Whole-Cell Catalyzed Generation of Alkyl Esters from Acid Oils

A thesis submitted in fulfillment

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DOCTOR OF PHILOSOPHY

In

Biotechnology

By

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Dedicated to My Parents

and

My Family

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Certificate

Certified that the thesis entitled "**Whole-cell catalyzed generation of alkyl esters from acid oils**" which is submitted by Mr. Anirudh Sharma in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy in the Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is a record of candidate's own independent and original research work carried out by him under our supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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Candidate's Declaration

I, hereby declare that the work presented in the thesis entitled "**Whole-cell catalyzed generation of alkyl esters from acid oils**" in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is an authentic record of my own work carried out under the supervision of Dr. Ranjana Prakash, Professor, School of Chemistry & Biochemistry, Thapar University, Patiala. India and Dr. N. Tejo Prakash, Professor, School of Energy & Environment, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Place: Patiala
Date: 29/09/18.

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List of Symbols/Abbreviations

ROH	Alcohol
$(NH_4)_2HPO_4$	Bi- ammonium hydrogen ortho-phosphate
B100	Biodiesel (100% + 0% diesel)
B20	Biodiesel (20% + 80% Diesel)
BAHP	Bi- ammonium hydrogen ortho-phosphate
BHB	Bushnell hass broth
BSPs	Biomass support particles
CFS	Cell free supernatant
cm	Centimetre
CO_2	Carbondioxide
°C	Degree Celsius
$CDCl_3$	Deuterated chloroform
DG	Diglycerides
DNA	Deoxyribonucleic acid
EE	Ethyl ester
etc.	Etcetera
FAAE	Fatty acid alkyl ester
FAME	Fatty acid methyl ester
FFA	Free fatty acid
e.g	For example
GC	Gas chromatography
g	gram
g/L	Gram per liter
g/mL	Gram per mililiter
Gy	Gray
h	Hour
M	Molar
MAG	Mono acyl glycerides
ME	Methyl ester
MG	Monoglycerides
mg/mL	Milligram per milliliter
$MgCl_2$	Magnesium Chloride
MHz	Mega hertz
min	Minute
mL	Millilitre
mL/min	Millilitre pre minute
mm	Milli meter

List of Symbols/Abbreviations

mM	Milli molar
MTCC	Microbial type culture collection
µg	Micro gram
µL	Microlitre
µm	Micro meter
µmol	Micro mole
SOx	Oxides of Sulphur
^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
%	Percentage
PDA	Potato dextrose agar
PDB	Potato dextrose broth
ppm	Part per million
PUF	Polyurethane foam
KOH	Potassium hydroxide
NaCl	Sodium Chloride
$NaHCO_3$	Sodium bicarbonate
$NaNO_3$	Sodium Nitrate
NaOH	Sodium hydroxide
TAG	Triacylglycerol
TLC	Thin layer chromatography
UV	Ultra violet
U/mL	Unit per millilitre
Ug^{-1}	U/ gram
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
w/w	Weight/weight
WCO	waste cooking oil

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1.0 Introduction

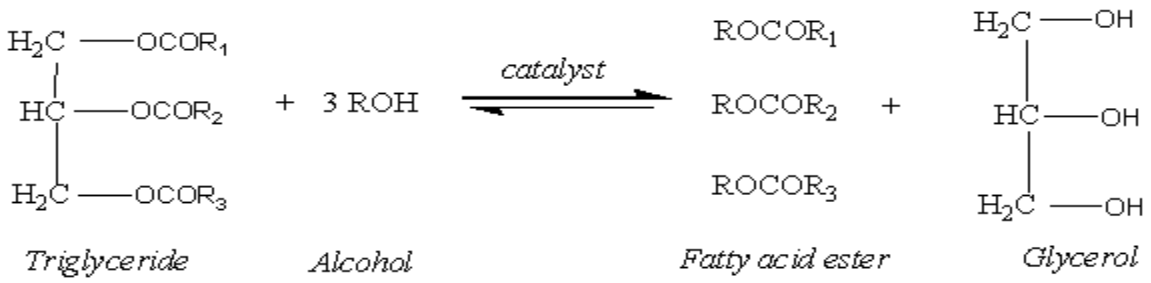
Fatty acid alkyl esters (FAAE), which are derived from triglycerides by transesterification with alcohol, has attracted considerable attention as alternative fuels. It can effectively supplement petroleum diesel as it is environmental friendly, renewable, biodegradable and non-toxic (Zhang 2003). The chemical composition of biodiesel is different from that of diesel fuel and does not contain any metal, sulfur, crude oil residues and aromatic hydrocarbons (Arkoudeas et al. 2003; Kalligeros et al. 2003). These characteristic properties of biodiesel facilitate in improving combustion efficiency and emission profile. As-on-date, fossil diesel blended with 20% of bio-diesel produced by conversion of oils is being used in some parts of the world. The European Union has set an objective to secure a market share of 20% of total motor fuel consumption by 2020 for motor bio-fuels (Körbitz 1999). According to international standard (ASTM D 6751), Bio-diesel is defined as a fuel comprising of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats and designated as B100. A blend of 20% bio-diesel with 80% petrodiesel, by volume, is termed B20 (Gerpen 2005). Among the attractive features of bio-diesel fuels are (Sams 1998; Schäfer 1998; Sheehan et al. 2000; Yamane et al. 2001):

1) It is plant derived and its combustion does not increase current net atmospheric levels of CO_2. In addition, relative to conventional diesel fuel, its combustion products have reduced levels of particulates, carbon monoxide, SO_X and, nitrogen oxides;
2) It can be domestically produced, offering the possibility of reducing petroleum imports; and
3) It is biodegradable.

Historically, it is believed that Rudolf Diesel himself started research with respect to the use of vegetable oils as fuel for diesel engines (Zaher 1990). In the following decades, the studies

became more systematic towards properties and application culminating in its use as alternative fuel in the present day. Despite obvious advantages, the direct use of vegetable oils in fuel engines is problematic. Due to the high viscosity and low volatility, they undergo incomplete combustion and form deposits in the diesel fuel injector of diesel engine.

For generation of fatty acid alkyl esters (FAAEs), the transesterification process has been considered to be a good choice, as the physical characteristics of fatty acid esters generated through this process are very close to those of diesel fuel and the process is relatively simple (Schwab et al. 1987). This process has been widely used to reduce the viscosity of triglycerides and enhancing the physical properties (Clark et al. 1984). Alcohols suitable for transesterification reaction include methanol, ethanol, propanol, butanol and amyl-alcohol. Methanol and ethanol are utilized more frequently, especially methanol because of its low cost and favorable physio-chemical properties. Two approaches for transesterification of vegetable oils have been explored extensively (Haas et al. 2002). The first being a chemical approach in which strong acid or base used to produce biodiesel and the second being bio-catalyzed transesterification (Fukuda et al. 2001).



Diverse varieties of acids have been used for transesterification of triglycerides. Acid catalysis facilitates very high yield of esters but the reaction is very slow (Marchetti et al. 2007). Although excess of alcohol can be used in the process to obtain better conversion of triglycerides, recovering of glycerol becomes more difficult. On the other hand serious environmental and corrosion related problems, make their use non-practical for biodiesel production at industrial scale

(Li, and Rudolph 2007; Van Gerpen et al. 1999). The alkali catalysts are commonly used for transesterification at industrial scale because of easy availability, less reaction time, less reaction temperature and economic viability. For alkali transesterification, the glycerides and alcohol must necessarily be anhydrous as presence of water result in saponification (Wright et al. 1944). The soap reduces the catalytic efficiency, and increases the viscosity resulting in difficult separation of glycerol (Fukuda et al. 2001). In addition, alkali catalyzed reaction necessitates low free fatty acid content in oil (≤0.5%) (Ma et al. 1998). The esters yield were observed to significantly reduce if the reactants did not meet these requirements (Freedman et al. 1984). A variety of heterogeneous catalysts have also been studied in recent past for biodiesel production. However, it requires high temperature and pressure with longer reaction time and higher energy consumption to catalyzed transesterification reaction (Meher et al. 2006).

Transesterification of the triglycerides has been studied by different research groups using lipase as a catalyst. Lipase catalyzed transesterification is carried out in aqueous or non-aqueous environment (Fukuda et al. 2001). Enzymatic methods have drawn greater attention for production of biodiesel, as they overcome the limitations of chemical approach due to advantages such as mild reaction conditions; insensitive to high FFA; regiospecificity; etc for wide variety of substrates (Akoh et al. 2007; Fjerbaek et al. 2009; Karimpil et al. 2012; Robles-Medina et al. 2009; Röttig et al. 2010)). However, the main hurdle of the commercializing lipase-catalyzed biodiesel production is the cost-intensive nature of lipase. The use of whole cell biocatalyst for biodiesel production is a cost-effective alternative that avoids the complicated processes of isolation, purification and immobilization of lipase (Ban et al. 2001; Ban et al. 2002). Lipase producing whole cell systems such as filamentous fungi, bacteria and yeast have been explored for this purpose in recent past. Transesterification has been carried out to a limited extent with immobilized cells of *Pseudomonas*

fluorescens (Devanesan et al. 2007*)*, *Bacillus subtilis* as a whole cell catalyst (Uthoff et al. 2009) and *Rhizopus oryzae* (Ban et al. 2001) within biomass supporting particles (BSPs) as whole cell biocatalysts. Another important aspect that promotes the use of whole cell catalysts is their potential to utilize waste edible and non-edible oils as well as by-products of oil-processing as feedstocks. However, there are limited reports on whole cell bio-catalyzed transesterification especially using fungi with most of them associated with *Rhizopus* species. Due to the 1, 3-regiospecificity of lipase from *Rhizopus*, only limited conversion of triglycerides to alkyl esters is possible in transesterification reactions of fats and oils.

Current industrial processes for biodiesel production use refined and edible grade vegetable oils for transesterification which affects the economic viability of fuel production. The feedstock cost comprises a very substantial portion for the overall cost of biodiesel production (Marchetti et al. 2008). The refined and edible vegetable oils are relatively expensive and their prices correspond to 70–80% of the total biodiesel production costs (Haas 2005). The selection of low cost raw material is essential for the production of biodiesel, this depends on the availability and affordability. Acid oil is an alternative cost-effective lipid-bearing, non-edible material for biodiesel production. The acid oil is obtained by the acidulation process of soapstock in oil refining industries (Watanabe et al. 2005). It comprises of long chain free fatty acids mixture, mineral acids, free moisture, phospholipids and sterols, neutral glycerides and unsaponifiable constituents of the oils and other impurities (Kulkarni et al. 2008; Wang et al. 2007). This by-product is unsafe to the environment if they cannot be used for any beneficial activity. Thus, acid oil is a very good alternative for biodiesel production as it reduces the cost and environment related problems. The conventional alkali catalysts are not suitable to acid oil, as the process results in soap formation due to high free fatty acids content (40-80%) (Fukuda et al. 2001).

To the best of our knowledge, observations on whole cell catalyzed transesterification of acid oil have been reported to a limited extent especially with fungi as catalysts, which formed the basis of this study.

2.0 Literature Review

The fossil fuels account over 80.3% of the primary energy consumed and 57.7% of that is used in transportation sector (IEA, 2006). On the other hand, the global consumption of diesel fuel is estimated to be 934 million tons per year (Kulkarni et al. 2006). According to the world energy forum, the fossil fuels that, are the sources of petroleum derived diesel fuel, will exhaust in the next few decades if new oil wells are not found (Sharma et al. 2009).The rapid increase in the population and industrial growth globally are the main reasons for extensive use and reduction in conventional energy resources (Pimentel et al. 2006). In the past decade, the renewable energy has been highlighted due to its potential to replace fossil fuel especially for transportation. Fuels are concentrated store of energy, combustible substances which are used to produce heat and energy. Amongst them, petrol and diesel are two of the most important commodities as they are used globally but are limited non-renewable resources in terms of availability and accessibility. So as to suffice the future needs of energy, researchers have been exploring various alternative fuels. Amongst them, biodiesel (fatty acid alkyl esters) has proven to be one of the promising energy materials (Ma et al. 1999). It is an alternative to petroleum diesel because it is environmentally friendly, renewable, biodegradable and non-toxic (Zhang 2003). The chemical composition of biodiesel is different from diesel fuel as it contains long chain of fatty acids generally 16-20 carbon chain and does not contain any metals, sulfur, crude oil residues and aromatic hydrocarbons (Arkoudeas et al. 2003; Kalligeros et al. 2003). These characteristic properties of biodiesel improve combustion efficiency and emission profile.

About a century ago, Rudolf Diesel tested vegetable oil as fuel for engine (Shay 1993). However, direct use of vegetable oils and animal fats as combustible fuels is observably not suitable due to high kinematic viscosity and low volatility. In addition, they undergo incomplete

combustion and form deposits in the fuel injector system of diesel engine (Muniyappa et al. 1996). During the World War II, the vegetable oils used as emergency fuel resulted in initiation of research and development on these oils as domestic fuels in India (Chowhury et al. 1942). However the research and development program on oils ceased as the petroleum based diesel fuel became getting easily available at affordable cost (Amrute 1947). After the World War II, Ohio State University (Columbus, USA), started a 'dual fuel' project to overcome the resultant fuel shortages. In this project, investigations were carried out on individual vegetable oils viz. cottonseed oil (Huguenard 1951), corn oil (Lem 1952), and also other blends with conventional diesel fuel. Different feedstocks like palm oil, soybean oil, cottonseed oil, castor oil, and a few less common oils, such as babassu (Pacheco Borges 1944) and crude rapeseed oil (Manzella 1936) were also under consistent investigations since the historic time.In addition, non-vegetable oil sources were also being investigated as fuel such as tallow (Lugaro et al. 1944) and fish oils (Kobayashi et al. 1921; Faragher et al. 1932).

Since the last decade, environmental concerns and energy security, have become driving forces for use of diesel fuels produced from vegetable oils (Shay 1993). In India, many vegetable oils were investigated as fuels viz; peanut, karanj, punnal, polang, castor, kapok, mahua, cottonseed, rapeseed, coconut, and sesame (Chowhury et al. 1942). In addition, Walton (1938) summarized twenty vegetable oils that were investigated as fuel such as castor, grapeseed, maize, camelina, pumpkinseed, beechnut, rapeseed, lupin, pea, poppy seed, peanut, hemp, linseed, chestnut, sunflower seed, palm, olive, soybean, cottonseed, and shea butter. In many notable publications, the satisfactory performance of vegetable oils as fuel and fuel sources have been discussed. However, their use was not found viable due to cost intensiveness and unfavourable physico-chemical properties as compared to petroleum derived fuels.

Walton (1938) said that "To get the utmost value from vegetable oils as fuel it is necessary to split off the triglycerides and to run on the residual fatty acid," which later paved way for research and development towards what we today term as 'biodiesel.' He recommended the removal of glycerol from fuel with no mention about esters. Some remarkable works have been performed in Belgium and Zaire-its former colony in the field of biodiesel and deserves the much recognition than it has received. The Belgian Patent 422,877, granted on Aug 31, 1937 to G. Chavanne (University of Brussels, Belgium) submitted the first report constituted on biodiesel and describes about using the ethyl ester of palm oil as diesel fuel, however other oils and methyl ester have also been mentioned (Chavanne 1937).

There are so many studies that have shown that vegetable oils hold promise as alternative fuel for diesel engine. However, direct use of vegetable oils is observed to be unsatisfactory and impractical for diesel engines. High viscosity, low cetane number, low flash point, natural gums (phosphatides), free fatty acid content in vegetable oil and improper injection timing cause many problems in fuel engines. The concerns include as short-term cold weather starting, plugging and gumming of filters, lines and injectors, engine knocking, carbon deposits on piston and head of engine, failure of engine lubricating oil due to polymerization (Ma et al. 1999; Srivastava et al. 2000). In order to reduce the high viscosity of vegetable oils, different approaches have been considered:

- Dilution of 25 parts of vegetable oil with 75 part of diesel fuel (Schwab et al. 1987).
- Pyrolysis- chemical change caused by thermal energy in the presence of air or nitrogen (Schwab et al. 1988).
- Micro-emulsions with short chain alcohols (e.g. ethanol or methanol) (Schwab et al. 1987).

- Thermal decomposition, which produces alkanes, alkenes, carboxylic acid and aromatic compounds (Schwab et al. 1988).
- Catalytic cracking, which produces alkanes, cycloalkanes and alkyl benzenes (da Rocha Filho et al. 1993).
- Transesterification with ethanol or methanol (Freedman et al. 1986).

2.1. Transesterification

Transesterification is a process in which the alkoxy group of an ester compound exchange with other alcohol, alcoholysis is an alternative term that is frequently used for transesterification. The transesterification reaction of fat or triglyceride with an alcohol to form fatty acid alkyl esters, it is the most popular method for producing methyl and ethyl esters of long chain fatty acids (Gerpen 2005). The methyl or ethyl esters of fatty acids can be burnt directly in diesel engines with low deposit formation (Graille et al. 1985; Mittelbach et al. 1988; Zaher 1990). This product is an excellent substitutes for diesel fuel and termed as biodiesel.

The overall process of transesterification reaction is a sequence of three consecutive and reversible reactions, in which TAG react with monohydric alcohol in the presence of catalyst to form fatty acid alkyl ester along with diglycerides and monoglycerides, which are intermediate compounds (Ma et al. 1999).

The stepwise process of conversion of TAG to biodiesel involves

- Initial reaction of alcohol with TAG as alkoxide anion to produce fatty acid alkyl esters (FAAE) and diacylglycerols
- Reaction of products with alkoxide to liberate another molecule of FAAE and production of monoacyl glycerol
- Alcoholysis of MAG (Mono acyl glycerides) to yield glycerol and FAAE

The FAAE is collectively known as bio-diesel. Every one mole of TAG produces three moles of biodiesel and one mole of glycerol after complete conversion (Freedman et al. 1986). Transesterification is reversible, although the reverse reaction (production of MAG from FAAE and glycerol) does not take place largely due to immiscibility of glycerol with FAAE. Alcohols that are suitable for use in carrying out transesterification reaction include methanol, ethanol, propanol, butanol and amyl-alcohol, with methanol and ethanol being utilized more frequently. The transesterification reaction brings drastic changes in viscosity of the triglycerides. The product of transesterification reaction has a lower viscosity due to removal of high viscous component (glycerol). The biodiesel produced is totally miscible with diesel in any proportion (Fukuda et al. 2001). The process of transesterification for biodiesel generation is affected by several process parameters viz; reaction time, reaction temperature, presence of moisture and free fatty acids (FFAs), molar ratio of alcohol and oil and catalyst type. There are two approaches for transesterification of vegetable oils for the production of biodiesel (Haas et al. 2002). The first and common being the alcoholysis of oil carried out with methyl or ethyl alcohol in the presence of strong acid or/and strong base to produce biodiesel. Another approach is the use of enzyme as catalyst, in which lipase catalyzed transesterification is carried out in aqueous and non-aqueous environment (Fukuda et al. 2001). Generally, transesterification reaction is carried out with homogenous or heterogeneous catalyst with varying reaction conditions.

2.2. Chemically catalyzed transesterification

The chemically catalyzed transesterification reaction with alcohol takes place in various steps, firstly being the conversion of triglycerides to diglycerides, followed by the conversion of diglycerides to monoglycerides and of monoglycerides to glycerol, yielding one methyl ester

molecule from each glyceride at each step. This reaction can be catalyzed chemically either by acid or alkali (Freedman et al. 1986; Noureddini et al. 1997).

Various types of acids have been used for transesterification which include sulfuric acid, phosphoric acid, hydrochloric acid and organic sulfonic acid (Freedman et al. 1984; Ma et al. 1999; Srivastava et al. 2000). Acid catalysts are insensitive to the presence of FFAs in the feedstock (Kulkarni et al. 2006) and can catalyze, esterification and transesterification simultaneously (Jacobson et al. 2008). Acid catalysis is more efficient when the amount of FFAs in the oil exceeds 1% (Van Gerpen et al. 1999; Zhang 2003). The use of acid catalysis is, however, not a popular choice for biodiesel production at industrial scale due to slower reaction rate, requirement of high reaction temperature, high molar ratio of alcohol to oil, separation of catalyst, serious environmental and corrosion related problems (Jacobson et al. 2008).

Biodiesel is commonly produced using base catalyst such as sodium hydroxide, potassium hydroxide, carbonates, and alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide and sodium butoxide (Felizardo et al. 2006). The rate of reaction of base-catalyzed reaction would be many times faster than that of acid-catalyzed reaction (Fukuda et al. 2001; Kulkarni et al. 2006). These base catalysts are commonly used in industries due to obvious reasons such as need of less reaction temperature to catalyze the reaction, high conversion of oil to ester in minimum time, easy availability and economic viability (Lotero et al. 2005). The use of base catalyst is limited only for refined vegetable oil with less than 1 wt% FFA (Wang et al. 2007). If the oil or fat contains high FFA, alkali catalyst will typically react with FFA to form soap, which is highly undesirable (Kulkarni et al. 2006; Yan et al. 2009).

A variety of heterogeneous catalysts have been examined in recent past for biodiesel production through transesterification. This include sulphated zirconia (Jitputti et al. 2006); tin

compounds supported in ion-exchange resins (Abreu et al. 2004); alkyl guanidine heterogenized on organic polymers (Gelbard et al. 1995); immobilized enzymes (Nelson et al. 1996; Shimada et al. 2002; Watanabe et al. 2000); calcium carbonate (Suppes et al. 2001); nanocrystalline nickel doped CaO (Kumar et al. 2016); tungsten supported Ti/SiO_2 nanoflowers (Kaur et al. 2018); γ-alumina-zirconia (Heydarzadeh et al. 2010); and Nb_2O_5/SO_4 (Loures et al. 2018). However, transesterification reactions catalyzed by heterogeneous catalysts require high temperature and pressure with longer reaction period and higher energy consumption (Meher et al. 2006).

