enzyme catalysis at demonstration scale production facility.

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APPENDICES

APPENDIX ONE

BG11 nutrient medium (Chapter 3)

Component	Quantity
$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
$NaCO_3$	0.02 g
Trace metal mix A5	1.0 ml
Agar (if needed)	10.0 g
Distilled water	1.0 L

Trace metal mix A5

Component	Quantity
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg
Distilled water	1.0 L

APPENDIX TWO

Fatty acid profiles of *S. obliquus* with different drying and cell disruption techniques (Chapter 3)

Lipid profile (% composition) obtained by the microwave technique (FM- freeze dried biomass, OM- oven dried biomass and SM- sun dried biomass)

	FM	OM	SM
C16:0	49.04 ± 0.16	55.18 ± 2.07	32.69 ± 1.91
C16:1	6.57 ± 0.03	4.40 ± 0.09	6.93 ± 0.76
C18:0	1.17 ± 0.07	0.88 ± 0.14	1.76 ± 0.02
C18:1	10.15 ± 0.11	6.87 ± 0.48	16.66 ± 0.85
C18:2	6.88 ± 0.17	10.17 ± 0.57	8.13 ± 0.73
C20:1	6.80 ± 0.14	3.00 ± 0.37	8.85 ± 2.48
C18:3	9.32 ± 0.41	11.96 ± 0.38	10.93 ± 0.85
C22:0	2.84±0.48	1.89 ± 0.25	6.01 ± 1.17
C24:0	2.01 ± 0.05	0.18 ± 0.04	1.80 ± 0.02
SFA	55.52 ± 0.30	58.44 ± 2.03	44.33 ± 0.38
MUFA	23.52 ± 0.05	14.27 ± 0.76	32.44 ± 0.86
PUFA	19.25 ± 0.35	25.34 ± 0.72	21.66 ± 0.46
OTHERS	1.71 ± 0.00	1.95 ± 0.55	1.57 ± 0.03

Lipid profile (% composition) obtained by the sonication technique (FS- freeze dried biomass, OS- oven dried biomass and SS- sun dried biomass).

	FS	OS	SS
C16:0	39.80 ± 0.33	44.72 ± 0.26	31.67 ± 0.32
C16:1	8.86 ± 0.32	6.75 ± 0.01	7.56 ±0.29
C18:0	1.64 ± 0.08	1.23 ± 0.20	2.53 ± 0.04
C18:1	13.66 ± 0.32	9.13 ± 0.33	15.96 ±0.37
C18:2	8.44 ± 0.30	12.72 ± 0.08	9.40 ± 0.00
C20:1	5.95 ± 0.33	1.41 ± 0.04	5.51 ± 0.04
C18:3	11.36 ± 0.07	15.30 ± 0.13	8.84 ± 0.12
C22:0	2.03 ± 0.05	2.39 ± 0.04	6.58 ± 0.67
C24:0	2.41 ± 0.49	1.92 ± 0.41	0.46 ± 0.07
SFA	46.43 ± 0.01	50.67 ± 0.39	44.98 ± 0.25
MUFA	28.48 ± 0.30	17.29 ± 0.31	29.03 ± 0.63
PUFA	22.13 ± 0.39	30.36 ± 0.07	23.82 ± 0.10
OTHERS	2.96 ± 0.10	1.68 ± 0.01	2.17 ± 0.28

APPENDIX THREE

Box-Behnken model results for reaction condition optimization and predicted values in immobilized *P. fluorescens* lipase application (Chapter 4)

Exp. No.	Coded values of reaction variables: Enzyme conc. (A)	Temp. (B)	Water content (C)	MeOH: Oil Molar ratio (D)	FAME Conversion (%)	Predicted values (%)
1	0	1	0	-1	7.62	8.59
2	1	-1	0	0	53.23	45.55
3	0	-1	0	1	15.51	12.09
4	-1	0	0	-1	12.06	17.87
5	0	0	-1	1	8.74	6.67
6	-1	0	0	1	8.61	7.22
7	1	0	1	0	18.03	18.66
8	0	1	-1	0	10.27	6.62
9	1	1	0	0	13.75	15.58
10	-1	0	-1	0	12.48	9.4
11	1	0	0	1	15.27	14.89
12	1	0	0	-1	32.39	39.18
13	0	1	1	0	22.17	12.82
14	0	1	0	1	14.70	20.17
15	0	0	0	0	12.21	12.34
16	0	0	1	-1	19.79	18.92
17	0	0	1	1	7.39	9.19
18	0	-1	1	0	13.31	22.37
19	1	0	-1	0	27.26	26.07
20	-1	0	1	0	7.63	6.36
21	-1	1	0	0	5.37	10.09
22	0	-1	0	-1	66.55	58.62
23	0	0	-1	-1	36.65	31.89
24	0	0	0	0	12.46	12.34
25	0	-1	-1	0	24.26	39.02
26	0	0	0	0	12.35	12.34
27	-1	-1	0	0	26.87	22.07