2.3. Enzyme catalyzed transesterification

Due to the limitations of chemically catalyzed transesterification, enzymatic transesterification, especially those using lipase, have drawn researcher's attention in last ten years. Enzymatic reactions are excellent alternatives to chemical catalysts due to the obvious advantages such as mild reaction conditions; insensitive to high FFA; regiospecificity; immobilization possibilities; improve efficiency by genetic engineering and mutation; acceptability for wide variety of substrates and better thermo-stability. Other important advantages include their higher efficiency, high selective property, less energy consumption component, lesser production of side products or waste and better efficacy in immobilized forms (Akoh et al. 2007; Fjerbaek et al. 2009; Karimpil et al. 2012; Robles-Medina et al. 2009; Röttig et al. 2010).

Transesterification of the triglycerides such as vegetable oil, animal fats, fish oil and grease with alcohol has been studied by different research groups using lipase as a catalyst. In general, lipases sourced from microbial systems have been used for transesterification reaction such as *Mucor miehei* (Lipozym IM 60), *Candida antarctica* (Novozym 435) (Nelson et al. 1996), *Pseudomonas cepacia* (*PS 30*) (Wu et al. 1999), *Bacillus subtilis* (Ying et al. 2007), *Rhizopus oryzae* (Chen et al. 2006), and *Penicillium expansum* (Li et al. 2009). Almost all types of alcohols

can be used for enzymatic catalysis, however alcohols like methanol, which is short chain alcohol, readily inactivates lipase. Ethanol is more appropriate for enzymatic catalysis because the degree of inactivation of lipase in inversely proportional to the number of carbon atoms in the short chain alcohol (Chen et al. 2003). Generally, higher reaction rates are observed with use of longer chain alcohols, however, FAAE yield ultimately depends on the substrate specificity of the lipase (Fukuda et al. 2001; Shimada et al. 2002). Not only the kind of alcohol but also the ratio of alcohol to oil plays a vital role with 3:1 alcohol to oil sufficing to high yields with no enzymatic inactivation. *C. antarctica* lipase, which is otherwise inactivated by methanol/oil ratios of 0.5:1 or higher, has however been prevented by stepwise addition of alcohol (Shimada et al. 1999; Watanabe et al. 2000; Xu et al. 2003). Further, increase of alcohol yields marginally higher FAAE but at the same time causes lipase inactivation.

Nelson et al. (1996) investigated the abilities of lipases in transesterification with short-chain alcohols to yield alkyl esters. The lipase from *M. miehei* was the most efficient for converting triglycerides to their alkyl esters with primary alcohols, whereas enzyme sourced from *C. antarctica* was the most efficient for transesterifying triglycerides with secondary alcohols to give branched alkyl esters. Nelson et al. (1996) and Wu et al. (1999) carried out studies on *P. cepacia* (*PS 30* enzyme) and attempted to optimize the transesterification reaction with primary alcohol such as ethanol and reported ester yield to an extent of 85%. Shimada et al. (1999) found that immobilized *C. antarctica* lipase (Novozym 435) was the most effective for methanolysis among lipases tested. Watanabe et al. (2000) demonstrated effective methanolysis using two-step batch and three-step flow reaction systems with Novozym 435. The effect of pretreatment of Novozym 435 on methanolysis for biodiesel fuel production was also investigated by Samukawa et al. (2000). Royon et al. (2007) carried out enzymatic production of biodiesel by methanolysis of

virgin cottonseed oil using immobilized *C. antarctica* lipase as catalyst in *t*-butanol solvent which yielded in 97% after 24 h at 50°C with a reaction mixture containing 32.5% *t*-butanol, 13.5% methanol, 54% oil and 0.017g enzyme/g oil. Immobilization of lipase enzyme on magnetic nanoparticles were also carried out by many researchers for transesterification reaction. Karimi (2016) reported the biodiesel synthesis from waste cooking oil (WCO) using *B. cepacia* lipase immobilized on superparamagnetic iron oxide nanoparticles (SPION). The conversion of WCO to biodiesel reached 91% in 35 h at 35°C temperature with 6:1 molar ratio of methanol: oil, 25 wt% immobilized lipase concentration, 10 wt% n-hexane content and 10 wt% water content. *C. antarctica* lipase B (CALB) was covalently cross-linked to magnetic nanoparticles of magnetite and form cross-linked enzyme aggregates (CLEAs) used for the synthesis of biodiesel from non-edible vegetable and waste frying oils. The 1% mCLEAs (w/w of oil) as catalysts yielded near 92% conversion at 30°C after 72 h of reaction (Cruz-Izquierdo et al. 2014). Studies on utilization of different lipases for biodiesel production is available from many reviews. Despite various advantages, industrialization of biodiesel production using pure enzymes as catalysts, have been hurdled by certain disadvantages like high production and purification cost of lipase, lipase inactivation by acyl acceptors such as methanol, inactivation by minor components in the crude oil and waste oils, desorption from immobilization support, and fouling in packed bed bioreactors. These hurdles have led to pave a new path in research of biodiesel production leading towards utilization of from whole cells which have been recently worked out only by a few research groups (Röttig et al. 2010).

2.4. Whole cell catalyzed transesterification

The limitation of using pure enzymes prompted researchers into exploring the potential use of whole cell systems such as bacteria, yeast and filamentous fungi that produce lipase use as whole cell catalysts for transesterification reactions. Among the established whole-cell biocatalyst systems, filamentous fungi have proven to be the most robust for industrial applications. The use of *R. oryzae, R. chinensis,* recombinant *S. cerevisiae* and most recently *A. niger* as whole cell biocatalysts, have been studied and reviewed by different research groups (Hama et al. 2008; Röttig et al. 2010; Xiao et al. 2010). Prakash et al. (2011) reported the conversion of used oil to alkyl esters using *Aspergillus* sp. as a whole cell catalyst. However, other than those mentioned, there are very few reports available on the use of whole-cells as catalysts especially with fungal systems for generation of alkyl esters by transesterification reaction.

To utilize whole cell biocatalysts, with ease and efficacy, different techniques have been studied such as use of porous biomass support particles (BSP's) developed by Atkinson et al. (1979). This technique has several advantages over other methods in terms of industrial applications such as (i) limited requirement of chemical additives; (ii) avoiding the step of preproduction of cells; (iv) large mass transfer rate of substrate and production within BSPs; (v) the reusability of the support particles; (vi) reduced cost; and (vii) enhanced durability of the particles (Fukuda et al. 2008).

Zeng et al. (2006) reported the effects of different oils such as refined soybean, refined olive, refined cottonseed, crude rapeseed and crude soybean oils on the transesterification reaction catalyzed by whole cell *R. oryzae* IFO 4697. Xiao et al. (2010) isolated *A. niger* from *Jatropha curcas* seed and used as whole cell biocatalyst for transesterification of palm oil. The fatty acid methyl ester yield was observed 87% after 72 h under standard conditions at 40°C temperature and

8% water content in the medium. Biomass support particles (BSPs) were used for immobilization of cell of *R. oryzae* (PTCC5174) to carry out transesterification of used cooking oil with stepwise addition of methanol. The 46% conversion of free fatty acid to methyl ester was achieved after 72 h at 35°C with using 3:1 molar ratio of methanol to fatty acid (Pazouki et al. 2010). Koda et al. (2010) reported the use of two types of whole cell biocatalysts viz; wild type *R. oryzae* producing tri-acyl glycerol lipase (w-ROL) and recombinant *A. oryzae* expressing *F. heterosporum* lipase (r-FHL) for transesterification of rapeseed oil with ethyl alcohol. The extent of ethyl ester was observed 79% when using wild type strain and 94% while using recombinant strain. Hama et al. (2008) also observed that *F. heterosporum* lipase producing *A. oryzae* attained better methyl ester production and higher lipase stability as compared to wild type *R. oryzae*. Different types of vegetable oils like palm, olive, soybean and rapeseed oils were used for lipase production from *R. oryzae* (IFO 4697). The authors also observed that the content of unsaturated fatty acid present in the oil would directly influence the production of intracellular lipase of the organism (Sun et al. 2010).

The use of *R. oryzae* cells immobilized within biomass support particles as a whole cell biocatalyst for transesterification reaction was first demonstrated by Ban et al. (2001) wherein optimal culture conditions for intracellular lipase production and the effect of water content on methanolysis were established. When methanolysis was carried out in the presence of 10-20% water, with stepwise addition of methanol using BSP-immobilized cells, methyl ester content in the reaction mixture was observed to reach 80-90% without any solvent pretreatment. This level of production of methyl ester was observed as similar to that achieved using extracellular lipase (Kaieda et al. 1999). The stability of a biocatalyst is particularly important in industrial biocatalysis aimed at the production of fuels and chemicals. Observations such as these indicate promising

means of biodiesel fuel production for industrial applications, using whole cell catalysts, because of simplicity of the lipase production process as well as the stability of lipase activity over a long period.

For the immobilization of *R. oryzae* fungus cells, a circulating packed-bed bioreactor system was developed using fibrous non-woven fabric as the immobilization matrix (Chen et al. 2011). It could further be exploited for the transesterification of soybean oil with methanol. The authors predicted the yield of 72.6% under the optimum conditions that is 10.97% (w/w) water content, 0.64% molar ratio of methanol to oil, 2.25 (w/w) cell weight and reaction time of 23 h. *R. oryzae* IFO4697 whole cells immobilized within BSPs, were used with tert-butanol as solvent and 72% yield of methyl ester from soybean oil. Li et al. (2007) suggested that tert-butanol as a solvent could eliminate the negative effects of excess methanol and by-product glycerol which were mainly responsible for the poor activity and stability of the lipase in biodiesel production. Sun et al. (2010) demonstrated the use of immobilized whole cells of *R. oryzae* IFO4697 on BSPs and treated with glutaraldehyde (GA) for cross-linking. The authors reported that the GA cross-linking provides the stability to cells for repeated uses, high methanol tolerance and high catalytic activity. The GA treated whole cells convert renewable oils into biodiesel through transesterification upto 94.1% in 24 h. Kyeong et al. (2014) used *R. oryzae* NBRC 4697 whole cells immobilized on polyurethane foam coated with activated carbon for catalyzing transesterification reaction. A packed-bed bioreactor (PBB) containing the immobilized whole cell biocatalyst was operated under circulating batch mode. Stepwise methanol feeding was used to mitigate methanol inhibition of the immobilized cells in the reactor. An increase in the feeding rate (circulating rate) of the reaction mixture barely affected biodiesel production, while an increase in the packing volume of the immobilized cells enhanced biodiesel production. Andrade et al. (2014) reported the

immobilization of *Mucor circinelloides* URM 4182 for transesterification of babassu oil with ethyl alcohol. The author attained 98.1% ester yield at 35°C using 1:6 oil/ethanol molar ratio. Yeom (2016) also observed the effect of immobilization of whole cells on polyurethane foam with activated carbon and treated with 0.1% chloroform. The author reported that the biodiesel conversion increased with increase in the number of polyurethane foam until it occupied 2.4% of reaction mixture and stepwise addition of 4.5 molar ratio of soybean oil to methanol prevented inhibition of the immobilized whole cells. The maximum conversion was achieved 92.7% at 3% water content in medium. A novel approach of immobilized *A. niger* whole cell lipase-catalyzed conversion of *Scenedesmus obliquus* lipids into biodiesel was investigated. Highest biodiesel conversion of 53.76% was achieved at 35°C, methanol to oil ratio of 5:1 and 2.5% water content based on oil weight with 6 BSPs. Step-wise methanol addition was applied to account for methanol tolerance, which improved biodiesel conversion upto 80.97% and gave 90.82 ± 1.43% yield. Immobilized *A. niger* lipase could be used for 2 batches without significant loss in conversion efficiency by Guldhe et al. (2016). Yan et al. (2014) used recombinant *Pichia pastoris* yeast whole cell catalyst (WCC) with functional intracellular expression of *Thermomyces lanuginosus* lipase (Tll) for biodiesel production from waste cooking oils. The authors obtained maximum yield of biodiesel 82% within 84 h at 6 wt.% concentration of whole cells as catalyst. Chen et al. (2016) reported the use of *Pseudomonas mendocina* cells immobilized into Fe_3O_4-chitosan microspheres (magnetic whole-cell biocatalysts, MWCBs) for biodiesel production from soybean oil. A biodiesel yield of 87.32% was obtained at 35°C in 48 h with 4:1 molar ratio of methanol to oil, 10 wt.% concentration of MWCBs and 10 wt.% concentration of water content in the reaction mixture. The transesterification of waste cooking oil (WCO) was also carried out in a magnetically

fluidized bed reactor (MFBR) by using *P. mendocina* cells immobilized in magnetic microspheres as catalysts. The biodiesel yield of 91.8% was obtained at 35°C after 48 h (Chen et al. 2017).

These past to recent studies have demonstrated the use of whole-cell systems and their immobilized forms for transesterification reactions, which gave impetus to our studies.

2.5. Acid oil as feedstock for biodiesel production

Commercialization of biodiesel is majorly concerned with and affected by the cost of feedstock. Therefore, different types of feedstocks are being attempted as raw materials for biodiesel production including vegetables oils, such as soyabean, canola, sunflower, corn, cottonseed, mustard, palm oil; animal fats (beet tallow or pork lard); restaurant waste cooking oils; float grease (from wastewater treatment plants), and by products of edible oil refining, like acid oil and soap stock (Gunstone et al. 2001).

Zhang (2003) reported that the cost of feedstock oil is one of the most significant factors which affect the economic viability of biodiesel manufacturing. Extensive use of biodiesel is mainly concerned with the economics of biodiesel production as demonstrated by Dorado et al. (2002). The high cost of virgin edible oils, especially in countries like India, has been a major hinderance in their use as raw materials for biodiesel production. The feedstock cost contributes more than 70-95% to the overall production cost of biodiesel (Connemann et al. 1998; Haas 2005; Krawczyk 1996). However, easily available and relatively cheap raw material like acid oils, waste cooking oil and non-edible feedstock may prove markably advantageous in cutting the production cost of biodiesel (Nisworo 2005; Zhang 2003). As an effective alternative, feedstocks like acid oils and waste edible oils are being extensively evaluated as possible substitutes (Marchetti et al. 2008). Amongst these feedstocks, acid oil, has, therefore, been considered to be a viable raw material, as it is cost-effective and readily available in significant quantities (Kulkarni et al. 2008).

Acid oil comprises of 40-80% long chain free fatty acids mixture, 1-2% mineral acids, 5-8% free moisture, 8-10% phospholipids and sterols, 20-50% neutral glycerides and unsaponifiables of the oils and other impurities. All these impart a characteristic pungent odour and dark brown colour to the acid oil. Acid oil's fuel properties are different from diesel fuel due to its oxygenated nature and presence of chain type of configurational compounds. The heating value of acid oils is slightly lower but viscosity and ignition values are higher than diesel fuel (Kulkarni et al. 2008; Tüter et al. 2004).

The conventional alkali catalysts are not suitable to acid oil, as the process results in soap formation due to high free fatty acids content (40-80%) (Fukuda et al. 2001). Haas et al. (2006) reported the combined nonenzymatic-enzymatic method for synthesis of fatty acid alkyl esters from the soapstock containing 10% FFA and 10.1% triglycerides. First step involved transesterification of glyceride and phosphoglyceride-linked fatty acids by using potassium hydroxide (KOH) as catalyst with 20:1 molar ratio of alcohol to lipid-linked fatty acids. The second step of the process involved use of lipase enzyme SP-435 (immobilized *C. antarctica* lipase B) as a catalyst. The maximum conversion obtained from this combined process was 81%. Haas et al. (2003) used acidulation process for production of acid oil with high acid content from soybean soapstock. Prior to acidulation, the acyl and phosphoacyl-FA glyceride esters bonds of soapstock were alkali-hydrolyzed to produce acid oil with high acid value. A maximum production of FAME was obtained 89% at molar ratio 1:1.8:0.17 FFA/methanol/acid and generated ethyl ester was subjected to washing with NaCl-$NaHCO_3$ resulting in complete removal of unreacted FFA. Besides acidulation, another approach was also used to recover acid oil from soapstock, wherein, isopropanol was used as a solvent and heated to 60°C with addition of sulphuric acid. The recovery yield of acid oil was about 97% (w/w) based on the total fatty acids of the soap stock (Reaney

2002). Wang et al. (2007) reported the generation of biodiesel (97.6%) from acid oil containing 50% free fatty acids. The acid oil was directly converted to biodiesel at 95°C under the pressurized condition (4 Kgf/cm^2) in reactor within 5 h in the presence of 1:1.5:0.1 ratio of acid oil/methanol/sulfuric acid. Higher reaction temperature was observed to facilitate shortening of the reaction time and required less amount of catalyst and methanol. Marchetti et al. (2010) used synthetic acid oil as a raw material for generation of biodiesel by using solid basic resin (Dowex monosphere 550A) as a catalyst. Solid basic resin was used as an alternative catalysts to produce fatty acid ethyl esters with final conversion over 90% at optimum molar ratio 6.1:1 of alcohol/mixture. The catalyst facilitated better conversion when the amount of initial free fatty acid increased with faster initial reaction rate. Thus, acid catalysis requires a time-consuming and cost-intensive multi-step approach than conventional technology.

The esterification of acid oil to alkyl esters using enzymatic and whole cell catalysis however, have been reported to limited extent. Transesterification of acid oil to alkyl esters has been attempted with pure enzymes by some research groups. Some of prominent observations of Ghosh et al. (1995) included the transesterification reaction of different types of acid oils such as coconut, soybean, mustard, sunflower, and rice bran with *C. cylindracea* lipase as a catalyst for hydrolysis followed by esterification or alcoholysis with *M. miehei* lipase. The acid oils were hydrolyzed almost completely within 48 h and the fatty acids were converted into fatty acid esters of short and long chain alcohols. The *C. antarctica* (Novozym 435) lipase B was also used for esterification of corn and sunflower acid oils with straight and branched chain alcohols resulting 50% and 70% conversion to fatty acid alkyl esters respectively (Tüter et al. 2004). Li et al. (2007) reported the use of immobilized whole cells of *R. oryzae* within the biomass support particles for production of biodiesel from acidified, refined and crude rapeseed oil in tert-butanol system, where

tert-butanol was used as media component to enhance the stability of *R. oryzae* whole cells. The authors obtained maximum yield of 70% methyl ester when rapeseed acid oil was used as a feedstock in place of refined and crude rapeseed oil. Zhou et al. (2015) uses a two-step biocatalytic process using lipase and whole cell catalysts for biodiesel production from unrefined jatropha oil. The hydrolysis reaction was carried out by using the commercial enzyme *C. rugosa* lipase followed by esterification reaction carried out by using *R. oryzae* IFO4697 cells immobilized within biomass support particles. The authors obtained maximum yield of 88.6% fatty acid methyl ester at 35°C in 42 h. The immobilized lipase Lipozyme TL IM was also employed for the transesterification reaction of rice bran acid oil in packed-bed reactor, resulting in maximum yield 92 % in 4 h (Choi et al. 2016). Aguieiras et al. (2014) developed a process of hydroesterification for biodiesel production from acid oil of *Acrocomia aculeata* with ethyl alcohol using lipase from *Rhizomucor miehei* to catalyzed esterification reaction. The authors obtained maximum yield of ethyl ester 92.2% in 72 h in dry fermented solid obtained by solid state fermentation. Further, one stage lipase (Novozym 435) catalyzed methanolysis was employed for generation of biodiesel from soapstock oil. A maximum FAME yield of 95.2% was obtained in 10 h with 4% (w/w) of lipase and methanol/oil molar ratio of 5:1 at 45°C with addition of tert-pentanol and use of a 10-fold 3 Å molecular sieve (Su et al. 2014). Soares et al. (2013) reported the biodiesel production from soybean soapstock acid oil using hydroesterification process and *B. cepacia* LTEB 11 lipase enzyme. The author obtained complete hydrolysis of feedstock to FFA in subcritical water followed by esterification in packed-batch reactor containing fermented solid with 92% conversion in 31 h at 50°C in a solvent-free system. Table 2.1 represents the use of various catalysts for generation of biodiesel from acid oil.

Table 2.1. Transesterification of acid oils by different catalysts

Catalysts	Temp (°C)	Alcohol	Molar ratio FFA/alcohol	Reaction time (h)	Yield (%)	FFA (%) in acid oil	References
Homogeneous acid catalyst							
H_2SO_4	35	Methanol	1:30	2-4	99	Soybean (26.6)	(Haas et al. 2000)
H_2SO_4	95	Methanol	1:1.5	5	94	Soybean (35)	(Wang et al. 2007)
H_2SO_4	65	Methanol	1:30	14	99	Soybean	(Haas 2005)
H_2SO_4	65	Methanol	1:15	26	89	Soybean (59.3)	(Haas et al. 2003)
H_2SO_4	-	Methanol	-	-	100	Soybean (72.33)	(Tripathi et al. 2017)
Heterogeneous catalyst							
Solid basic resin	55	Ethanol	1:6.1	3	90	Mixture of refined oil and oleic acid (19.49)	(Marchetti et al. 2010)
Sulphonated carbon catalyst	180	Methanol	1:30	5	99	*Calophyllum inophyllum* (15)	(Dawodu et al. 2014)
Solid superacid catalyst (SO_4^{2-} /ZrO_2-TiO_2/La^{3+})	200	Methanol	15:1	2	96.24	Acid oil (60)	(Li et al. 2010)
Bifunctional Solid Catalyst	200	Methanol	15 (wt%)	4	80	High acid oil (50)	(Jeong et al. 2017)
Enzymatic catalyst							
C.antarctica lipase	30	Methanol	5.5 wt%	48	98.5		(Watanabe et al. 2005)
C.antarctica lipase B	40	Methanol	1:1	1.5	63.6	Sunflower (55.6)	(Tüter et al. 2004)
M.miehei lipase	60	Methanol	1:1	4	77 & 90	Sunflower (38) & Mustard (76.5)	(Ghosh et al. 1995)
C. cylindracea lipase	35	Methanol	1:1	48			(Ghosh et al. 1995)
Novozym 435, Lipozyme RM IM	40	Ethanol	1:5		92	Rice bran (53.7)	(Choi, Lee, et al. 2016)
Lipozyme TL IM	20	Ethanol	1:4	4	92	Rice bran	(Choi, Kim, et al. 2016)
Rhizopus oryzae lipase	30	Methanol	-	36	100		(Bonet-Ragel et al. 2015)
Whole-cell biocatalyst							
Rhizopus oryzae IFO4697	35	Methanol	1:1.2	42	88.6	Jatropha	(Zhou et al. 2015)
Rhizopus oryzae	35	Methanol	4:1	24	70	Acidified rapeseed	(Li et al. 2007)

2.6. Biodiesel properties and specifications

Successful commercialization, market acceptance of biodiesel and its increasing use as fuel, is possible only with assurance of desirable fuel properties and quality. In order to make sure that only high quality biodiesel reaches the market place, Biodiesel standards are in place in a number of countries. Further, biodiesel standards are being established or developed in various countries, for the products derived from various feed stocks. The three important fuel standards, ASTM D6751 (ASTM 2006) in United States, EN 14214 (European Committee for Standardization, CEN) (CEN2003a) in the European Union and IS 15607:2005 (Indian Standard) are followed for determining the quality parameters of biodiesel. It is mandatory for the biodiesel component to satisfy the requisites of these standards before inclusion as an alternative fuel to petroleum diesel.

It is therefore, imperative to ensure that the quality of biodiesel, produced by biocatalytic approach and using renewable feedstocks, as per the parameters outlined under international standards, so as to make the product competitive to conventional diesel and chemically catalyzed biodiesel. As-on-date, this aspect has not been seriously considered for further application of biodiesel at scale-up production or application levels.

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The use of biodiesel has become more attractive because of its environmental benefits and sourcing from renewable resources. The main hurdle for the production of biodiesel is its cost and limited availability of fats and oil resources. The cost of raw material accounts upto 90% of the total cost of biodiesel fuel. This can be overcome by the choice of raw material used, type of catalyst, and easily recoverable by-product. The use of acid oil can significantly reduce the economic implication involved the production. The process of transesterification and by-product

recovery contributes another important share in reducing the cost associated with production of biodiesel. In the literature, biocatalysis has been noted to gain importance with increasing focus on whole cell catalyzed transesterification that may reduce the cost of these processes.

Keeping in view, the limited observations on biocatalyzed transesterification especially with fungal system and acid oils as raw material, the study was envisaged with following objectives.

Objectives

1. Screening of fungal isolates for their potential to mediate transesterification/esterification of acid oil;
2. Optimization of culture conditions to enhance biomass production and generation of alkyl esters from acid oil; and
3. Characterization and evaluation of properties of the alkyl esters.

3.0 Materials and Methods

3.1. Screening of fungal strains on the basis of acid oil tolerance and hydrolytic activity

The five fungal strains; *Aspergillus flavus* (MTCC 5436), *Aspergillus aculeatus, Aspergillus sydowii* (MTCC 10397), *Curvularia pallescens* (MTCC 10390) and *Periconia sp* (MTCC 10391) were isolated from the contaminated clarified butter. *Rhizopus oryzae* was obtained from Bhabha Atomic Research Centre, Mumbai. These strains were screened on the basis of hydrolysis and tolerance of acid oil. The active culture of strains were inoculated in mineral salt medium containing magnesium sulphate (0.20 g/l), calcium chloride (0.02 g/l), monopotassium phosphate (1.0 g/l), di-potassium phosphate (1.00 g/l) and ferric chloride (0.05 g/l) and acid oil was used as carbon source for screening purpose.

Inocula of different strains, were subjected to growth in two different levels (50% (v/v) and 70% (v/v)) of acid oil supplementation in the basal medium and incubated for 72 h at 30ºC and 120 rpm, to examine the acid oil tolerance. Viability of the strains were checked after 72 h of growth by plating the biomass on PDA (potato dextrose agar) and observing the re-growth of the strain. Hydrolytic activity of the fungal strains were checked by determining the free fatty acid (FFA) produced after 72 h of incubation.

3.2. Improvement of strain through gamma irradiation and screening of variants

Spore suspension of *A. flavus* (RBD-01; MTCC 5436), was irradiated at different doses (i.e. 50Gy, 100Gy, 200Gy, 300Gy, 400Gy) of gamma radiation using gamma irradiator with dose rate of 4.8Gy/min. The irradiated spores were serially diluted to 10^{-4} and grown on PDA-Ox gall medium along with spores from non-irradiated culture. The effect of irradiation on the growth time and morphology of spores was noted. The irradiated varaints (i.e.50Gy, 100Gy, 200Gy, 300Gy,

400Gy) were further screened for their lipolytic activity on the basis of colour zone formation on spirit-blue agar medium. The spirit blue agar media was prepared in distilled water followed by addition of 3% olive oil as carbon source for fungal growth. The irradiated variants were inoculated on spirit blue agar medium and incubated at 30°C, for 48 h. After screening on the basis of zone formation, the 300Gy variants (AKS-01 to AKS-18) were further screened for their lipase production/activity through shake flask fermentation.

3.2.1. Preparation of inoculum

The colonies of selected variants were inoculated aseptically in 500ml Erlenmeyer flask containing 200 ml of sterile PDB (potato dextrose broth) and incubated at 30°C, 120 rpm for 3 days. The culture were used for streaking over potato dextrose agar plate and incubate at 30°C for 24 h.

3.2.2. Cultivation and acclimatization of culture

The active culture of selected variants was inoculated in 100 mL of minimal medium (Bhushnell-Hass broth (BHB)) containing $MgSO_4$ (0.2 g/l), $CaCl_2$ (0.02 g/l), KH_2PO_4 (1.0 g/l), K_2HPO_4 (1.0 g/l) and $FeCl_3$ (0.05 g/l). The medium was further supplemented with mycological peptone (0.5% w/v), di-ammonium hydrogen orthophosphate $(NH_4)_2HPO_4$ (0.5% w/v) as nitrogen sources and cottonseed oil (10% v/v) as main carbon source. The culture was incubated at 120 rpm and 30°C for 72 h. The active culture obtained was further used for experimentation.

3.2.3. Preparation of dried biomass

The acclimatized biomass (section 3.2.2) was separated by filtering through Whatman filter paper. The filtered biomass was washed with hexane to remove the excess of oil and FFA (free fatty acid) and dried overnight at 30°C. The dried biomass was crushed in liquid nitrogen using

pestle-mortar to prepare homogenous powder. The powdered biomass was used for estimating intracellular lipase activity and the cell free supernatant (CFS) was further separated from oil using a separating funnel. The CFS was centrifuged to remove the debris and used for estimating extracellular lipase activity.

3.2.4. Determination of intracellular enzyme activity

The intracellular lipase enzyme activity was determined by adding 1.0 g of dried biomass to a reaction mixture containing 0.8 mL of 0.05 M phosphate buffer (pH 7.0) and 0.1 mL of 0.05 M *p*NP (*p*-nitrophenol laurate) in ethanol. The mixture was incubated at 30°C for 30 min, followed by addition of 0.25 mL of ethanol, on cooling, to stop the reaction. The activity was determined at 420 nm. One unit of lipase activity is defined as the amount of enzyme that liberates 1 µg *p*-nitrophenol with *p*NP-laurate as substrate under standard assay conditions in 30 min (Sigurgísladóttir et al. 1993). The calculations of lipase units were carried out by preparing a standard curve using commercial lipase enzyme [Sigma-Aldrich L1754-25G].

3.2.5. Determination of extracellular enzyme activity

Nine variants (AKS-05, AKS-06, AKS-07, AKS-08, AKS-09, AKS-10, AKS-11, AKS-17 and AKS-18) were selected and termed as sub-variants on the basis of intracellular lipase activity. These were further screened for extracellular lipase activity. The extracellular enzyme activity was also determined by above method outlined (section 3.2.4). 0.1 mL of CFS was added to the reaction, instead of dried biomass.

3.2.6. Screening on the basis of acid oil tolerance and hydrolytic activity

Of the nine sub-variants, five sub-variants (AKS-08, AKS-10, AKS-11, AKS-17 and AKS-18) were further screened on the basis of hydrolysis and tolerance of acid oil. The active culture

of variants were inoculated in mineral salt medium containing acid oil as carbon source for screening purpose. Inocula of different irradiated variants, were grown in different levels of acid oil supplementation (50% v/v - 80% v/v) in the basal medium and incubated for 72 h at 30ºC and 120 rpm, to examine the acid oil tolerance. Hydrolytic activity of the fungal strains was checked by determining the FFA produced after 72 h of incubation.

3.3. Optimization of culture conditions for lipase production

Gamma irradiation did not significantly improve the lipase activity of the strain therefore, wild type strain (*A. flavus*) was used for optimization of culture conditions and subsequent further studies. Mineral salt medium supplemented with acid oil (carbon source as well as lipase inducer) was used for optimization study. Various step-wise modifications were carried out in growth conditions (Table 3.1) so as to standardize parameters for obtaining optimum lipase activity. The enzyme activity was determined after 72 h in all the steps outlined. In the step-1, *A. flavus* was grown in mineral salt medium along with 10% acid oil by varying percentage of different organic (peptone and urea) and inorganic ($NaNO_3$, $(NH_4)_2HPO_4$) nitrogen sources (0.5% w/v - 2.5% w/v) maintaining pH 7.5 ± 0.2 at 30ºC. In step-2, pH of the growth medium was modulated from 5.0 to 8.5 by maintaining acid oil supplementation at 10% and using peptone as nitrogen source (0.5% w/v) with growth conditions set at 30ºC. In step-3, modulation was carried out in the percent supplementation of acid oil (40% v/v -90% v/v) to growth medium that contained mineral salt medium and peptone (0.5% w/v) and growth condition set at 30ºC with pH 7.5.

Table 3.1. Experimental layout for lipase production

	Constant Parameter	Variable Parameter	Range
Step 1	Minimal salt medium + 10% acid oil + pH 7.5 + temperature 30°C	Nitrogen source: Peptone; bi-ammonium hydrogen orthophosphate (BAHP); urea; or sodium nitrate;	0.5% to 2.5% (w/v)
Step 2	Minimal salt medium + 10% acid oil + 0.5% peptone + temperature 30°C	pH	5.0 to 8.5
Step 3	Minimal salt medium + 0.5% peptone + pH 7.5 + temperature 30°C	Percentage of acid oil in the growth medium	40- 90%

3.4. Physiochemical properties, FFA content and composition of acid oils

Physical (density, viscosity, moisture content) and chemical analysis (iodine value, saponification value, ash content, acidity) of the acid oil were carried out according to the Indian standard method SP: 18 [P:13]-1984.

Free fatty acids (FFA) content in acid oil was determined by the standard method proposed by AOCS Ca5a-40 (AOCS, 1989). 2.0 gm of sample was dissolved in iso-propyl alcohol and toluene (1:1 ratio). It was titrated against standardized 0.1 M NaOH solution and phenolphthalein used as an indicator. The results were presented in percent FFA expressed as oleic acid wherein the molecular weight of oleic acid (282) divided by the sample weight.

$$\%FFA = (Alkali\ Vol.(mL) \times (Alkali\ Normality \times 28.2)/(Sample\ Wt.(g)) \quad (1)$$

The fatty acid composition of acid oil was determined by preparation of fatty acid methyl ester (FAME) of the acid oils through transesterification using hydrochloric (HCl) acid as catalyst. The generated FAME was analyzed by using gas chromatograph GC-5765 (Nucon, India)

equipped with a fused silica capillary column (30-m length, 0.25-µm film thickness and 0.25-mm internal diameter), wall coated with EC wax (polyethene glycol) and a flame-ionization detector. Nitrogen was used as a carrier gas with flow rate 30 mL/min. Hydrogen gas and zero air were used for ignition with flow rates 30 mL/min and 300 mL/min respectively. The injector and detector temperature were maintained at 230°C and 240°C respectively. The initial oven temperature was 140°C with 1 min hold time while final oven temperature was 240°C. Temperature was increased at the rate of 4°C/min and the programme was completed in 30 min.

3.5. Transesterification studies

3.5.1. Transesterification of different acid oils

Inoculum preparation (section 3.2.1. and 3.2.2.) and standardization of maximum lipase activity was carried out in section 3.3 (Table 3.1). For the transesterification reaction, before inoculation, Bushnell-Hass broth (minimal medium) supplemented with CS (cottonseed acid oil) (70 % v/v acid oil and 30% v/v media), was sterilized at 121^{o}C, 15 psi for 15 min. The active culture, was further inoculated in medium and incubated for 72 h at 120 rpm and 30˚C for hydrolytic reaction. Subsequent to the hydrolytic reaction, time interval of ethanol addition was optimized through one-time addition (0 h), stepwise mode (4 times) at an intervals of 4 h and stepwise mode (3 times) at an intervals of 12 h. Followed by incubation for 24 h, the biomass was filtered from the reaction mixture using Whatman filter paper and the fitered fatty acid ethyl ester (FAEE) was dissolved in hexane to separate out from aqueous layer. The hexane was evaporated out using Rota-evaporator (IKA RV10) and the ethyl ester, thus obtained was used for further analysis.

Similar procedure was followed for carrying out the transesterification reactions with other acid oils viz; SO (soybean acid oil), SUN (sunflower acid oil) and RB (rice bran acid oil) (70% v/v acid oil and 30% v/v media).

3.5.2. Time dependent variations in transesterification reaction

To examine the total reaction time of transesterification, observations on time dependent variations in the ester generation were recorded using whole cell catalyst. The culture, cultivated in soybean acid oil, was incubated in conditions as mentioned earlier. Through this period of incubation, samples were collected at periodic intervals of 4 h and subjected to thin layer chromatography (TLC) and further analyzed by ^{1}H NMR. Subsequent to the hydrolytic reaction over 12 h, ethanol was added (one-time addition) at 1:4 molar ratio (oil to alcohol) in the reaction. Followed by alcohol addition, samples were collected at an interval of 1 h upto 24 h and washed with hexane to separate out the aqueous layer. In extension of transesterification reaction, to enhance the yield of ester, after hydrolytic reaction of 12 h duration, esterification was also carried out with stepwise (3 times) addition of ethanol at regular intervals of 12 h, followed by 12 h incubation. The obtained ethyl ester was washed with hexane to separate out the aqueous layer. Hexane was evaporated out on water bath and the esters, thus obtained, were confirmed using TLC with silica gel-G and then quantified using ^{1}H NMR. The FAEE, thus obtained from transesterification reaction, was washed with 28 vol% batches of 5% (w/v) NaCl in tap water followed by 20 vol% of 11% (w/v) $NaHCO_3$ in tap water to remove residual unreacted FFA. The FAEE recovered after washing was quantified by using ^{1}H NMR.

3.6. Transesterification of acid and virgin cottonseed oil: Influence of different acyl acceptors

3.6.1. Transesterification with biomass suspension

500 mg of freshly grown biomass, was inoculated in 100 mL mineral media containing 30% (v/v) BHB supplemented with 70% (v/v) acid oil/virgin oil in each flask, separately and incubated at 30°C and 120 rpm for carrying out the hydrolytic reaction. Through this period of incubation, samples were collected at periodic intervals of 4 h and subjected to thin layer chromatography (TLC) to monitor the formation of FFA. The enzyme units of biomass were determined (section 3.2.4.) after complete hydrolysis of oil. Subsequent to the hydrolytic reaction, the stepwise (12 h interval) addition of different alcohols (1:4 molar ratio) viz., methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol and decanol was carried out in each flask separately. After the addition of alcohol and further incubation for 12 h, the alkyl esters were separated from the reaction mixture and quantified using ^{1}H NMR. All reactions were carried out in triplicates with both feedstocks.

3.6.2. Transesterification with dried biomass

211 mg of dried powdered biomass (prepared as in section 3.2.3) was inoculated in round bottomed flask containing 10 mL of acid oil/virgin oil. The addition (one-time addition) of 1:4 molar ratio of oil to alcohol (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol and decanol) was carried out in each flask separately and the mixture was stirred for 36 h at 30°C. The progress of the reactions were checked regularly by thin layer chromatography. After 36 h, the reaction mixtures were washed three times with hexane to separate the product and quantified by using ^{1}H NMR.

3.7. Transesterification reaction of acid oil with immobilized biomass

3.7.1. Immobilization and cross linking of culture on polyurethane foam

The cells were immobilized on commercial grade polyurethane foam (PUF) (cut into cubical pieces of dimensions 1×1×1 cm). Polyurethane pieces were washed with deionized water and taken in 1000 mL Erlenmeyer flasks containing 500 mL PDB, followed by sterilization at 121°C, 15 psi for 15 min. The freshly grown spores of *A. flavus* were inoculated and incubated for 72 h at 30ºC and 180 rpm. After incubation, the biomass filled matrices were removed from the culture flasks and shaken with phosphate buffer (50 mM, pH 7.0) for 10 min. The immobilized PUF was washed twice with phosphate buffer and dried for 24 h at room temperature. The immobilized cells on the PUF were treated with 0.1% glutaraldehyde solution for 1 h for cross-linking, followed by washing with phosphate buffer afterward drying at room temperature for 24 h. The extent of immobilization of the cells on the support was assessed by scanning electron microscope (JEOL JSM 6510 LV), which clearly show proper immobilization of the cells on the surface of PUF in high density.

3.7.2. Bradford assay

The protein content of immobilized culture was determined by Bradford assay using BSA (Bovine Serum Albumin) protein as the standard. An immobilized PUF piece (containing 10±0.7 mg biomass) was dipped in Bradford reagent in a test tube and incubated at room temperature for 5 min followed by the recording of absorbance at 595 nm.

3.7.3. Time dependent variations in transesterification reaction with immobilized biomass: Shake flask conditions

Fifteen pieces of immobilized matrices were inoculated separately in BHB medium supplemented with acid oil (70 %v/v oil and 30 %v/v media) and incubated at 30˚C at 120 rpm for

carrying out hydrolytic reaction. Through this period of incubation, samples were collected at periodic intervals of 4 h and subjected to thin layer chromatography (TLC) and further analyzed by ^{1}H NMR. Subsequent to the hydrolytic reaction over 12 h, ethanol was added (one-time addition, 1:4 molar ratio) to the reaction. Followed by ethanol addition, samples were collected at an interval of 1 h upto 24 h and the ethyl ester was separate out from reaction mixture and quantified by using ^{1}H NMR.

3.8. Optimization of reaction conditions using response surface methodology

The Box-Behnken design (BBD) of response surface methodology was used for optimization of lipase activity vis-a-vis transesterification reaction by modifying various parameters associated with the reaction conditions.

3.8.1. Design of Experiment

In this study, a 3-level-4-factor BBD was employed, requiring 30 experiments. The variables and their levels selected to enhance lipase enzyme activity were: temperature range (26°C to 36°C), pH (5.5 to 8.5), peptone concentration (0.5% to 2.5%) and number of immobilized PUF pieces (5 to 15). Table 3.2 and 3.3 represents the factors in three different coded levels: low (−1), middle (0) and high (+1) and 30-trial experimental designs. All experiments were performed in 100 mL Bushnell-Hass broth (BHB) minimal medium with 70% (v/v) acid oil as main carbon source/lipase inducer and incubated at 120 rpm for 24 h.

Table 3.2. Variables and experimental design levels for response surface

S.no		Factors	Levels		
			-1	0	+1
1	A	pH	5.5	7.0	8.5
2	B	Temperature (°C)	26	31	36
3	C	Peptone (%)	0.5	1.5	2.5
4	D	Immobilized pieces (No.)	05	10	15

Table 3.3. Box-Behnken design matrix and responses of independent variables

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Std	Run	A:pH	B:Temperature (°C)	C: Peptone (%w/v)	D:pieces (No.)	Activity (U/mL)
1	1	5.5	26	1.5	10	0.81
2	2	8.5	26	1.5	10	0.971
3	3	5.5	36	1.5	10	1.111
4	4	8.5	36	1.5	10	1.569
5	5	7	31	0.5	5	0.802
6	6	7	31	2.5	5	0.841
7	7	7	31	0.5	15	2.381
8	8	7	31	2.5	15	2.544
9	**9**	**5.5**	**31**	**1.5**	**5**	**0.534**
10	10	8.5	31	1.5	5	0.679
11	11	5.5	31	1.5	15	1.435
12	12	8.5	31	1.5	15	2.088
13	13	7	26	0.5	10	1.852
14	14	7	36	0.5	10	1.942
15	15	7	26	2.5	10	1.972
16	16	7	36	2.5	10	2.109
17	17	5.5	31	0.5	10	1.435
18	18	8.5	31	0.5	10	1.81
19	19	5.5	31	2.5	10	2.1
20	20	8.5	31	2.5	10	2.215
21	21	7	26	1.5	5	0.682
22	22	7	36	1.5	5	0.748
23	23	7	26	1.5	15	2.12
24	24	7	36	1.5	15	1.818
25	**25**	**7**	**31**	**1.5**	**10**	**3.102**
26	26	7	31	1.5	10	3.102
27	27	7	31	1.5	10	3.102
28	28	7	31	1.5	10	3.102
29	29	7	31	1.5	10	3.102
30	30	7	31	1.5	10	3.102

3.8.2. Lipase activity of immobilized culture

The enzyme activity of immobilized culture was determined by method described earlier in section 3.2.5. Once the lipase activity (U/mL) was measured, the second order polynomial coefficients were calculated and investigated using the 'Design Expert' software (Version 10, Stat-Ease Inc., Minneapolis, USA) trial package. The second degree polynomial equation is in the following form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_4 X_4^2 \quad (2)$$

Where Y is the predicted response, β_0 is the model constant; X_1, X_2, X_3 and X_4 are independent variables; β_1, β_2, β_3 and β_4 are linear coefficients and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients.