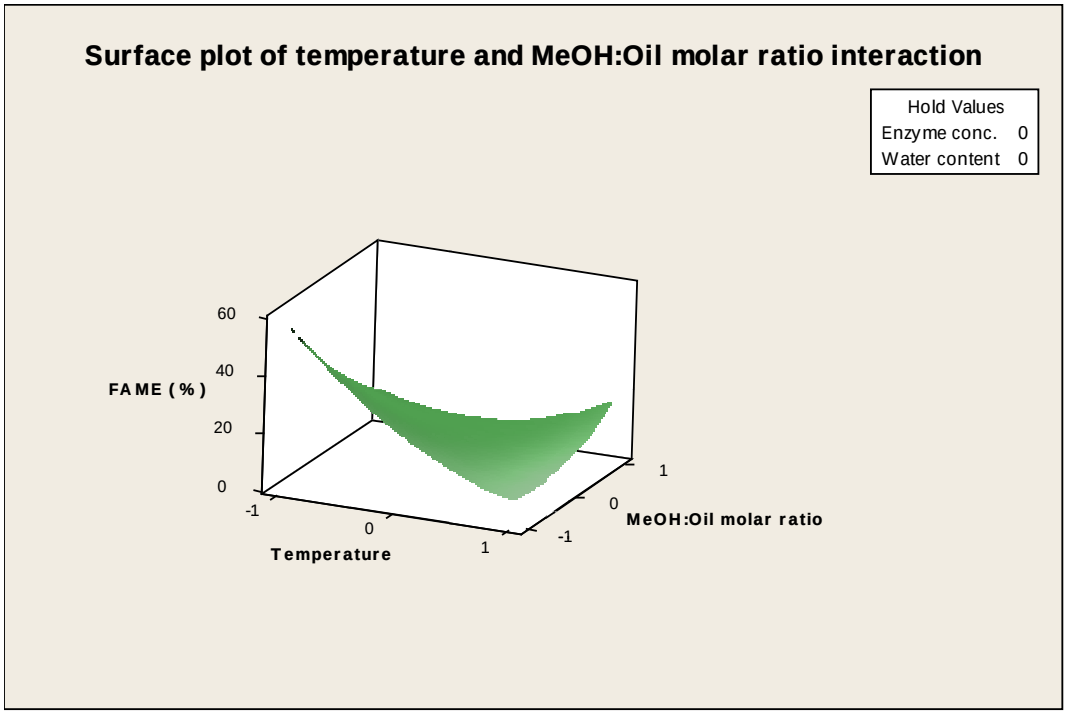
APPENDIX FOUR

Analysis of variance (ANOVA) for response surface quadratic model for immobilized *P. fluorescens* lipase application with squared terms and interactions. (Chapter 4)

	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	4549.92	4549.92	324.99	5.27	0.003
Linear	4	2947.38	2947.38	736.85	11.95	<0.001
Enzyme concentration (A)	1	629.52	629.52	629.52	10.21	0.008
Temperature (B)	1	1319.87	1319.87	1319.87	21.41	0.001
Water content (C)	1	81.89	81.89	81.89	1.33	0.272
MeOH: Oil Molar ratio (D)	1	916.09	916.09	916.09	14.86	0.002
Square	4	435.22	435.22	108.8	1.76	0.201
A*A	1	1.53	46.45	46.45	0.75	0.402
B*B	1	309.28	344.19	344.19	5.58	0.036
C*C	1	16.65	0.15	0.15	0	0.961
D*D	1	107.76	107.76	107.76	1.75	0.211
Interaction	6	1167.32	1167.32	194.55	3.16	0.043
A*B	1	80.84	80.84	80.84	1.31	0.274
A*C	1	4.79	4.79	4.79	0.08	0.785
A*D	1	46.63	46.63	46.63	0.76	0.402
B*C	1	130.54	130.54	130.54	2.12	0.171
B*D	1	844.39	844.39	844.39	13.7	0.003
C*D	1	60.12	60.12	60.12	0.98	0.343
Residual Error	12	739.81	739.81	61.65		
Lack-of-Fit	10	739.78	739.78	739.78	4774.89	<0.001
Pure Error	2	0.03	0.03	0.02		
Total	26	5289.73				