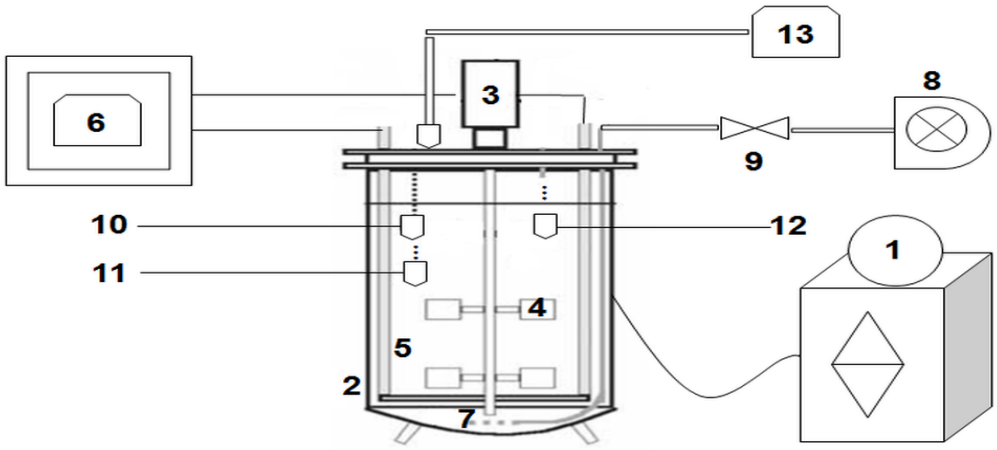
3.8.3. Statistical analysis

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis comprised Fisher's F-test; its associated probability p(F); determination coefficient R^2 which measure the goodness of fit of regression model; and correlation coefficient (R). The response surface curves were generated using Design Expert software (Version 10, Stat-Ease Inc., Minneapolis, USA) trial package and the quadratic model were represented as 3D contour plots.

3.9. Transesterification reaction of acid oil in bioreactor

A common cylinder-shaped glass vessel with a working volume of 3.6 L (total volume 5 L) was used for transesterification process. The reactor incorporated a stirring controller that allowed a stirring rate between 0 and 1000 rpm and a set of stainless steel soft edge cross-blades for proper agitation. A thermostatic bath was connected to an internal heat exchanger to control

the temperature of the reaction. The aeration was performed, by an external air pump, through a porous diffuser located underneath of glass vessel. The gas discharged through another air filter connected to CO_2 sensor. This system was also fixed with a pH sensor and dissolved oxygen sensor (Figure 3.1). The transesterification reaction was carried out in cylindrical vessel containing 1.5 L of mineral medium BHB (30%v/v) supplemented with acid oil (70%v/v) as feedstock. The medium was further supplemented with mycological peptone (1.2% w/v) as nitrogen source followed by sterilization at 121°C, 15 psi for 15 min. The 150 pieces (optimized) of immobilized PUF cubes were inoculated aseptically into glass vessel of reactor, for hydrolysis of acid oil to free fatty acids. A stirring of 150 rpm was kept constant throughout the experiment. The agitation speed was selected to provide suitable mixing without disturbing the pieces of PUF. The optimum temperature of the reaction system was kept at 31°C. The reactor was covered tightly throughout the experiments to prevent evaporation. Subsequent to the hydrolytic reaction over 6 h, alcoholysis took place through pulse feeding of alcohol (1:4 molar ratio of oil to alcohol) at the rate of 2.4 mL/min through peristaltic pump. Followed by alcohol addition, 5 ml sample was withdrawn from the reactor by a sampling port, at regular time intervals of 1 h upto 12 h for subsequent analysis and the reaction was continued upto 24 h. The collected samples were washed thrice with hexane to remove the aqueous layer from ester. Hexane was then evaporated out using rotary evaporator (IKA RV10) and obtained ethyl ester analyzed by ^{1}H NMR. The reusability potential of immobilized whole cells was studied upto 5 cycles. The matrices were used over 5 cycles by replacing the used medium with fresh medium and acid oil after each cycle.



1. Fermenter controller
2. Vessel
3. Stirrer
4. Impeller
5. Internal heat exchanger
6. Cryo-thermostat
7. Air diffuser
8. Air pump
9. Air filter (0.22 µm)
10. pH probe
11. Dissolve oxygen probe
12. Temperature probe
13. Carbon dioxide sensor

Figure 3.1. Schematic diagram of bioreactor

3.10. Kinetic Model

The rate of reaction for product (ethyl ester) formation and substrate (FFA) consumption were fitted to Michealis-Menten kinetics model.

$$v = \frac{V_{max}\,[S]}{K_M + [S]} \qquad (3)$$

Various notations are: v-initial velocity of the reaction, S-substrate concentration, V_{max} -maximum reaction velocity or rate of product formation, K_M-Michealis constant. The parameters K_M and V_{max} in the Michealis-Menten kinetic expression have physical significance. The Michealis constant, K_M, represents the substrate concentration at which half the enzyme active sites are filled by substrate molecules. V_{max} represents the maximum rate attainable; it is the rate at which the total enzyme concentration is present as the enzyme-substrate complex. Numerical values of these

parameters of Michealis-Menten kinetics model can be determined by measuring initial reaction velocity of ethyl ester generation for the initial substrate concentrations and solving the Michealis kinetic equation with these values. The transesterification rate calculated using reduction in concentration of FFA or increase in concentration of the final product (ethyl ester).

3.11. Identification and quantification of the transesterified products

3.11.1. Identification of alkyl esters

The alkyl esters separated from reactions were analysed by using thin layer chromatography with silica gel G as stationery phase and hexane: ethyl acetate: acetic acid (90:10:1) as a mobile phase. The chromatogram was developed in the iodine chamber (Samukawa et al. 2000).

The esters were further quantified using proton nuclear magnetic resonance spectroscopy (^{1}H NMR) (400 MHz; JEOL JNM-ECS 400). Deuterated chloroform ($CDCl_3$) with tetra methyl silane (TMS) as internal standard, was used as solvent. ^{1}H NMR spectra were recorded with pulse duration of 2.18 sec with a relaxation delay of 4 sec and 64 scans.

3.11.2. Quantification of methyl ester

Methyl ester content in the reaction mixture was quantified by using the equation proposed by Gelbard et al. (1995), wherein the signals at 4.1– 4.3 ppm are caused by the protons attached to the glycerol moiety of mono-, di-, or triacylglycerol (TAG). The strong singlet at 3.6 ppm indicates methyl ester (–COOC**H**$_3$) formation. The signals at 2.3 ppm result from the protons on the CH_2 groups adjacent to the methyl or glyceryl ester moieties (–C**H**$_2$$CO_2CH_3$ for methyl esters).

$$C = 100 \times (2\ A_{ME} / 3A_{\alpha CH_2}) \quad (4)$$

Wherein,

C - conversion of TAG to the corresponding methyl ester.

A_{ME} - integration value of the protons of the methyl esters (the strong singlet peak).

$A_{\alpha CH_2}$ - integration value of the methylene protons.

The factors **2** and **3** have been derived from the fact that the methylene carbon possesses two protons and methanol carbon has three attached protons.

3.11.3. Quantification of other alkyl esters

Ethyl ester quantification by ^{1}H NMR spectroscopy is more complex than methyl ester quantification due to a superimposition of the glyceryl methylenic hydrogens in TAG and the -OC**H**$_2$ from ethyl ester in biodiesel, where partial conversion was obtained. However, the reaction where peak due to glyceryl methylenic hydrogens in TAG at 4.25-4.35 ppm completely disappeared, the process of transesterification was considered to be nearly complete. Following derivation was used to determine the FFA, derived by Satyarthi et al. (2009).

$$\%FFA = \frac{(Area\ of\ Triplet\ of\ \alpha CH_2 of\ FFA \times 100)}{(Total\ Area\ of \alpha CH_2\ of\ FFA\ and\ Ester)} \quad (5)$$

Wherein,

αCH_2 is α-acyl methylenic hydrogens in TAG and FFA at 2.20-2.40 ppm.

The product (alkyl esters) formed from the transesterification reactions were quantified by using ^{1}H NMR derivation **formulated during this study** (Sharma et al. 2013).

$$C = \frac{(AE_{\alpha-CH_2})}{(A_{\alpha-CH_2})} \times 100 \quad (6)$$

C - Conversion of oil to the alkyl ester;

$AE_{\alpha CH_2}$ - Integration value of the methylene protons of the alkyl esters;

$A_{\alpha CH_2}$ - Integration value of the methylene protons.

3.11.4. Quantification of alkyl ester by gas chromatography

Quantification of alkyl esters were further validated using gas chromatograph. Percentage of alkyl ester of fatty acid present in sample was determined according to EN ISO 5508 with internal calibration (methyl heptadecanoate, 10 mg/mL). Sample was prepared by weighing 250 mg of alkyl ester in a 10 ml vial, followed by the addition of 5 ml of methyl heptadecanoate (10 mg/mL). 1.0 µl of sample was injected into GC-5765 (Nucon, India) equipped with a flame-ionization detector. A fused silica capillary column (0.25-mm internal diameter, 30-m length and 0.25-µm film thickness, wall coated with EC wax (polyethene glycol) was used to separate FAEE. The flow rates of nitrogen as carrier gas and hydrogen gas were 30 ml/min while that of zero air was 300 ml/min was used. The injector and detector temperature were maintained at 230 and 240°C respectively. The oven initial temperature (160°C) hold time was 1 min and final oven temperature was 240°C. The rate of increase in temperature was 4°C/min and complete programme duration was 30 min. Split injection ratio 1:30 and split flow rate 30 ml/min were maintained.

The ester content C, expressed as a mass fraction in percent, was calculated using the following formula.

$$C = \frac{(\sum A) - A_{EI}}{A_{EI}} \times \frac{C_{EI} \times V_{EI}}{m} \times 100 \qquad (7)$$

Wherein,

$\sum A$- the total peak area from the ethyl ester in C_{14};

A_{EI} - the peak area corresponding to methyl heptadecanoate;

C_{EI} - the concentration in milligram per millilitre of the methy heptadecanoate solution;

V_{EI} - the volume in millilitres of methyl heptadecanoate solution being used; and

m – mass (mg) of the sample

3.12. Fatty acids composition of alkyl ester

The fatty acid composition of alkyl ester obtained from acid oil was analyzed by gas chromatograph as described in section 3.4.

3.13. Physical and chemical properties of ethyl ester

The FAEE (B100) recovered from transesterification reactions, was used for the determination of fuel properties and blending with petroleum diesel. Further, a 20% blend of biodiesel (B20) was prepared by mixing the appropriate quantity of biodiesel (B100) and petroleum diesel (PD). Fuel properties were determined for the biodiesel (B100) as well as the blend (B20); and compared with those of commercial diesel/petroleum diesel (PD). The fuel characteristics of biodiesel produced from acid oil were analyzed for density, kinematic viscosity, cloud and pour point, flash point, calorific value, sulphur content and ash content. The density measurement was made according to the capillary stoppered pyknometer method (Indian Standard 1448 [P:18]-1990). The kinematic viscosity were determined using the Brookfield viscometer according to Indian Standard IS: 1448 [P:25]-1976. The cloud point and pour point were determined using the Indian standard 1448 [P:10]-1970. Method outlined in the Indian Standard 1448 [P:21]:1992, (Pensky-Martens closed cup tester) was used to determination of flash point. The calorific value of the biodiesel sample was determined using bomb calorimeter (Indian Standard 1350 [P:2]-1970). The sulphur content and ash content were measured according to the Indian standards 1448 [P:33]-1991 and 1448 [P:4] respectively. The properties of biodiesel were compared with ASTM standards (D6751).

4.0 Results & Discussion

4.1. Identification of fungal strains

The fungal strains isolated from the contaminated clarified butter were identified as *Aspergillus flavus* (MTCC 5436), *Aspergillus aculeatus, Aspergillus sydowii* (MTCC 10397), *Curvularia pallescens* (MTCC 10390) and *Periconia sp* (MTCC 10391) by Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. *Aspergillus aculeatus* was not deposited at MTCC. *Rhizopus oryzae* was obtained from Bhabha Atomic Research Centre, Mumbai.

4.2. Screening of fungal strains on the basis of hydrolytic activity and acid oil tolerance

Isolated fungal strains were further screened on the basis of hydrolytic activity and acid oil tolerance. Three strains, *A. flavus, A. aculeatus* and *R. oryzae* showed growth in medium supplemented with 70% (v/v) acid oil, whereas, *A. sydowii* (MTCC 10397) showed growth at 50% (v/v) acid oil supplementation. *C. pallescens* (MTCC 10390) and *Perconia sp*. (MTCC 10391) showed no viability at the mentioned levels of acid oil in medium (Table 4.1).

Study on hydrolytic activity of these strains revealed that *A. flavus* exhibited maximum activity by converting acid oil containing 55% FFA to 100% FFA at 70% (v/v) acid oil supplementation in the growth media. On the other hand, *R. oryzae* completely (100%) hydrolyzed acid oil to FFA at 50% (v/v) supplementation. *A. aculeatus* hydrolyzed acid oil to 77% FFA and 53% FFA at 50% (v/v) and 70% (v/v) acid oil supplementation respectively (Table 4.1) followed by *A. sydowii*, *C. pallescens* and *Periconia sp.* wherein 29%, 15% and 22% hydrolysis of acid oil to FFA were noted respectively at 50% (v/v) acid oil supplementation in the medium. As the *A.flavus* showed maximum tolerance and completely hydrolyzed acid oil at higher concentration of acid oil in the medium, this strain was used for further experiments.

Table 4.1. Growth (+/-) of strains in acid oil supplementation and FFA (%) produced after growth for 72h

Acid oil (% v/v)	*Growth/FFA (%)*					
	Aspergillus flavus	***Rhizopus oryzae***	***Aspergillus aculeatus***	***Aspergillus sydowii***	***Curvularia pallescens***	***Periconia sp***
50	**+ ve/100**	**+ ve/100**	**+ ve**/77	**+ ve**/29	- ve	- ve
70	**+ ve/100**	**+ve**/92	**+ ve**/53	- ve	- ve	- ve

Limited observations have been reported till-date where oil was used as carbon source and lipase inducer. Sidra et al. (2016) reported maximum lipase production (25 U/mL) using *A. niger* at 1% sunflower oil supplementation in medium. The mycelium-bound lipase of *A. niger* was active at pH range from 2.0 to 9.0 when growth medium was supplemented with 2% olive oil. Olive oil induced 20% mycelium-bound activity at pH 4.0 and pH 7.0 (Romero et al. 2007). A 1.0% sesame oil supplementation induced the lipase activity of *B. subtilis* with 80% and 98% enhancements (Takaç et al. 2008). Wang et al. (2008) reported the effects of oil and oil related subtrates on lipase activity of *R. chinensis*. The authors used three types of oils viz; olive, soybean and sunflower with 2% concentation in broth medium. A maximum biomass production (17.6 g L^{-1}) with a relative synthetic activity (322%) and relative hydrolytic activity (165%) were observed in case of olive oil supplementation. However, fungus mediated transesterification of acid oil has been observed to a limited extent, as fungi, reported till date, are observed to be less tolerant to acid oil as a substrate.

Enhancement of tolerance to such substrates, through induced mutations can effectively facilitate a promising route for exploiting variants for transesterification reactions. The present study focused on enhancing transesterification through induced mutation in fungi using gamma radiation with a focus on reducing the time period for lipase production and transesterification

reaction. Improvement of microbial strain for the enhanced production of industrial products has been the hallmark of all commercial fermentation processes. Conventionally, strain improvement has been achieved through mutation, selection, or genetic recombination. The objective of mutagenesis for developing improved strain is to maximize the frequency of desired mutation in a population.

Radiations such as ultra-violet rays, x-rays and gamma rays are most common mutagens used for the improvement of strain as they induce single or double strand breakage of DNA by deletion or by structural change with high frequency (Reisz et al. 2014). Gamma rays induced mutation of *Pseudomonas aeruginosa* strain S8, capable of hyper-production of biosurfactant from hydrocarbons, was isolated and named as EBN-8 (Iqbal et al. 1995). The mutant showed 3-4 times more hydrocarbons emulsification/conversion as compared to the parent strain when grown on Khaskheli crude oil in minimal media. Gamma radiation mutagenic techniques have also been successfully employed for the improvement of *A. niger* strains for citric acid production. An intracellular glucose oxidase was produced from mycelium extract of a gamma rays mutant strain of *A. niger* BCG5 showed 172.87 U/ml of activity while enzyme produced from parent strain showed activity of 38.17 U/ml (Zia et al. 2007).

Similar studies were also reported with UV and chemically induced mutagenesis to enhance the production of commercially important lipase by fungal strain *A. japonicus* MTCC 1975. The best UV selectant showed 127% higher lipase activity than the parent strain. The results indicated that UV, HNO_2 and NTG treatment were effective physical and chemical mutagenic agents for strain improvement of *A. japonicus* for enhanced lipase productivity (Karanam et al. 2008). *Aspergillus* sp. CJ22-326 subjected to successive UV-irradiation followed by NTG treatment, also indicated increased chitosanase activities (Chen et al. 2008). Su et al. (2006)

obtained a mutant strain with higher chitosanase yield by mutation of the wild strain, *Bacillus sp.* S65, with nitrogen ion beam, and at the same time the fermentation time was shortened significantly which greatly increased efficiency.

Keeping the potential application of radiation-induced strain improvement in view, the next step was focused on improving wild type *A. flavus* through induced mutation using gamma radiation.

4.3. Exposure and effect of gamma radiation on fungal strain

4.3.1 Effect of irradiation on growth of strains

In order to increase the production of extracellular as well as intracellular lipase, doses of γ-radiations (50Gy, 100Gy, 200Gy, 300Gy and 400Gy) were applied to induce mutation in the cells of wild type fungal strain *A. flavus.* The irradiated variants were then screened for growth on PDA-Ox gall plate (a selective fungal medium) with respect to wild type (control) strain. The extent of growth and number of colonies of irradiated spores varied with the doses of irradiation. The spores which were irradiated with 50Gy appeared in distinct green colour with growth similar to that of control and the spores which were irradiated with 100Gy, 200Gy and 400Gy appeared in marginal green colour with slow growth rate (Table 4.2). The spores that were irradiated with 300Gy also appeared with green colour but exhibited growth earlier (with respect to time taken) than control. The irradiated variants along with control strain were then screened for lipase activity on the basis of substrate consumption and colour zone formation on spirit blue agar medium.

Table 4.2. Effect of irradiation on growth of variants

S.No.	Dose of irradiation (Gy)	Growth
1.	Control	++
2.	50	+
3.	100	+
4.	200	+
5.	**300**	**+++**
6.	400	+

The spirit blue agar medium, containing olive oil as lipase inducer, was employed to screen lipase producing strains (Gloor et al. 2002). The irradiated variants, with dose of 300Gy, showed maximum lipase activity with the larger zone of appearance with respect to control along with distinct dark blue colour around the colonies indicating significant lipolytic activity (Figure 4.1). The spores irradiated with 50Gy showed a small zone of appearance of blue colour which did not clearly indicate the lipase activity of the variants. There was no difference between irradiated variants with dose of 100Gy and 200Gy although they exhibited blue colour with small zone of appearance with respect to control. In contrast, the colonies of spores irradiated with 400Gy did not grow on spirit blue agar medium showing complete mortality of cells due to irradiation (Table 4.3). The variants at different doses exhibited different results in terms of viability and enzyme activity presumably due to association of the enzyme activity with the cell growth and the fact that products of the reactions caused by ionizing radiations, damage bases and sugars (Gutarra et al. 2007; Zhiqiang 2005). Bapiraju et al. (2004) reported that induced mutation in *Rhizopus* sp., exhibited higher lipase activity in mutant strains than the parent strain.

The irradiated variants, with dose of 300Gy were further screened for its intracellular and extracellular lipase enzyme activity by spectrophotometric method due to high lipase activity on spirit blue agar medium.

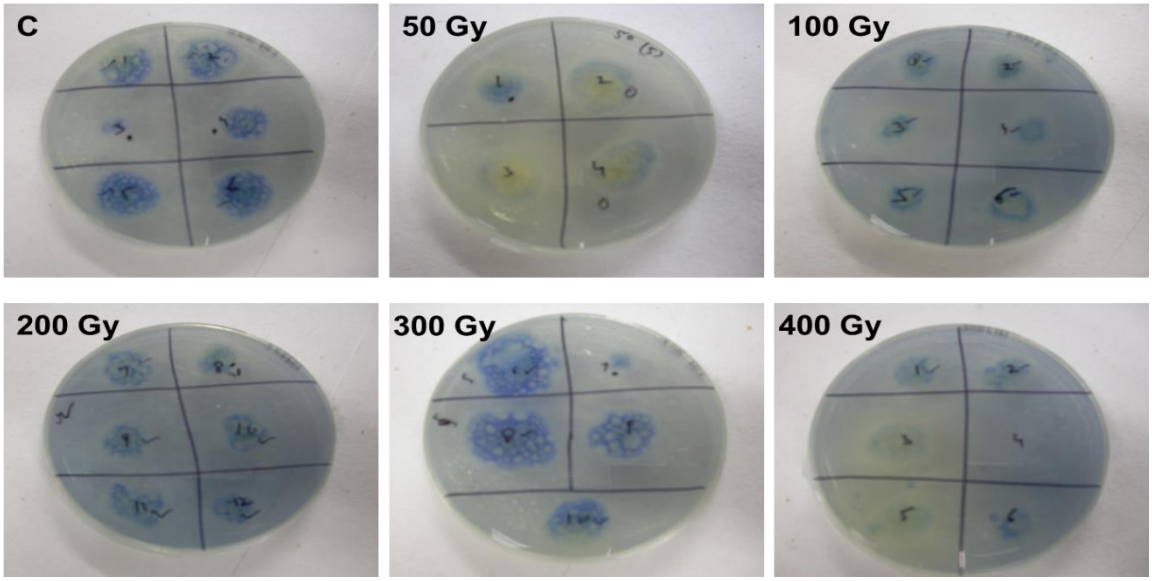


Figure 4.1. Variations in lipolytic activity in colonies subjected to different doses of irradiation as defined by formation of blue zones

Table 4.3. Variations in lipolytic activity of variants

S.No	Irradiation doses (Gy)	Zone with respect to control
1.	Control	**+ve**
2.	50	-ve
3.	100	-ve
4.	200	-ve/-ve
5.	**300**	**++ve**
6.	400	No zone

4.3.2. Intracellular and extracellular lipase activity of irradiated variants

The variants that exhibited larger blue zone in the previous experiment were screened for the synthesis of extracellular and intracellular lipase enzyme using shake flask fermentation process. There were total 18 variants (AKS-01 to AKS-18) obtained from 300Gy irradiation, screened for lipase activity on the basis of colour zone formation. Out of these variants (AKS-01 to AKS-18), AKS-05, AKS-06, AKS-07, AKS-08, AKS-09, AKS-10, AKS-11, AKS-17 and AKS-18 exhibited somewhat similar/lower [$P<0.001$] intracellular lipase activity with respect to control (Figure 4.2). These nine sub-variants (AKS-05, AKS-06, AKS-07, AKS-08, AKS-09, AKS-10, AKS-11, AKS-17 and AKS-18) were, selected on the basis of intracellular lipase activity, further

screened for extracellular lipase activity. Figure 4.3 presents the extracellular lipase activity of these sub-variants. The sub-variants AKS-08, AKS-10, AKS-11, AKS-17 and AKS-18 showed nearly similar [P<0.001; P<0.05] extracellular lipase activity with respect to control. The variation in lipase activity in these selected sub-variants may be due to destruction of functional orientation of lipase by gamma-irradiation (Diehl 1995), as gamma radiation in some of them may cause changes in tertiary structure of lipase resulting in loss of its functionality leading to inactivation of enzyme (Jha et al. 2013). Further, the radiation might have deregulated the transcription of the mRNA corresponding to lipase enzymes, resulting in decreased production of the enzyme (Nicolás-Santiago et al. 2006), in these sub-variants.

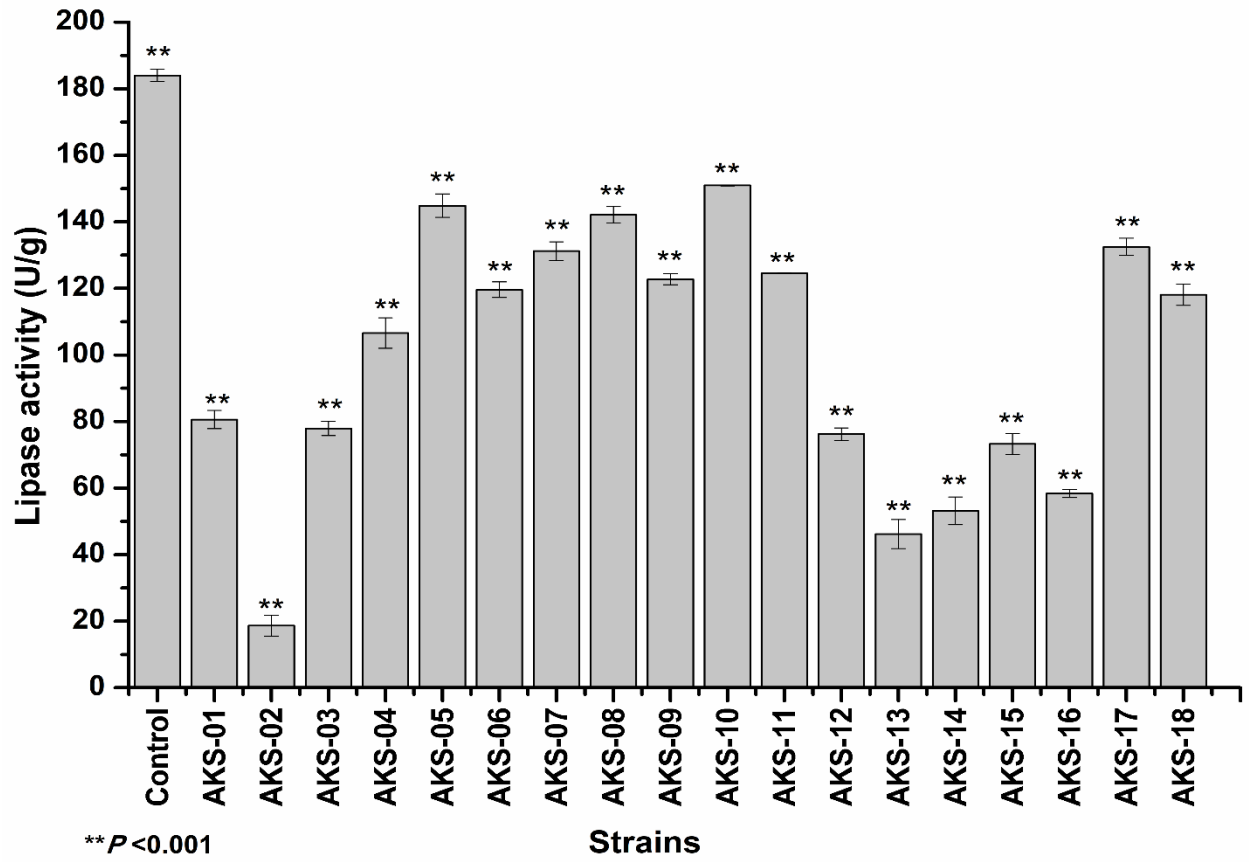


Figure 4.2. Intracellular lipolytic activity of variants

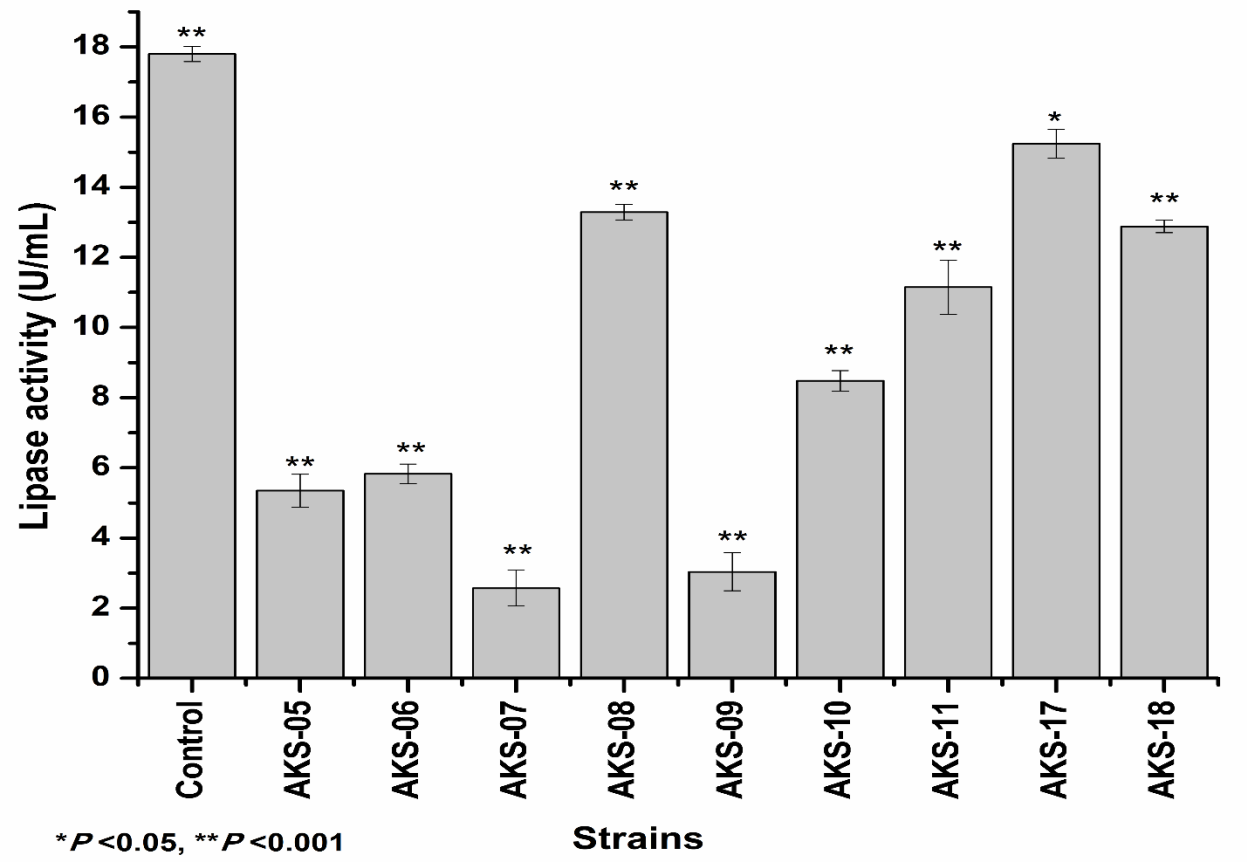


Figure 4.3. Extracellular lipolytic activity of selected sub-variants

These five sub-variants AKS-08, AKS-10, AKS-11, AKS-17 and AKS-18, were further screened for hydrolytic activity of acid oil and their viability at different concentration of acid oil in medium.

4.3.3. Screening of sub-variants on the basis of acid oil tolerance and hydrolytic activity

The parent strain (wild type, *A. flavus)* and selected irradiated sub-variants (AKS-08, AKS-10, AKS-11, AKS-17 and AKS-18) were further screened on the basis of acid oil tolerance. *A. flavus,* AKS-08 and AKS-17 showed growth in medium supplemented with 80% (v/v) of acid oil, whereas, AKS-18 showed growth in medium at 70% (v/v) acid oil supplementation. AKS-10 and AKS-11 were viable in medium supplemented with 60% (v/v) and 50% (v/v) acid oil respectively (Table 4.4).

Table 4.4. Growth (+/-) of wild type strain and irradiated sub-variants at different level of acid oil supplementation and FFA (%) produced after growth of 72 h

	Growth/FFA (%)					
Acid oil (% v/v)	***Aspergillus flavus***	***AKS-08***	***AKS-10***	***AKS-11***	***AKS-17***	***AKS-18***
50	**+ ve /100**	**+ ve /100**	**+ ve**/37.8	**+ ve**/28.3	**+ve/100**	**+ ve**/92.6
60	**+ ve /100**	**+ ve /91**	**+ ve**/29	- ve	**+ve**/79.8	**+ ve**/84
70	**+ ve /100**	**+ ve** /72.7	- ve	- ve	**+ ve**/76.7	**+ ve**/57.9
80	**+ ve /89.3**	**+ ve** /62.6	- ve	- ve	**+ ve**/61	- ve

Study on hydrolytic activity revealed that the wild type, *A. flavus*, exhibited maximum hydrolytic activity (100% FFA) at 70% (v/v) acid oil supplementation in the growth media. On the other hand, AKS-08 and AKS-17 showed maximum hydrolysis (100% FFA) at 50% (v/v) acid oil (Table 4.4) followed by AKS-18 showing upto 92.6% at 50% (v/v) acid oil supplementation. AKS-10 and AKS-11 showed tolerance only upto 60% (v/v) and 50% (v/v) acid oil resulting in 37.8% and 28.3% FFA generation respectively.

Observations on use of acid oil as an inducer of lipase activity have been reported to a limited extent till date. However, variety of virgin oils have been used to induce and optimize lipase production by various micro-organisms. Colen et al. (2006) reported that the fungus, *Colletotrichum gloeosporioides,* exhibited hydrolytic activity of 18.8 U/mL after 72 h of incubation using olive oil as supplement. Jonsson et al. (1974) used 2% olive oil in growth medium to optimize the lipase production of four different microorganisms *Saccharomycopsis lipolytica* (39 µmol/mL/min), *Micrococcus caseolyticus* (17 µmol/mL/min), *B. licheniformis* (6.3 µmol/mL/min) and *Staphylococcus* species (3.5 µmol/mL/min). Similar observations were reported by Fadıloğlu et al. (1999) with *R. oryzae* lipase at 3% olive oil supplementation in the growth media to obtained maximum lipase activity (14.38 U/mL) and biomass production (2.76

mg/mL) and concluded that olive oil is a better carbon source than glucose or lactose. Thus, this section of the study demonstrated acid oil could also effectively induce lipase production.

The wild type strain, A. flavus, was observed to be a better strain for lipase production and hydrolysis of acid oil over other irradiated variants, based on the maximum hydrolytic activity and acid oil tolerance wild type strain, A. flavus (RBD01, MTCC 5436) was further used for the standardization of lipase activity.

4.4. Optimization of culture conditions for lipase production

4.4.1. Effect of different concentration of nitrogen sources

The strain, *A. flavus*, was grown in increasing concentration of various organic and inorganic nitrogen sources, with 10% (v/v) acid oil as carbon source to evaluate the effect of nitrogen source and its concentration on the enzyme activity. Nitrogen sources, including organic nitrogen sources (peptone and urea) and inorganic nitrogen sources (bi-ammonium hydrogen orthophosphate (BAHP) and sodium nitrate), play an important role in the synthesis of the enzyme. The influence of various nitrogen sources and their concentrations on enzyme activity is shown in Figure 4.4. The organic nitrogen sources supplementation was most effective in case of 0.5% (w/v) of peptone with lipolytic activity 2.28 U/mL obtained after 72 h. With further increase in peptone concentration to 1.0% (w/v), the enzyme activity marginally decreased and remained constant (Figure 4.4(a)). It is, therefore, presumed that concentration of peptone at 0.5% (w/v) fulfilled the nitrogen requirement of the medium and is sufficient for growth of culture as compared to other nitrogen sources (Sidra et al. 2016). Amongst the inorganic nitrogen sources, BAHP was most effective for lipase production. Maximum lipolytic activity (2.53 U/mL) was observed in the presence of 0.5% (w/v) BAHP (Figure 4.4(c)). However, presence of inorganic ($NaNO_3$) as well as organic (Urea) nitrogen sources, beyond 1.5% (w/v) and 1.0% (w/v) respectively, showed sharp

decrease in enzyme activity (Figure 4.4 (b&d)). Both organic and inorganic nitrogen sources were used to observe their regulatory role in lipase production as inorganic nitrogen sources are consumed faster and normally cause repression of enzyme synthesis due to formation of ammonium repressible entity (AreA) protein (Borges et al. 2005), while organic nitrogen sources are substrates for various cell growth factors and amino acids which play a role in cell metabolism and protein synthesis (Rajoka 2008). Aulakh et al. (2010) and Chander et al. (1980) also reported that peptone was highly efficient nitrogen source. These authors also stated that the lipase activity is dependent on the source of nitrogen and not the nitrogen content of the medium.

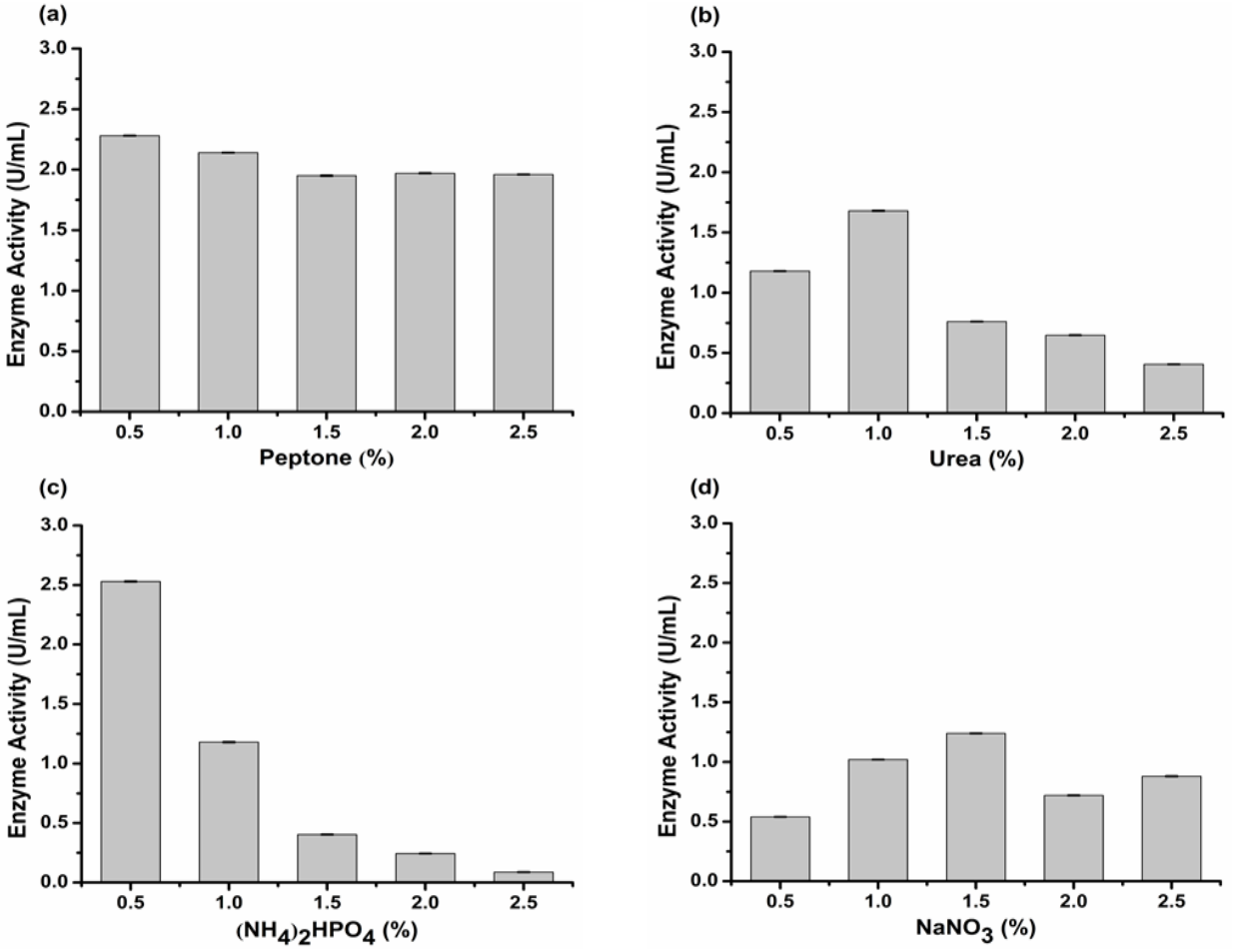


Figure 4.4. Effect of concentration of different nitrogen sources on lipase activity (a) Peptone; (b) Urea; (c) BAHP; (d) Sodium nitrate

4.4.2. Effect of pH

The pH of the culture medium was one of the most important critical environmental parameters that affected the growth and enzyme production of *A. flavus*. To examine the effect of pH on the lipase production, the culture was grown in conditions as outlined in Step 2 (Table 3.1, section 3.3.) with optimized levels of organic (peptone) and inorganic (BAHP) nitrogen sources. Lipase production increased from 0.58 to 1.98 U/ml with corresponding increase in pH from 5.0 to 7.5 (Figure 4.5(a)). Further increase in pH from 8.0 to 8.5 resulted in reduction of activity i.e. from 1.10 U/mL to 0.98 U/mL. Thus, pH 7.5 was selected for further studies. The fungi require slightly basic pH for production of lipases and its metabolic processes (Kiran et al. 2008), whereas Rapp (1995) has also reported good enzyme production in acidic range. Gombert et al. (1999) reported that the enzyme biosynthesis generally decreases with change in pH of the medium, because most active sites function as general acids and bases in catalysis. The enzymes usually have an optimal working pH, higher or lower of which may lead to deactivation of enzymes (Romero et al. 2007). Shankar et al. (2013) reported the maximum activity of *C. antarctica* lipase B to be at pH 7.0. A similar range of optimum pH (7.0-8.0) have also been reported for three other lipases from different microbial sources (Abbas et al. 2003; Dosanjh et al. 2002). Corzo et al. (1999) reported maximum lipase activity at pH 6-7 with activity decreasing significantly beyond pH 8. The optimum pH for lipase activity of *Geotrichum*-like R59 was observed to be near neutral with reduction in activity at pH above 8.0 (Ginalska et al. 2007).