APPENDIX FIVE

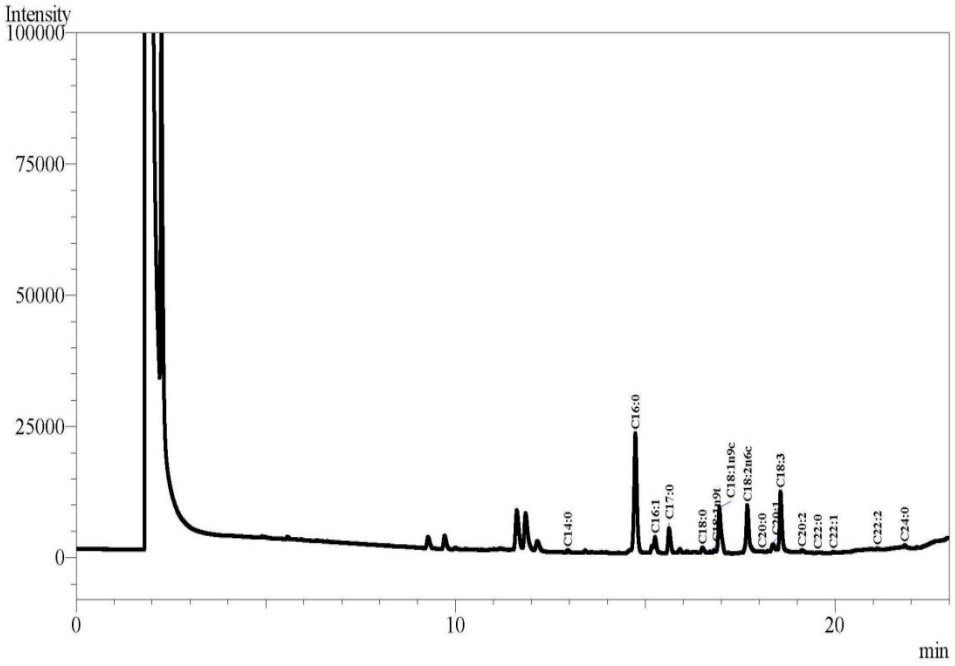
Surface plot of significant interaction for immobilized *P. fluorescens* lipase application. (Chapter 4)



Surface plot of temperature and methanol to oil molar ratio interaction

APPENDIX SIX

Chromatogram of *S. obliquus* biodiesel produced by immobilized *P. fluorescens* lipases (Chapters 4)



Chromatogram of *S. obliquus* biodiesel produced by immobilized *P. fluorescens* lipase

APPENDIX SEVEN

Box-Behnken model results for reaction condition optimization and predicted values in immobilized *A. niger* whole cell lipase application. (Chapter 5)

Exp. No.	Coded values of reaction variables				FAME Conversion (%)	Predicted values (%)
	Temp. (A)	MeOH: Oil Molar ratio (B)	Water content (C)	No. of BSPs (D)		
1	0	-1	1	0	30.64	28.65
2	0	-1	0	-1	28.49	34.34
3	-1	1	0	0	27.25	29.15
4	0	-1	-1	0	37.44	27.99
5	1	0	0	-1	38.41	38.73
6	-1	0	0	-1	39.41	37.24
7	1	0	1	0	25.46	26.89
8	1	1	0	0	14.58	11.90
9	0	0	-1	1	19.90	25.98
10	-1	-1	0	0	28.43	32.29
11	0	1	0	1	24.77	23.19
12	0	1	1	0	20.21	24.21
13	0	0	0	0	31.85	32.22
14	0	1	0	-1	21.28	23.10
15	0	1	-1	0	22.19	18.72
16	1	0	0	1	22.70	19.41
17	0	0	-1	-1	45.42	44.5
18	0	0	1	1	41.17	43.28
19	0	0	0	0	32.44	32.22
20	1	-1	0	0	23.19	22.47
21	0	0	1	-1	38.25	33.35
22	-1	0	1	0	45.15	44.50
23	-1	0	0	1	53.76	47.98
24	1	0	-1	0	22.97	27.89
25	0	-1	0	1	23.23	25.67
26	-1	0	-1	0	34.51	37.35
27	0	0	0	0	32.38	32.22