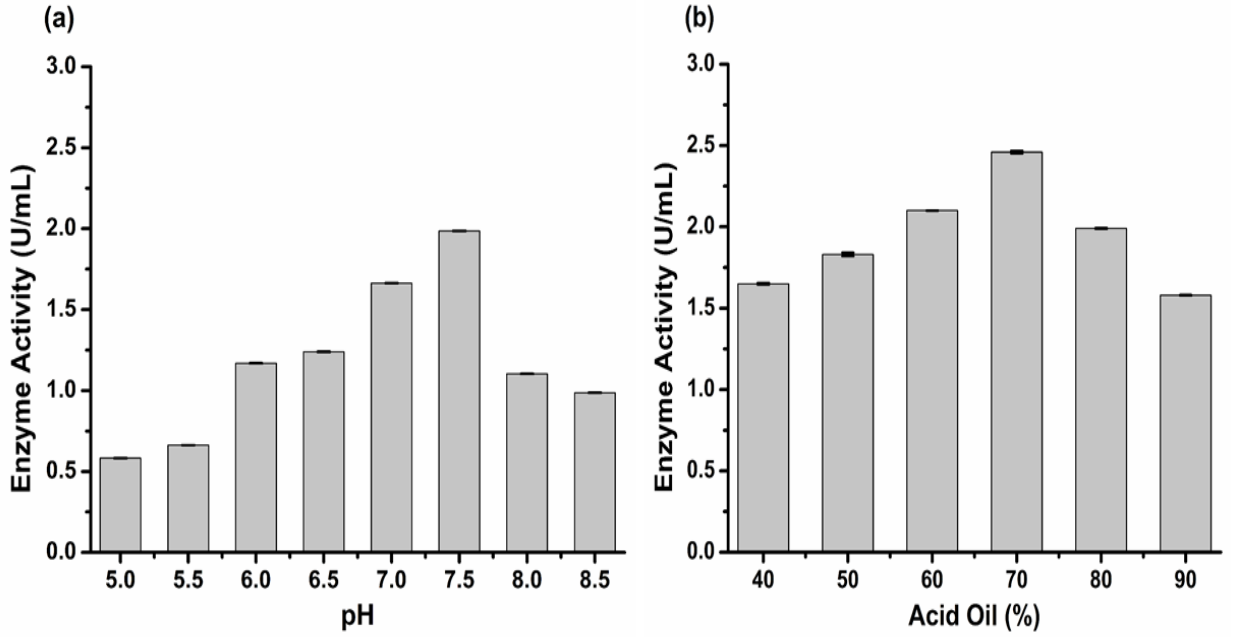


Figure 4.5. Effect of (a) pH; and (b) Acid oil concentration on lipase activity

4.4.3. Effect of different acid oil concentration on lipase activity

The type and concentration of carbon source also influenced the lipase production and hence lipolytic activity. Different levels of acid oil supplementation (40% v/v – 90% v/v) were taken as carbon source with other optimum parameters remaining unaltered (pH 7.5, peptone (0.5% w/v) and BAHP (0.5% w/v)). Maximum lipase activity 2.46 U/ml was obtained at 70% (v/v) acid oil supplementation in the growth medium (Figure 4.5(b)). As mentioned earlier, influence of acid oil on lipase activity and production has been reported to limited extent, when compared to those with virgin oils. Elibol et al. (2000) indicated that the synthesis of lipase was inhibited by the higher concentration of olive oil in the medium, it could be due to poorer oxygen transfer in the medium. Low oxygen supplies can alter fungal metabolism, and consequently, the production of lipases. The favourable effect of olive oil and oleic acid on lipase production has been observed by various research groups (Chahinian et al. 2000; Odibo et al. 1995; Pimentel et al. 1994). Fatty acids such as oleic acid, linoleic acid and linolenic acid as well as triglycerides such as olive,

groundnut and cotton seed oils stimulate lipase production by *P. mephitica* (Jonsson et al. 1974). A supplementation of 1% (v/v) sunflower oil gave the maximum lipase production and did not affect the nutrient uptake by fungus (Martinez et al. 1993). Gao et al. (1995) compared different vegetable oils (olive, soybean, sunflower, sesame, cotton seed, corn, and peanut oil) as carbon sources for lipase production from the sapwood staining fungus, *Ophiostoma piceae*. High levels of lipase activity were obtained when olive oil was used as main carbon source. Essamri et al. (1998) reported that the rapeseed and corn oil were the most suitable carbon sources for cell growth and lipase production.

Majority of the reports, to-date, have only used virgin oil as an inducer for microbial synthesis of lipases, with limited reports available on use of acid oil as the main carbon source. The extent of acid oil supplementation as the main carbon source i.e. 70% (v/v) of the growth medium and enhanced lipase activity of 2.46 U/mL achieved here, is higher than any observation reported till date.

4.5. Physical and chemical properties of acid oils

4.5.1. Physical and chemical properties

Table 4.5 presents the chemical and physical properties of acid oils viz; cottonseed (CS), sunflower (SUN), rice bran (RB) and soybean (SO). The acid oils constituting high free fatty acid contents and some amount of moisture, are notably favourable for transesterification process with whole cell biocatalyst. The moisture content in the feed stock increases the reaction rate significantly in such systems (Li et al. 2007). Acid oils were extremely viscous, due to high content of free fatty acids. The conventional alkali catalysts are not amenable to high free fatty acid as the reaction results in soap formation. The density of acid oils were near to the density of ASTM standard of biodiesel. The saponification values of acid oils were 123 (CS), 190.3 (SUN), 191.7

(RB) and 193.6 (SO). As the saponification value depends on the molecular weight of oil, rise in molecular weight reduces the saponification value. Another main factor of fuel quality is iodine value, which depends on the carbon-carbon double bond content in the oils, as the higher iodine value of oil decreases the heat content of the fuel (Demirbaş 1998). The CS also had very low amount of ash content (0.003%). ***From the viewpoint of physio-chemical properties of acid oils examined in this study, it is presumed that the acid oil is an effective feed stock for generation of good quality fuels and alkyl esters.***

Table 4.5. Physio-chemical properties of acid oils

S.No.	Parameters	CS	SUN	RB	SO
1.	Free fatty acids (%)	55	62.2	59.2	54.6
2.	Acidity (mg KOH/gm oil)	109.6	124	118	109
3.	Density@15°C (Kg/m^3)	926	913	936	920
4.	Viscosity@40°C (cSt)	39.2	28.4	50.8	27.8
5.	Saponification value	123	190.3	191.7	193.6
6.	Moisture (%)	0.50	0.4	0.4	0.4
7.	Iodine value	111	109	113	115
8.	Ash (%)	0.003	Not detected	Not detected	Not detected

4.5.2. Components and FFA composition of acid oil

Table 4.6 presents the composition of acid oil as reported by Kulkarni et al. (2008) and Tüter et al. (2004). Constituents such as long chain free fatty acids mixture, mineral acids, moisture, phospholipids, sterols, neutral glycerides and unsaponifiable constituents, impart a typical pungent odour and dark brown color to the acid oil. Acid oil's fuel properties are different from diesel fuel due to presence of chain type of configurational compounds and its oxygenated nature. In addition, the heating value of acid oil's fuel is slightly lower and the viscosity vis-à-vis the ignition values are higher than diesel fuel.

Table 4.6. Components of acid oil

S.No.	Components	Concentrations (%)	References
1.	Mineral acids	1-2	Kulkarni et al. 2008
2.	Free moisture	5-8	
3.	Phospholipids & Sterols	8-10	
4.	Neutral glycerides & Unsaponifiable constituents	20-50	Tüter et al. 2004

Fatty acid composition of acid oil constitutes a mixture of saturated and unsaturated fatty acids classified according to the number of unsaturated bonds. Table 4.7 indicates the fatty acid composition of the acid oils. All the acid oils considered in this study contained two most common saturated fatty acids viz, palmitic acid (16:0) and stearic acid (18:0). Similarly, oleic acid (18: 1) and linoleic acid (18:2) were the most common monounsaturated and polyunsaturated fatty acids. [Annexure I; Fig: 4.5.2 (I-V)].

Table 4.7. Fatty acids composition of acid oils

Fatty acids	CS (wt %)	SUN (wt %)	RB (wt %)	SO (wt %)
Palmitic acid (C16:0)	24.24	13.25	12.39	13
Stearic acid (C18:0)	3.31	2.83	3.05	3.21
cis-9-Oleic acid (C18:1)	36.78	34.43	26.96	27.54
Linoleic acid (C18:2)	35.42	49.17	56.96	55.57

4.6. Transesterification studies

4.6.1. Transesterification reaction of different acid oils

The transesterification reaction of different acid oils was optimized after 72 h of hydrolysis, by varying the time interval of ethanol addition ranging from 0 to 12 h. With increase in time interval between alcohol additions, the extent of transesterification increased in terms of

conversion of acid oil to ethyl ester. The one-time addition (0 h) resulted in very limited yield of only 50%, 53%, 59% and 68% of ethyl ester (EE) from rice bran (RB), soybean (SO), sunflower (SUN) and cottonseed (CS) acid oil, respectively (Figure 4.6). With step-wise addition of ethanol at an interval of 4 h (4 times), the yield of ethyl ester slightly increased with all the acid oils. Whereas, a distributed addition of 1:4 molar ratio of ethanol at regular intervals of 12 h (3 times) facilitated maximum yield of 88% ethyl ester in case of CS. A better result with step-wise addition of ethanol, as compared to one time addition, is attributed to the fact that when polar substrate gets accumulated in the microenvironment of the enzyme it may cause the denaturation of the proteins and thus, reduced activity (Jin et al. 2008; Trubiano et al. 2007; Villeneuve et al. 2000). Shimada et al. (1999) also carried out the transesterification reaction with sequential addition of alcohol to reduce the inhibition of enzyme activity. The same was also proposed by Azocar et al. (2010) to avoid the loss of activity of the enzyme caused by inhibition of alcohol.

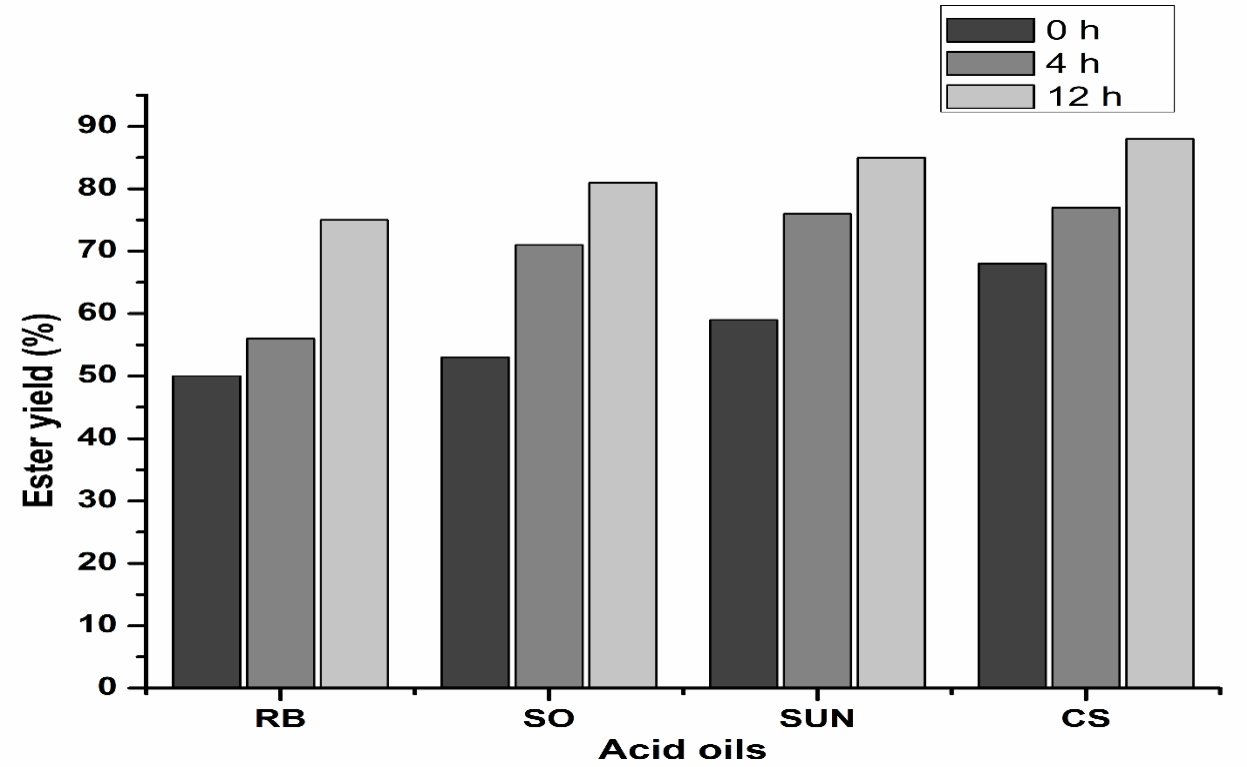


Figure 4.6. Effect of time interval of alcohol addition on extent of transesterification

4.6.2. Time dependent variations in transesterification reaction

To examine the total reaction time of transesterification, observations on time dependent variation in the ester generation was recorded using whole cell catalyst. The complete conversion of acid oil to FFA was observed in 12 h duration during the hydrolytic reaction. The conversion of acid oil to FFA was confirmed through ^{1}H NMR wherein, the peak due to glyceryl methylenic hydrogen at 4.25-4.35 ppm disappeared on the hydrolysis of acid oil to FFA confirming the complete conversion of oil to FFA (Figure 4.7(a,b)).

Further, esterification was carried out with addition of 1:4 molar ratio of ethanol (one-time addition), followed by 1 h incubation, resulting in the generation of ethyl esters with corresponding decrease in FFA content. The ethyl ester formation was evident with appearance of quartet of ethoxy hydrogens (–OC**H**$_2$CH$_3$) of ester at 4.10 to 4.20 ppm (Figure 4.7(c)).

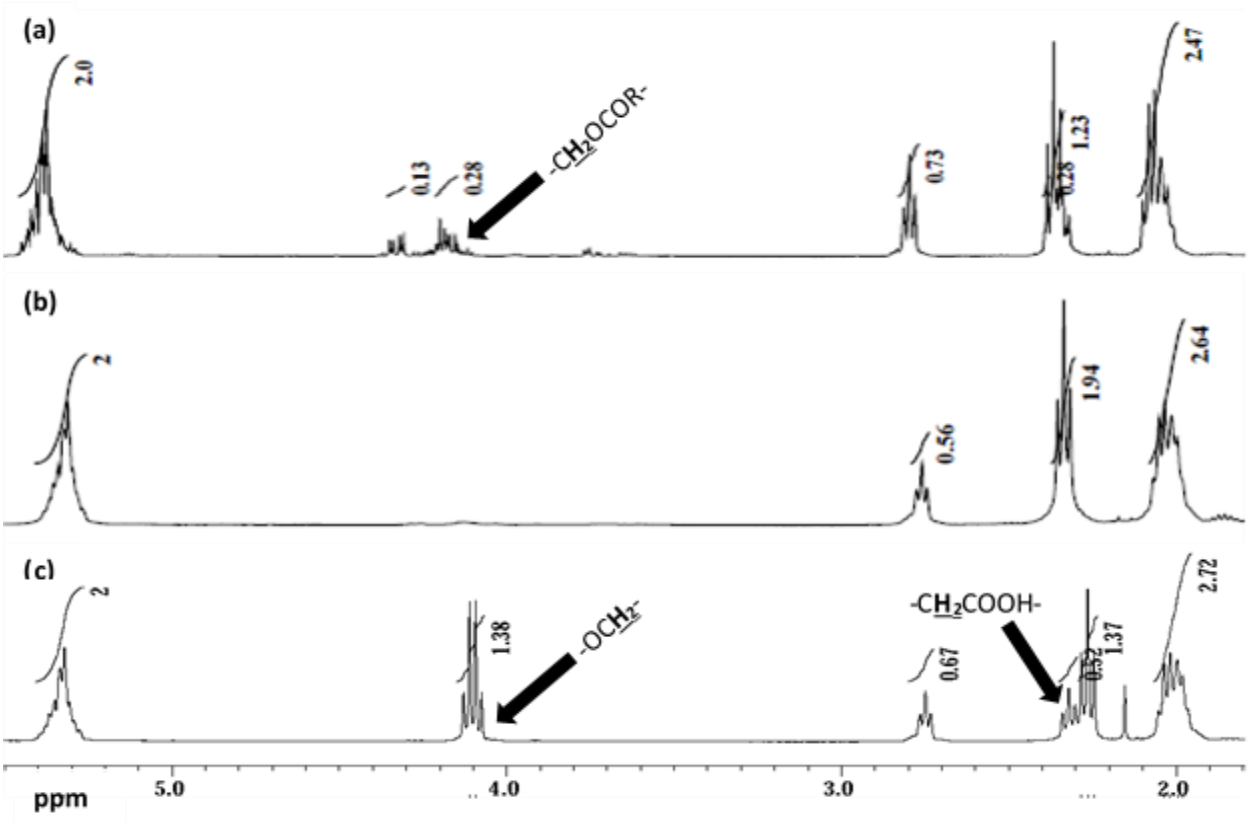


Figure 4.7. ^{1}H NMR of (a) Acid oil; (b) Conversion of acid oil to FFA; (c) Ethyl ester

The trend depicts the formation of ethyl esters with decrease in FFA content from 100% to 45% within 1 h of alcohol addition leading to ester yield of 55%. The yield of ester further increased to 66% and 70% over duration of 4 h and 16 h respectively. The reaction which was continued up to 24 h resulted in maximum ester yield of 72% with 28% corresponding FFA (Figure 4.8). The hydrolysis reaction completed in 12 h duration and the maximum yield of ester obtained was 72% in 24 h of reaction with one-time addition of ethanol. As mentioned in the section 4.6.1, the one-time addition of ethanol expectedly gave limited yield of ester. [Annexure II; Fig: 4.6.2]

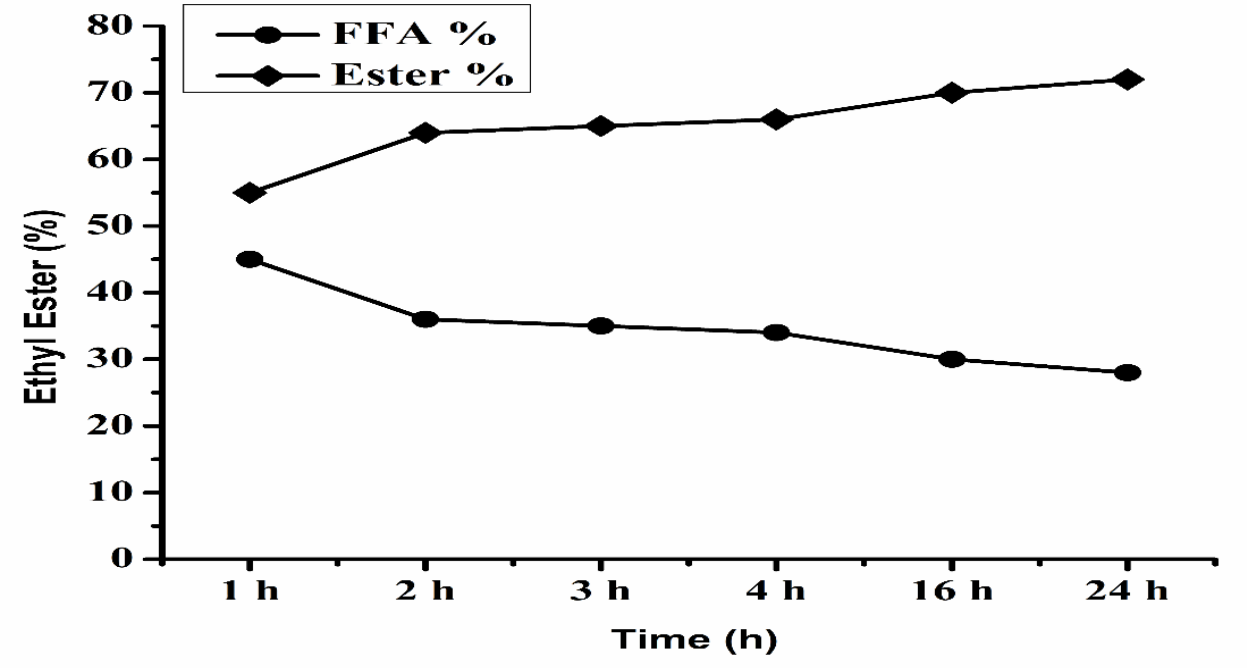


Figure 4.8. Profile of ethyl ester generation over time with one-time addition of alcohol at 1:4 molar ratio

To enhance the yield of ester, after hydrolytic reaction of 12 h duration, esterification was carried out with stepwise (3 times) addition of ethanol at regular intervals of 12 h, followed by 12 h incubation. In this condition, the average yield of ethyl ester obtained was 92% ± 0.8% with 8.0% ± 0.8% of corresponding FFA. ***Step-wise addition of ethanol observably gave better yield as compared to one-time addition of ethanol.***

The generated ethyl ester was subjected to washing with NaCl-$NaHCO_3$ resulting in complete removal of unreacted FFA. The ^1H NMR obtained after transesterification confirmed the formation of ethyl ester with the peak due to -OC**H**$_2$ of ethoxy hydrogens of ester appearing at 4.10 ppm (Figure 4.9(a)). A small triplet at 2.34 ppm indicated the presence of unreacted FFA (-C**H**$_2$COOH) in ethyl ester, which completely disappeared after washing of ester with NaCl-$NaHCO_3$ (Figure 4.9(b)). The signals at 2.30 ppm result from the protons on the -C**H**$_2$ groups adjacent to the alkyl or glyceryl ester moieties (–**CH**$_2$$CO_2$ CH_2 (CH_2)x CH_3 for alkyl esters).