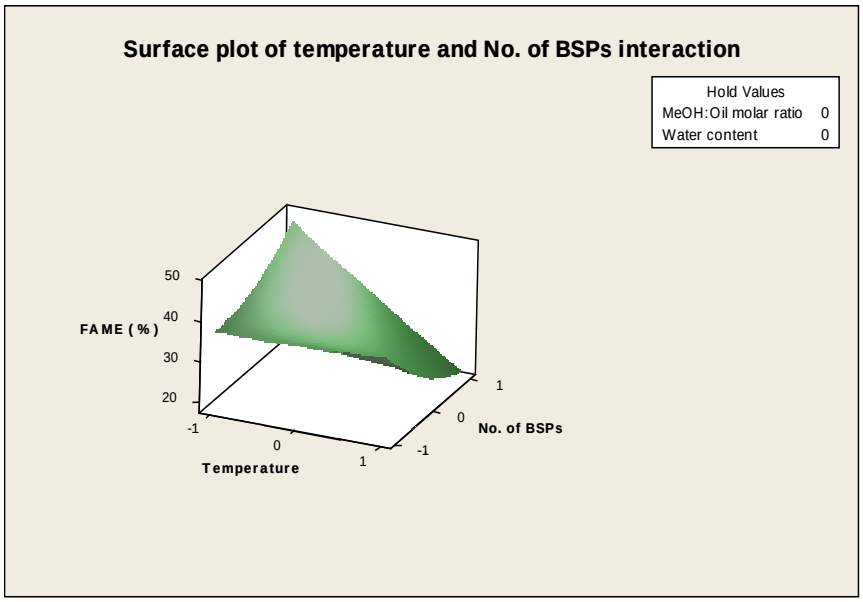
APPENDIX EIGHT

Analysis of variance (ANOVA) for response surface quadratic model for immobilized *A. niger* whole cell lipase application with squared terms and interactions. (Chapter 5)

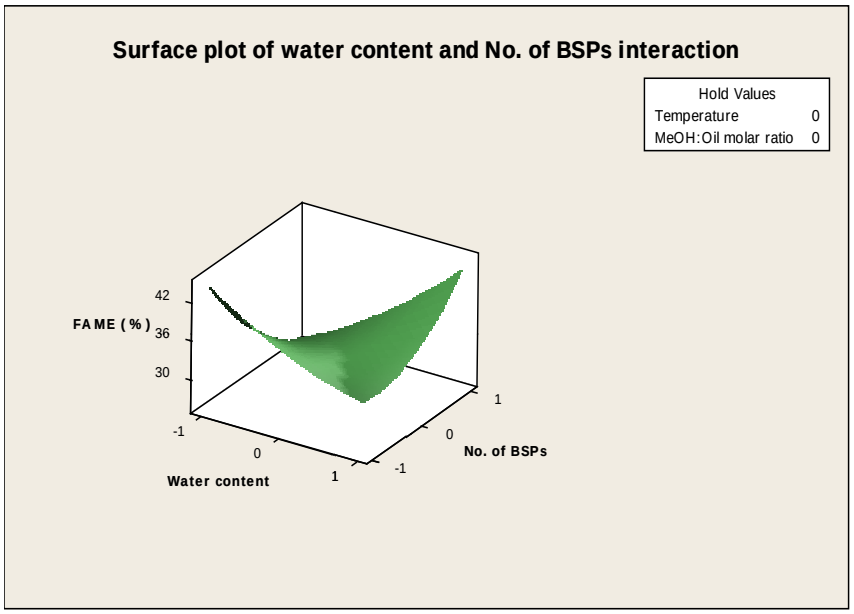
	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	1950.55	1950.55	139.32	4.87	0.005
Linear	4	774.22	774.22	193.55	6.76	0.004
Temperature (A)	1	549.53	549.53	549.53	19.19	0.001
MeOH: Oil Molar ratio (B)	1	141.1	141.1	141.1	4.93	0.046
Water content (C)	1	28.39	28.39	28.39	0.99	0.34
No. of BSPs (D)	1	55.2	55.2	55.2	1.93	0.19
Square	4	692.74	692.74	173.19	6.05	0.007
A*A	1	11.97	1.32	1.32	0.05	0.83
B*B	1	627.97	409.93	409.93	14.31	0.003
C*C	1	0.94	10.98	10.98	0.38	0.547
D*D	1	51.88	51.88	51.88	1.81	0.203
Interaction	6	483.59	483.59	80.6	2.81	0.06
A*B	1	13.81	13.81	13.81	0.48	0.501
A*C	1	16.57	16.57	16.57	0.58	0.461
A*D	1	225.93	225.93	225.93	7.89	0.016
B*C	1	5.82	5.82	5.82	0.2	0.66
B*D	1	19.17	19.17	19.17	0.67	0.429
C*D	1	202.29	202.29	202.29	7.06	0.021
Residual Error	12	343.65	343.65	28.64		
Lack-of-Fit	10	343.43	343.43	34.34	321.41	0.003
Pure Error	2	0.21	0.21	0.107		
Total	26	2294.2				

APPENDIX NINE

Surface plot of significant interactions for immobilized *A. niger* whole cell lipase application (Chapter 5)



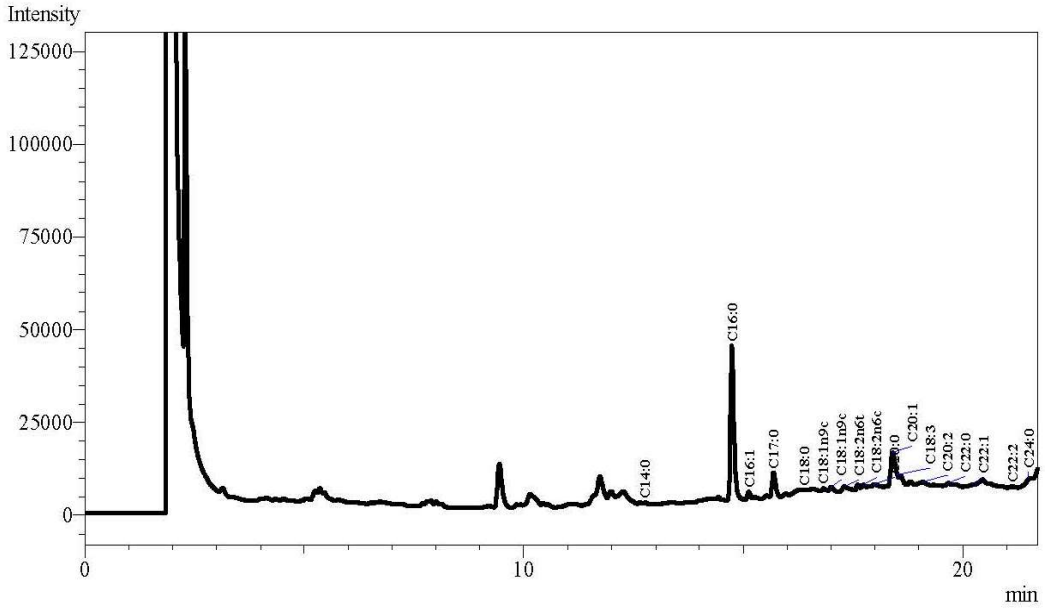
Surface plot of temperature and No. of BSPs interaction



Surface plot of water content and No. of BSPs interaction

APPENDIX TEN

Chromatogram of *S. obliquus* biodiesel produced by immobilized *A. niger* whole cell lipases (Chapters 5)



Chromatogram of *S. obliquus* biodiesel produced by immobilized *A. niger* whole cell lipase

APPENDIX ELEVEN

Power output of instruments (Chapter 6)