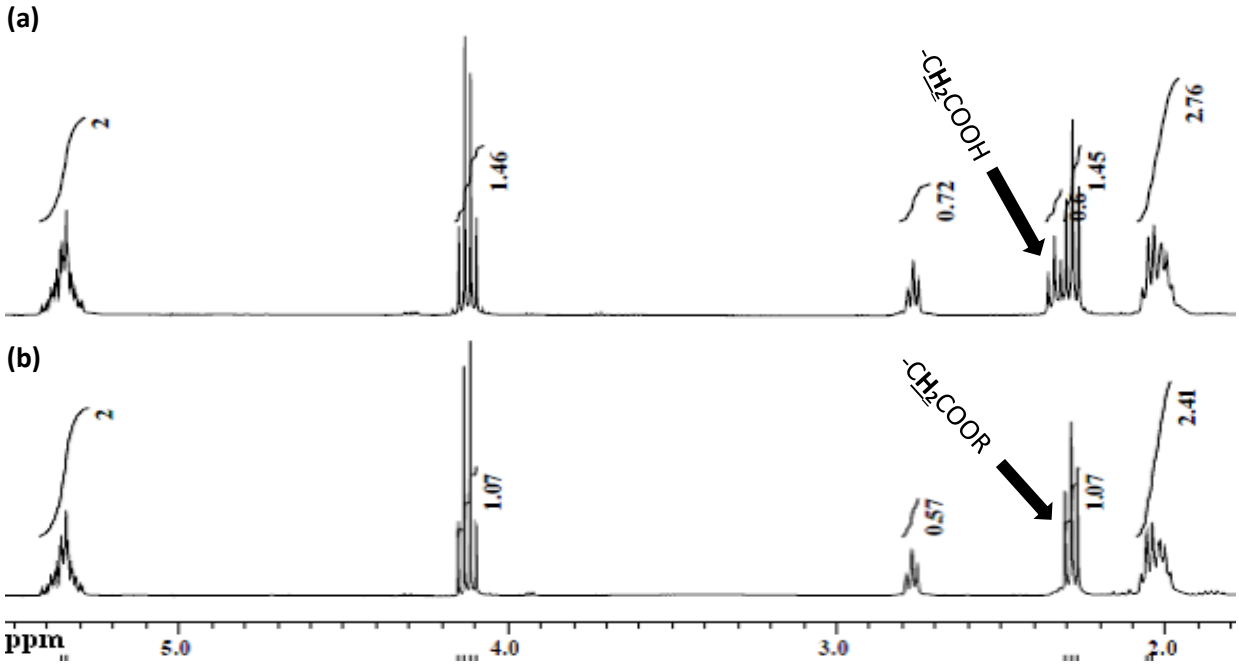


Figure 4.9. ^1H NMR of (a) Ethyl ester with unreacted FFA at 2.34 ppm; and (b) Ethyl ester obtained after washing.

To the best of our knowledge, there are limited reports on generation of ethyl ester from acid oil especially with fungal whole cells as catalysts. However, transesterification of acid oil to alkyl esters has been attempted with pure enzymes by some research groups. The complete hydrolysis of feedstock to fatty acids was carried out in subcritical water followed by the use of a packed-bed reactor that contained fermented solids with *Burkholderia cepacia* LTEB11 enzyme.

The maximum conversion of fatty acids to alkyl esters, in this study, was 92% in 31 h (Soares et al. 2013). Ghosh et al. (1995) reported the transesterification reaction of different types of acid oils such as coconut, soybean, mustard, sunflower, and rice bran with *C. cylindracea* lipase as a catalyst for hydrolysis followed by esterification or alcoholysis reaction with *M. miehei* lipase. The acid oils were hydrolyzed almost completely within 48 h and the fatty acids were converted into fatty acid esters of short and long chain alcohols. The *C. antarctica* (Novozym 435) lipase B enzyme was also used for esterification of corn and sunflower acid oils using straight and branched chain alcohols. The maximum yield of alkyl ester was 50% and 70% with corn acid oil and sunflower acid oil respectively (Tüter et al. 2004). Further, Haas et al. (1996) reported that 81% conversion of the fatty acids in soapstock to simple alkyl esters by two step process, in which, second step of the process involved use of lipase enzyme instead of KOH as a catalyst. Thus, the use of whole cell systems, as reported in the present study, provide an alternative to enzymatic catalysis resulting in similar or better yield of alkyl esters.

4.7. Transesterification of acid and virgin oils with wet and dried biomass: Influence of different acyl acceptors

This part of study outlines the observations on whole-cell catalyzed transesterification reaction of cottonseed oil and its corresponding acid oil by using biomass suspension and dried biomass of *A. flavus* with short and medium chain alcohols (methanol to decanol) as acyl acceptors. The enzyme activities of biomass suspension and dried biomass were calculated from the standard graph prepared by using commercial lipase enzyme (*Candida rugosa*) [Sigma-Aldrich L1754-25G]. The enzyme activity in biomass suspension and dried biomass was found to be 118 U g^{-1} and 280 U g^{-1} respectively. Further, both forms of biomass viz., suspension and dried were normalized to 59 units of enzyme in biomass by taking 500 mg and 211 mg respectively, to carry

out transesterification reaction.

4.7.1. Transesterification with biomass suspension

The hydrolytic reaction carried out with fungal biomass suspension resulted in gradual increase of FFA from initial levels of 55% to 100% within 12 h in case of acid oil. In case of cottonseed oil (virgin), however, the time taken for conversion to FFA (100%) was 72 h. This is expectedly due to higher FFA content in acid oil (55%) than cottonseed oil (0.5% FFA). The enzyme activity of biomass (suspension), observed after hydrolysis, was 440 U g^{-1} and 93 U g^{-1} in case of acid oil and cottonseed oil respectively. It is presumed that the constituents such as phospholipids, mineral acids and sterols present in the acid oil may enhance the lipase activity and rate of reaction. Li et al. (2007) also reported that the phospholipids present in the substrate promote the reaction and influence the rate of reaction to certain extent. Cottonseed oil, on the other hand contains higher amount of triacylglycerol (TAG) (99.5%) than acid oil (45%). The TAG gets completely converted to FFA (substrate) and glycerol (by-product) in 12 h and 72 h during the hydrolysis in case of acid oil and cottonseed oil respectively. On hydrolysis, the concentration of substrate and glycerol is expectedly higher in medium in case of virgin oil when compared to acid oil. It is presumed that the catalytic activity of lipase in case of cottonseed oil gets inhibited/reduced due to high concentration of substrate and glycerol in the medium, resulting in blockage the active sites. The lipase is observed to get inhibited due to lose in catalytic activity with build up of FFA, a phenomenon that possibly depicts atypical or non-Michealis-Menten kinetics (Du et al. 2004; Elliott et al. 1991; Lencki et al. 1998; Musser et al. 1997). ***Absence of such inhibitory effects during the use of acid oil as a substrate would possibly provide added advantage in comparison of cottonseed oil.***

Esterification reaction was carried out with addition of different acyl acceptors, followed by 12 h incubation, resulting in the generation of alkyl esters with corresponding decrease in FFA content. The conversion of oil to FFA and alkyl esters was confirmed through ^{1}H NMR, with the appearance of singlet of methoxy hydrogen (-OC**H**$_3$) in the region of 3.60 ppm due to the proton of methyl ester (Figure 4.10(c)). The ethyl ester formation was evident with appearance of quartet of ethoxy hydrogens (–OC**H**$_2$CH$_3$) of ester at 4.10 to 4.20 ppm (Figure 4.10(d)). With reference to other alkyl esters (propanol to decanol), the formation of ester was confirmed with the appearance of triplet of alkoxy hydrogens (-OC**H**$_2$CH$_2$-) at integration value of around 4.10 ppm (Figure 4.10(e)).

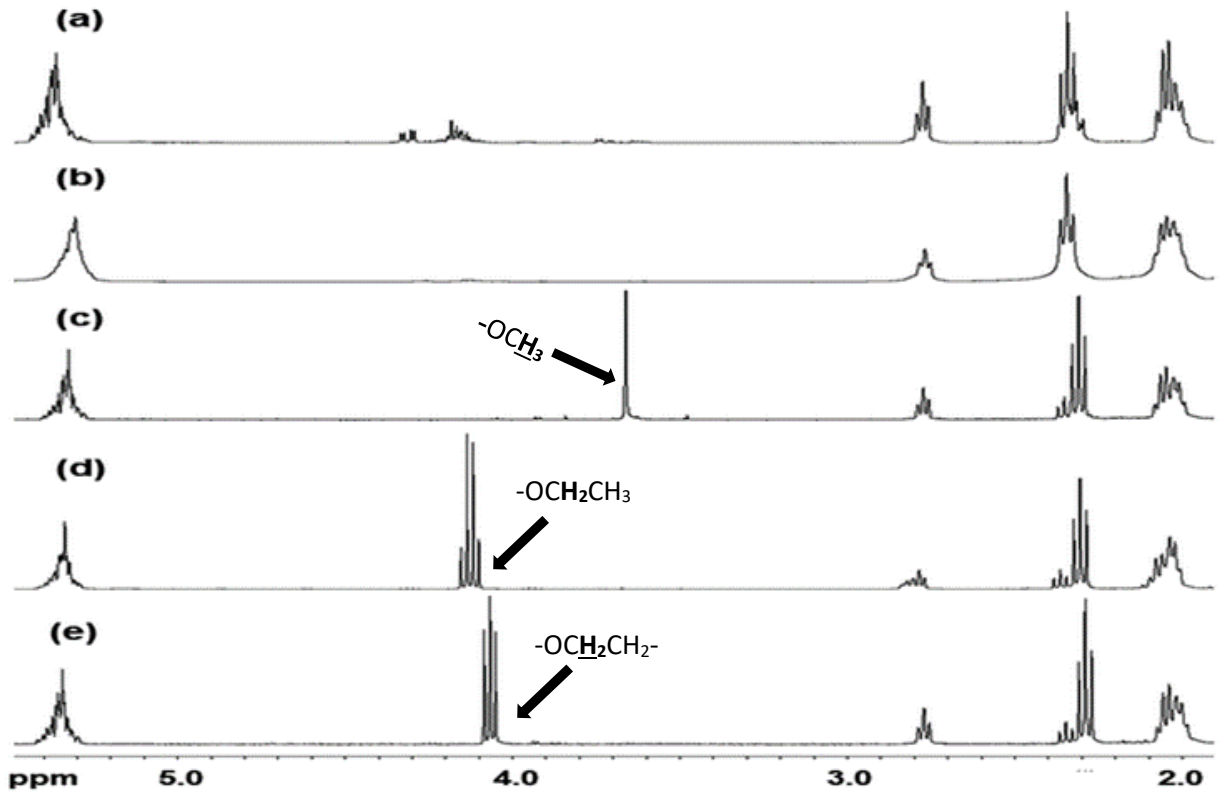


Figure. 4.10. NMR spectra (a) Acid oil/cottonseed oil; (b) Hydrolysis of acid oil/cottonseed oil; (c) Methyl ester; (d) Ethyl ester; and (e) Other alkyl esters (Propanol to Decanol)

The yield of methyl and ethyl esters were somewhat similar [P>0.05] in both the substrates (acid oil and cottonseed oil) while using biomass suspension as catalysts. The extent of transesterification of acid oil with acyl acceptors (propanol to decanol) was significantly higher [P<0.05] as compared to ester yield from cottonseed oil (Figure 4.11). In case of cottonseed oil, the maximum yield of 81% was obtained when ethanol was used as acyl acceptor. It is probably due to loss of enzyme's catalytic activity during the period of hydrolysis. The yield of alkyl esters increased from methanol to pentanol (C1 to C5) i.e. 79% to 87%, followed by decrease from hexanol to decanol (C6 to C10) i.e. 80% to 55% in case of acid oil.

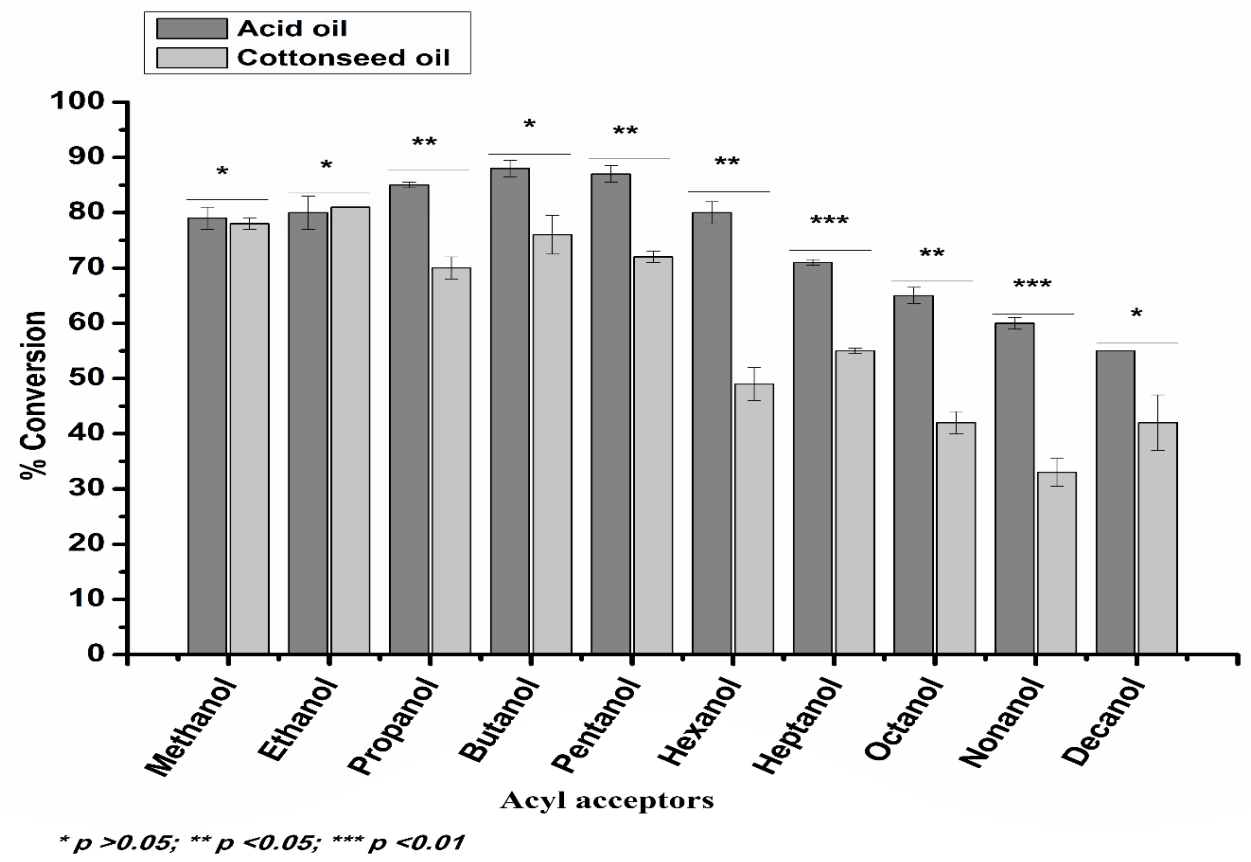


Figure 4.11. Extent of transesterification with biomass suspension as a catalyst

The esterification process occurs dominantly at the polar-nonpolar interfacial region. Thus, the variations in yield of ester, with increasing chain length of alcohols from ethanol to decanol,

is expected to be due to decrease in the polarity of alcohol (Aulakh et al. 2017; Sridharan et al. 1974). Observations reported by Gatfield (1986) and Romero et al. (2003) also indicated that the chain length of alcohol influences the ester yield only to a certain extent beyond which it is independent of chain length. In the case of transesterification reaction employing methanol, alcoholysis takes place between two immiscible liquids (Doell et al. 2008; Stavarache et al. 2008). On formation of diacyl and monoacyl glyceridic intermediates in sufficient quantities, they serve as surfactants that improve mass transfer of triacylglycerides into methanol phase (Moser 2009). Ethanol is of particular interest primarily as it is less expensive than methanol in some regions (such as Brazil) of the world. However, ethanolysis proceeds at a slower rate than methanolysis because of the lower reactivity of the ethoxide anion in comparison to methoxide. As the length of the carbon chain of the alkoxide anion increases, a corresponding decrease in nucleophilicity occurs, resulting in reduced reactivity of ethoxide in comparison to methoxide (Sridharan et al. 1974). However, there are limited reports on the use of other alcohols as acyl acceptors in whole cell catalyzed transesterification. Butanol may also be obtained from biological materials thus yielding completely bio-based biodiesel as well (Qureshi et al. 2008). Butanol is completely miscible with vegetable oils and animal fats as it is less polar than methanol and ethanol (Boocock et al. 1996). Consequently, transesterification reactions employing butanol are monophasic throughout enhancing the rate and extent of the reaction (Zhou et al. 2006).

4.7.2. Transesterification with dried biomass

In the case of dried biomass as catalyst, the observations were contrastingly different within the types of substrates (cottonseed oil/acid oil) tested. The trend in the ester yield in case of acid oil, was relatively similar to that of cell suspension with maximum ester yield peaking at pentanol (96%), followed by decrease till decanol (64%) (Figure 4.12). Whereas, with cottonseed oil, the

yield of esters marginally increased from 51% to 59% in presence of hexanol to nonanol with no significant conversion observed between methanol (5%) to propanol (5%). The lower yield of the reaction with methanol and ethanol could be due to the inhibition and inactivation of the lipase. Amongst the substrates, the extent of transesterification were significant higher [P<0.01] in case of acid oil with acyl acceptors (ethanol to nonanol) as compared to ester yield from cottonseed oil, which is expected to be due to high FFA and moisture content (0.5%) in acid oil, as the moisture content is expect to increase the rate of reaction and enhanced yield of esters during transesterification (Li et al. 2007).

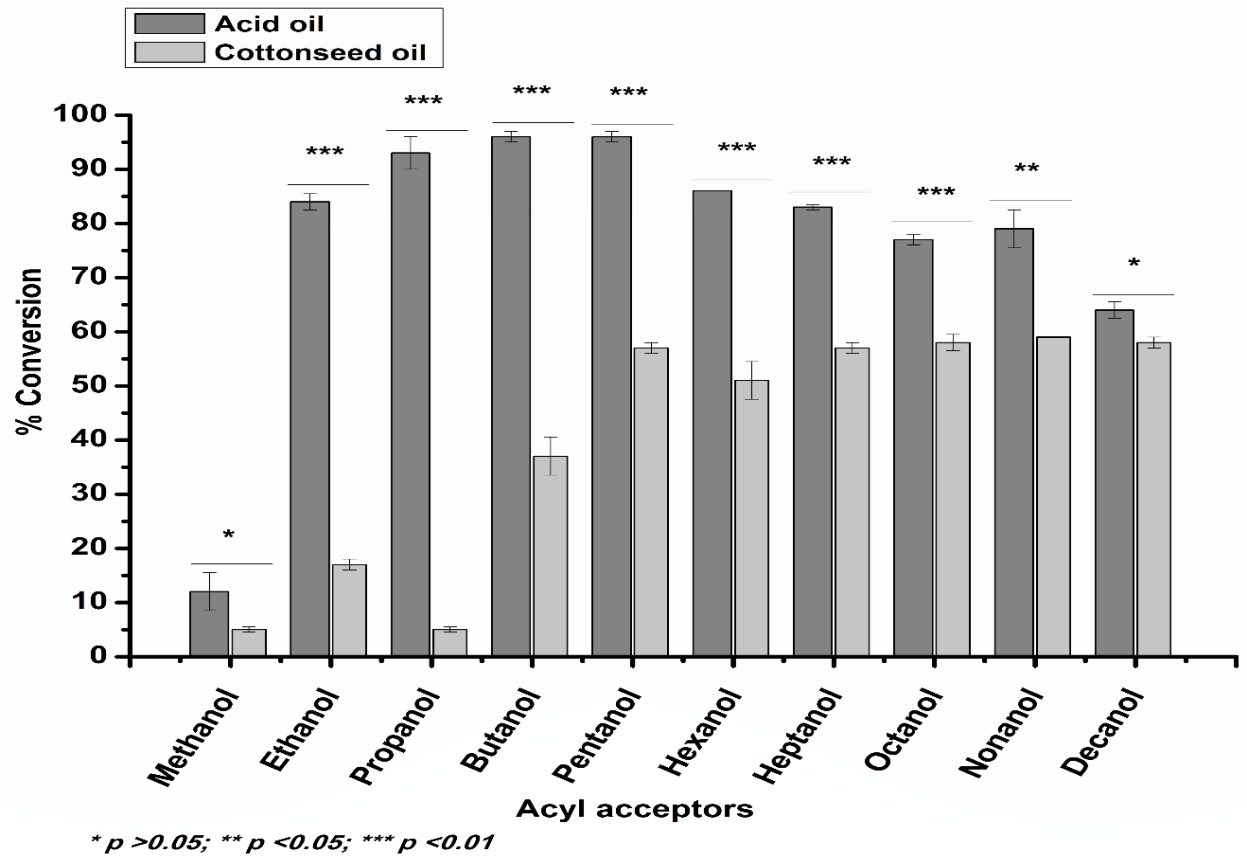


Figure 4.12. **Extent of transesterification with dried biomass as catalyst**

With reference to the yield of esters from both substrates across the two forms of catalysts and different acyl acceptors, the long-chain alcohols (2-propanol and n-butanol), are reported to

have lesser inhibitory effect on lipase stability, and also improved low-temperature properties of the fuel (Salis et al. 2005). The increase in the yield of the reactions with propanol, butanol and pentanol (C3-C5) is presumably due to the gradual increase in hydrophobicity of the alcohol resulting in lesser inhibitory and inactivating effect on the lipase (Shintre et al. 2002). The present observations match with that of Langrand et al. (1990) and Posorske (1984) wherein decrease in ester yield were observed with increasing length of C-chain in acyl acceptors, beyond pentanol.

The variation in the conversion of oil to ester with the chain length of the acyl acceptors, is due to the influence of various factors such as the miscibility of long chain acyl acceptors in oil, molecular size of the acyl acceptors, interaction between the enzyme and acyl acceptors and affinity of the lipase for the particular acyl acceptors. This differential affinity of lipase enzyme for different alcohols can possibly be explained in terms of the binding energy that is released when a substrate binds at the active site (Dixon et al. 1966; Koshland 1959; Malcata et al. 1992). Substrates such as methanol, ethanol, propanol, which are relatively smaller in size, may not be able to release energy enough to facilitate change in conformation of the native lipase to the desired catalytically active form, resulting in reaction proceeding slowly. In case of butanol and pentanol, it is presumed that there is an optimal release of binding energy required for conformational change and subsequent catalytic activity (Malcata et al. 1992). Substrates that are longer (>C5), are also expected to release binding energy that ought to be sufficient to effect the desired conformational change. However, some of this energy may be required to change the conformation of the substrate so as to make it fit into the active site. Hence, only a small amount of the energy released by the binding process will actually be made available to derive the conformational change of the enzyme (Gandhi et al. 1995). Consequently, the optimum activity is not being achieved. Thus, the ester yield in this study is presumed to be influenced by these

variations in binding energy. ***The observations clearly indicated that acid oil is a better alternative to its corresponding virgin oil, as a substrate for alkyl ester generation, when using both forms of catalyst viz, cell suspension and dried biomass.***

4.8. Transesterification reaction of acid oil with immobilized biomass

This part of the study was focused on scale-up of ethyl ester production in bioreactor by using immobilized whole cell as catalyst through transesterification of acid oil.