Instrument	Output power (W)
Microwave	1000
Sonication	100
Freezer	750
Lyophilizer	1080
Centrifuge	4000
Incubator for acid catalysis	259
Incubator for enzyme catalysis	84
Rotary evaporator	1360

APPENDIX TWELVE

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Advances in synthesis of biodiesel via enzyme catalysis: Novel and sustainable approaches

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ABSTRACT

Biodiesel, a renewable fuel has a great potential in fulfilling an ever-increasing transport fuel demand. The enzymatic conversion process of feedstock oil to biodiesel is greener when compared to the conventional approach of chemical conversion due to mild reaction conditions and less wastewater generation. Lipases obtained from various microbial sources have been widely applied as catalysts for the conversion of oil to biodiesel. Biodiesel and glycerol obtained by enzymatic conversion have shown a higher purity as compared to that obtained by other conversion techniques. Enzymatic conversion of oil to biodiesel is less energy intensive because of milder reaction conditions and fewer purification steps involved in processing. Lipases, due to their catalytic efficiency and specificity, have emerged as a great tool for converting a wide range of feedstock oils to biodiesel. This manuscript presents an overview of the use of enzymatic conversion for making biodiesel production sustainable and environmentally-friendly. The constraints of enzymatic conversion are the high cost of the enzyme and its inhibition by alcohol and glycerol. The possible solutions to overcome these constraints are discussed. Recent advances to develop an effective process for enzymatic conversion of feedstock oils into biodiesel are critically evaluated. Prospective and challenges in scaling up of this technology are also discussed.

Contents

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Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production

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HIGHLIGHTS

- Microwave and sonication techniques are compared for efficient lipid extraction.
- Qualitative lipid variation caused by cell disruption and drying methods is studied.
- Energy consumption calculated for drying and lipid extraction processes.
- Lipid quality has been assessed for its suitability for biodiesel production.
- Sun drying with efficient microwave extraction could be possible processing step.

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ABSTRACT

Downstream processing of microalgal biomass presents a significant challenge to large scale biodiesel production. *Scenedesmus* sp. which is known to be a potential feedstock for biodiesel production was cultivated in an open circular pond. The biomass productivity peaked at day 21 with a yield of 1.16 g L^{-1}. Biomass was harvested by gravitational settling followed by centrifugation. Harvested biomass was dried using the freeze drying, oven drying and sun drying processes followed by lipid extraction which yielded 29.65%, 28.63% and 28.33% lipid g^{-1} DCW (dry cell weight) respectively. Lipids were extracted from microalgal biomass dried by selected drying techniques using microwave and sonication for cell disruption in the presence of mixture of chloroform and ethanol (1:1). Microwave assisted extraction of sun dried biomass yielded 28.33% lipid g^{-1} DCW, as compared to 18.9% lipid g^{-1} DCW achieved by sonication assisted extraction. The saponification and acid values of the lipid obtained from *Scenedesmus* sp. dried by selected drying techniques showed high saponification and acid value indicating presence of high free fatty acid content. Effect of different drying and cell disruption technique on fatty acid profile of lipids extracted from *Scenedesmus* sp. biomass was also studied. These values indicate promising potential of the oil produced for conversion into biodiesel.

1. Introduction

Microalgae have emerged as promising feedstocks for biodiesel production. Microalgal biodiesel has several advantages over crop based biodiesel such as high potential for biomass production with minimal land and freshwater requirement; no arable land requirement, no food security concerns; a higher carbon-dioxide sequestration potential; wastewater utilization during cultivation; and the production of value-added products from de-oiled microalgal biomass. The production of a sustainable and economically viable biodiesel from microalgae is however still a challenge [1–6].

Biodiesel production from microalgae is a multi-step process including cultivation, harvesting and dewatering of microalgal biomass, extraction of lipids from biomass, and conversion of lipids to biodiesel. Photoautotrophic cultivation of microalgae is widely accepted as a more economically viable method for large scale microalgal biomass production. Microalgae essentially require light, carbon dioxide, inorganic nutrients, and water for their growth [7]. Microalgae are either cultivated in open raceway ponds or in photo-bioreactors for a high biomass and lipid production. The choice of technique for cultivation and production of microalgal biomass with high quantity of lipids depends upon microalgal strain selection, culture conditions, land area availability, natural light and production scale [2].

Harvesting and dewatering of microalgal biomass are crucial steps in commercial production of microalgae. The harvesting

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Towards a sustainable approach for development of biodiesel from plant and microalgae

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ABSTRACT

The production of biodiesel can be accomplished using a variety of feedstock sources. Plant and microalgae based feedstocks are prominent and are studied extensively. Plant based feedstocks cultivated as monoculture on wastelands and trees in forests can cater towards partial fulfillment of feedstock requirements for biodiesel industry. Synthesis of biodiesel from microalgal oil has gathered immense interest and has potential to cater to the increasing feedstocks demands of the biodiesel industry. The major advantage offered by microalgal oil, as compared to plant based oils, is its potential for culture on non-arable land. Despite of the advantages of microalgal oil as a feedstock for biodiesel, there are constraints that have to be overcome in order to make it economical and sustainable. Sustainable approaches for both the plant and microalgae as feedstocks have been drawn. Despite there being several plant species, few have been found to be desirable as feedstocks for biodiesel production based on their lipid profiles. Among the microalgae, there are thousands of species and several of these have been cultured for extracting the oil to explore their feasibility in utilization as biodiesel feedstocks. Though, several of the microalgal species have shown potential for high biomass growth and lipid productivity, only a few have been found to provide a high biodiesel yield and conversion. Due to the several steps involved in the extraction of oil which are energy intensive, the cost of biodiesel from microalgal oil is high as compared with that obtained from the plant oils. A sustainable approach for utilizing plant and microalgal oils as feedstocks for biodiesel have been discussed. The emerging cost effective methods in production of biodiesel have been described. The energy return and greenhouse gas emissions from biodiesel have been outlined. Together, the plant oil and microalgal oil can offer potential source of feedstocks for the production of biodiesel.

Contents

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Electrochemical harvesting process for microalgae by using nonsacrificial carbon electrode: A sustainable approach for biodiesel production

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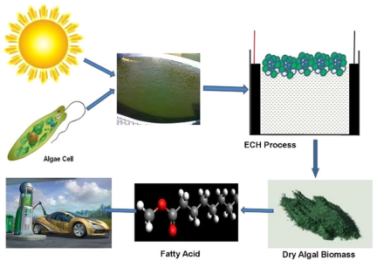
HIGHLIGHTS

- Electrochemical harvesting of algae employing non-sacrificial carbon electrode.
- Anode depletion and accompanying metallic contamination can be completely avoided.
- Effect of operating condition on lipid recovery and composition are evaluated.
- Electrochemical process has no adverse effect on the lipid extraction process.
- Striking recovery at low energy consumption by applying non-sacrificial electrodes.

GRAPHICAL ABSTRACT



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ABSTRACT

Microalgal biodiesel has to overcome a cost incurring harvesting bottleneck for its commercial scale production. In this study electrochemical harvesting (ECH) using *Chlorella sorokiniana* and *Scenedesmus obliquus* was investigated. Nonsacrificial carbon electrodes were used to overcome the cost and metallic contamination implications. The effect of applied current and addition of electrolyte on harvesting efficiency was investigated. Addition of electrolyte (NaCl) increased the recovery efficiency of *C. sorokiniana* from 65.99% to 94.52%. ECH process has not shown any deteriorating effect on the lipid extraction process as well as fatty acid composition. ECH process for *C. sorokiniana* with optimum conditions showed 94.52% recovery efficiency with energy consumption of 1.6 kWh kg^{-1}. This study for the first time validates application of nonsacrificial carbon electrodes in ECH process of microalgae. Attractive high recovery efficiency, low energy consumption and use of nonsacrificial electrodes could make ECH a possible step in commercial microalgal biomass and biodiesel production.

1. Introduction

In the quest for new energy sources, biofuels are one of the more attractive principal energy carriers. The production of biofuels is likely to offer new prospects to expand fuel supply sources and can help to reduce the adverse effects of the fossil fuel shortages [1]. Biodiesel can be produce by utilizing oil sources such as edible, non-edible, microalgal and waste cooking oil [2,3]. Due to their high growth rate and lipid accumulation capability, microalgae have attracted much attention as a feedstock for biodiesel production [4]. Microalgae can also be exploited for other biofuels

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