4.8.1. Whole cell immobilization on polyurethane foam (PUF)

The micrographs of the matrix with biomass immobilization indicated dense mycelial growth uniformly distributed on the fibrous network of the matrix. Incubation of spores under submerged condition for 72 h, to facilitate colonization, resulted in biomass of 10 (±0.7) mg embedded within polyurethane foam. A uniform network of fungal mycelia was observed within the matrix (Figure 4.13).

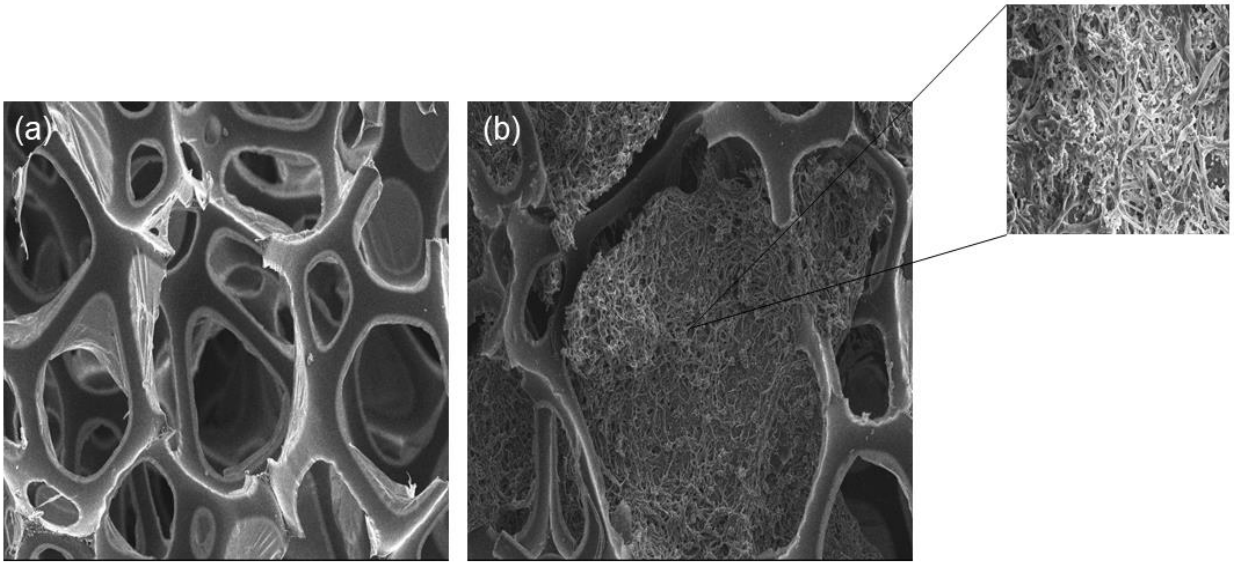


Figure 4.13. Microscopic Images (a) Polyurethane foam (PUF) [Magnification: 100X]; (b) Immobilized whole cells on PUF [Magnification: 100X and Magnification: 500X (inset)]

4.8.2. Protein content and lipase activity

The protein levels of immobilized culture was higher than that of free form i.e. 0.362 mg/mL and 0.084 mg/mL respectively. This may be due to the high concentration of cells localized in immobilized form, while, the free form of culture turns out to be diluted when inoculated in liquid medium, leading to a limited enzyme-substrate interaction. It is known that during the course of reaction, number of intermediate products are generated, which may attach to the surface of cells and inhibit the enzyme activity, the immobilization support preventing the attachment of these products and stabilizes the enzyme activity (de Ory et al. 2006; Malani et al. 2013).

4.8.3. Time dependent variations in transesterification reaction with immobilized biomass: Shake flask conditions

The transesterification reaction of acid oil was carried out with immobilized culture with conditions optimized with free cell suspension. Immobilized whole cell catalyzed hydrolysis of acid oil showed gradual increase in FFA through conversion of acid oil to FFA in 12 h. The esterification with 1:4 molar ratio of acid oil/ethanol, resulted in the decrease of FFA content from 100% to 63% within 1 h of alcohol addition (one-time addition) and ester yield obtained was 37% which increased gradually to 59% in 2 h and 64% in 4 h with corresponding decrease in FFA. There was slight decrease in ester content i.e. 62% in 16 h which can be attributed to reversibility of the reaction (Freedman et al. 1986). The reaction continued upto 24 h with maximum yield of ester being 72% with 28% of corresponding FFA (Figure 4.14) [Annexure III; Fig: 4.8.3].

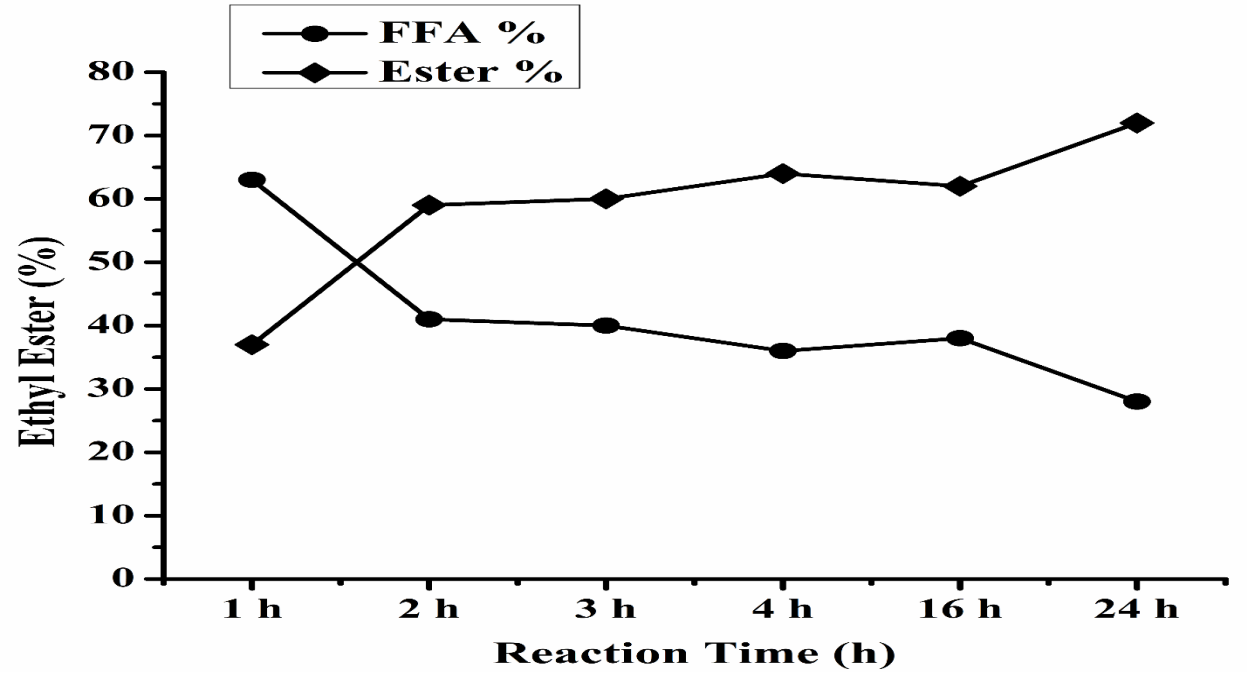


Figure 4.14. Profile of ethyl ester generation over time using immobilized culture

It was therefore presumed that the reaction conditions could be optimized for the use of immobilized culture to carry out transesterification reaction in bioreactor. Various modifications such as peptone concentration, various pH and different temperature range were carried out in growth medium so as to standardize parameters for obtaining maximum lipase activity. The Box-Behnken design of response surface methodology was used for optimizing lipase activity vis-a-vis transesterification reaction.

4.8.4. Response surface methodology

Box-Behnken design (BBD) was employed to investigate the effects of selected factors (temperature, pH, peptone concentration and number of pieces of immobilized PUF) in the culture medium and also to determine their optimum levels for maximum lipase enzyme activity. Table 4.8 (Section 3.8.1) showed considerable variation in the lipase activity under different reaction conditions. The lowest lipase activity of 0.53 U/mL was observed at reaction temperature 31°C,

pH 5.5 and 1.5% (w/v) peptone concentration with 5 immobilized PUF pieces **(run 9)**. Highest lipase activity of 3.10 U/mL was observed at temperature 31°C, 7.0 pH and 1.5% (w/v) peptone with 10 immobilized PUF pieces **(run 25)**.

Table 4.8. Box-Behnken design matrix and responses of independent variables

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Std	Run	A:pH	B:Temperature (°C)	C: Peptone (% w/v)	D:pieces (No.)	Activity (U/mL)
1	1	5.5	26	1.5	10	0.81
2	2	8.5	26	1.5	10	0.971
3	3	5.5	36	1.5	10	1.111
4	4	8.5	36	1.5	10	1.569
5	5	7	31	0.5	5	0.802
6	6	7	31	2.5	5	0.841
7	7	7	31	0.5	15	2.381
8	8	7	31	2.5	15	2.544
9	**9**	**5.5**	**31**	**1.5**	**5**	**0.534**
10	10	8.5	31	1.5	5	0.679
11	11	5.5	31	1.5	15	1.435
12	12	8.5	31	1.5	15	2.088
13	13	7	26	0.5	10	1.852
14	14	7	36	0.5	10	1.942
15	15	7	26	2.5	10	1.972
16	16	7	36	2.5	10	2.109
17	17	5.5	31	0.5	10	1.435
18	18	8.5	31	0.5	10	1.81
19	19	5.5	31	2.5	10	2.1
20	20	8.5	31	2.5	10	2.215
21	21	7	26	1.5	5	0.682
22	22	7	36	1.5	5	0.748
23	23	7	26	1.5	15	2.12
24	24	7	36	1.5	15	1.818
25	**25**	**7**	**31**	**1.5**	**10**	**3.102**
26	26	7	31	1.5	10	3.102

27	27	7	31	1.5	10	3.102
28	28	7	31	1.5	10	3.102
29	29	7	31	1.5	10	3.102
30	30	7	31	1.5	10	3.102

Table 4.9. Analysis of variance of the fitted quadratic polynomial model for optimization of reaction conditions

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Model	20.53	14	1.47	32.34	< 0.0001	significant
A-pH	*0.30*	*1*	*0.30*	*6.69*	*0.0207*	
B-Temperature	*0.066*	*1*	*0.066*	*1.46*	*0.2462*	
C-Peptone	*0.20*	*1*	*0.20*	*4.47*	*0.0517*	
D-pieces	*5.47*	*1*	*5.47*	*120.62*	*< 0.0001*	
AB	*0.022*	*1*	*0.022*	*0.49*	*0.4962*	
AC	*0.017*	*1*	*0.017*	*0.37*	*0.5506*	
AD	*0.065*	*1*	*0.065*	*1.42*	*0.2514*	
BC	*5.523E-004*	*1*	*5.523E-004*	*0.012*	*0.9136*	
BD	*0.034*	*1*	*0.034*	*0.75*	*0.4011*	
CD	*3.844E-003*	*1*	*3.844E-003*	*0.085*	*0.7749*	
A^2	*6.59*	*1*	*6.59*	*145.30*	*< 0.0001*	
B^2	*5.09*	*1*	*5.09*	*112.32*	*< 0.0001*	
C^2	*0.72*	*1*	*0.72*	*15.91*	*0.0012*	
D^2	*6.73*	*1*	*6.73*	*148.46*	*< 0.0001*	
Residual	0.68	15	0.045			
Lack of Fit	*0.68*	*10*	*0.068*			
Pure Error	*0.000*	*5*	*0.000*			
Cor Total	21.21	29				

Table 4.9 represents the adequacy of the model which was checked using analysis of variance (ANOVA) and tested using Fisher's (F-test) statistical analysis. The Model F-value, 32.34 with very low probability value [(P>F) = 0.0001] exhibits a very high significance for the regression model. The multiple correlation coefficients (R^2) values, predicted R^2 = 0.8153 and adjusted R^2 = 0.9380 denoted better correlation between the observed and predicted responses

(Figure 4.15). The adjusted determination coefficient value was also very high implying high significance of the model (Khuri et al. 1987). The coefficient of variation (CV) value 11.58%, indicates the reliability of the experiment and degree of precision with which the experiments were compared. The *p* values (<0.0001) are used to check the significance of the coefficients and mutual interactions between the variables. The results obtained from the Box-Behnken Design were fitted to a second order polynomial equation to explain the dependency of maximum lipase activity on the medium components.

$Y = 3.10 + 0.16A + 0.074B + 0.13C + 0.68D + 0.074AB - 0.065AC + 0.13AD + 0.012BC - 0.092BD + 0.031CD - 0.98A^2 - 0.86B^2 - 0.32C^2 - 0.99D^2$

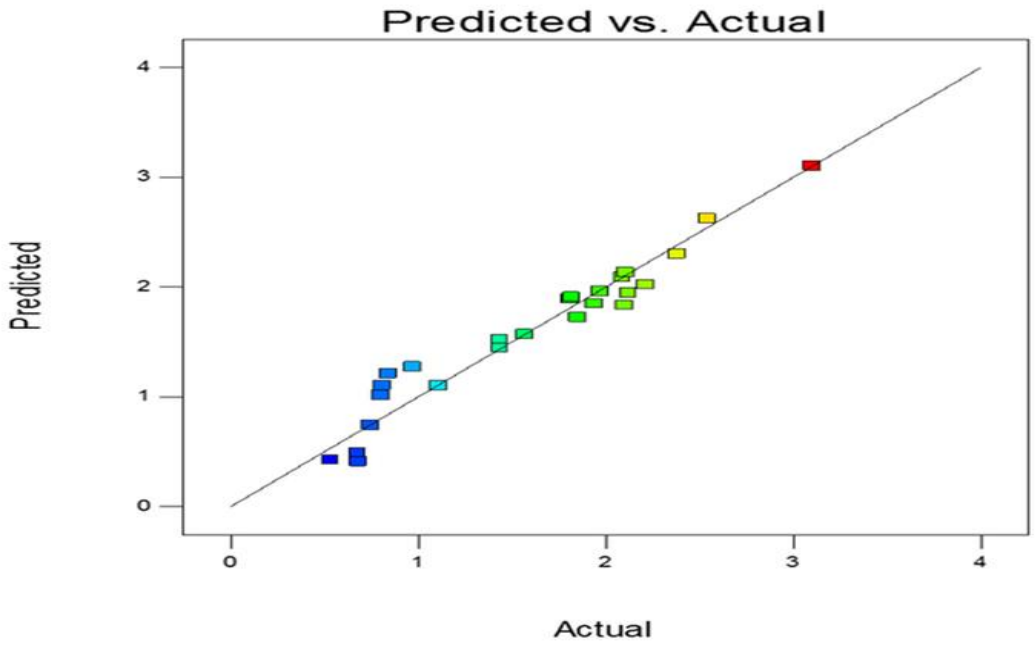


Figure 4.15. Regression plot of predicted versus actual data

The fitted response for the regression model was plotted in 3D graphs, which display the effects of the independent variable and composites on the response variable. The 3D plots are the

graphical representation of the regression equation for the optimization of reaction conditions (Figure 4.16).

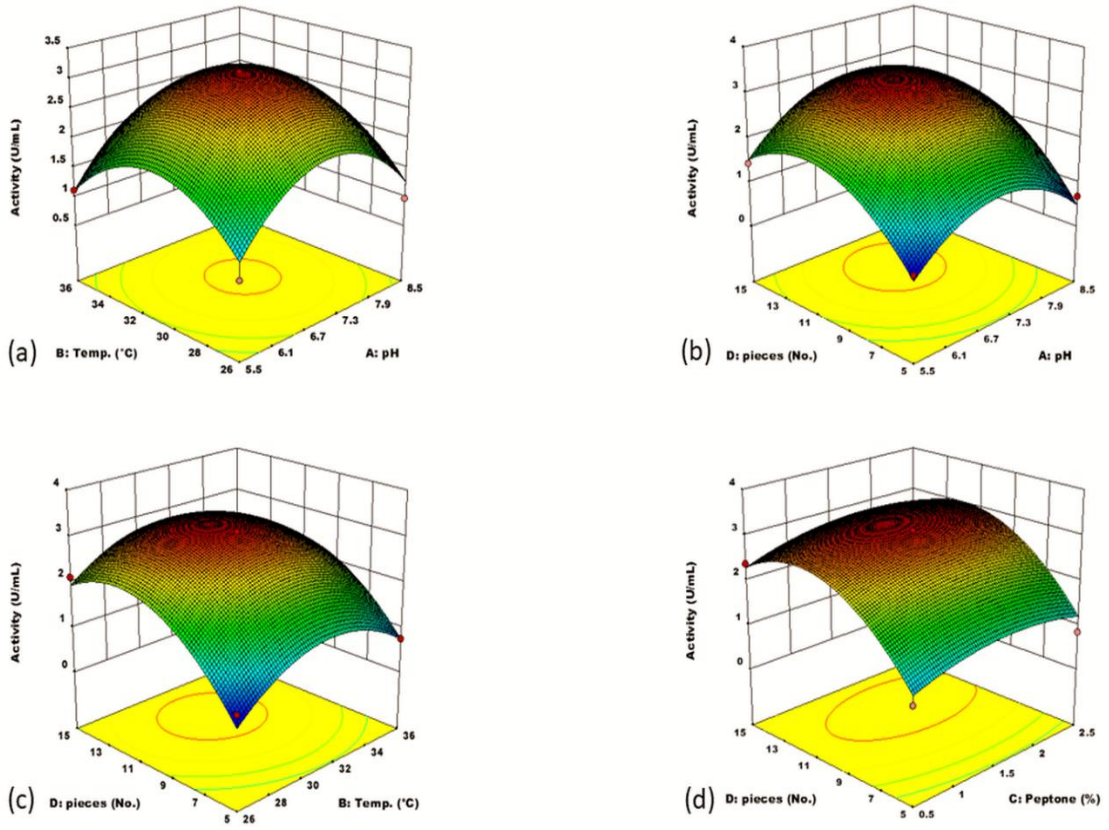


Figure 4.16. 3D plots of combined effects of factors (a) Temperature and pH; (b) PUF pieces and pH; (c) PUF pieces and temperature; (d) PUF pieces and peptone concentration

Figure 4.16 represents the effects of each factor, on the lipase activity in three dimensional plots. Figure 4.16 (a) represents the effect of the significant interaction between factors; temperature and pH. It shows that the pH (7.1) and reaction temperature (31°C) were optimum for the maximum lipase activity. The activities of many enzymes vary with pH, because most active sites function as general acids and general bases in catalysis. As mentioned in section 4.4.2 the enzymes usually have an optimal working pH, higher or lower value of pH can lead to deactivation of enzyme (Romero et al. 2007).

Temperature also plays a critical role in enzyme catalysis. The plot represents that the temperature 31°C was optimum for the lipase activity. Further increase in temperature, decreased the activity of enzyme, expectedly due to denaturation of protein. Most of the fungal lipases exhibit maximum activity in the range of 22°C-35°C. Chander et al. (1980) also reported the maximum lipase activity of *A. wentii* at 30°C.

Figure 4.16 (b) shows that the maximum lipase activity was attained at moderate levels of immobilized PUF pieces (10) and pH (7.1) of the medium. The lipase activity increased to certain extent with increasing number of immobilized PUF pieces and further increase in the number of immobilized PUF pieces (15), resulted in marginally decrease of lipase activity. This may be due to the presence of a high level of enzyme resulting in aggregation of enzyme active sites (Foresti et al. 2005; Liou et al. 1998). Similar observation was reported by Shankar et al. (2013) with *C. antarctica* lipase B catalyzed esterification reaction of lauric acid.

Figure 4.16 (c) represents the interaction between number of immobilized pieces and temperature. The enzyme activity of immobilized pieces slightly increase with increase in temperature to the maximum of 31°C and then decreased again as the temperature went beyond 31°C. The decrease in lipase activity, marginally after 31°C, which may be owing that denaturation process of enzyme, caused by the movement of chemical bonds in the lipase structure beyond a critical temperature (Luo et al. 2006).

Figure 4.16 (d) shows the effect of lipase activity with respect to number of immobilized PUF pieces and peptone concentration. Peptone was selected based on reports which showed increase in lipase activity of microorganisms, such as *R. oligosporus* (Nahas 1988); *Candida* and *Yarrowia* (Novotný et al. 1988) and *A. flavus* (Long et al. 1996) in the presence of this nitrogen

source. Results showed that there was an increase in lipase activity with increase in the concentration of peptone up to 1.2% (w/v). With further increase in peptone concentration to 2.5% (w/v), the enzyme activity marginally decreased and remained constant. The high concentration of peptone in medium showed inhibitory effect on lipase activity as shown in section 4.4.1. This is expected to be due to complex nature of peptone and other constituents that might have toxic effect on lipase activity (Sooch et al. 2013). The immobilization provides the stability to culture and remain active in high concentration of peptone in the medium. Sharma et al. (2017) reported a similar observation, wherein the lipase activity increased with increase in peptone concentration in medium to some extent followed by decrease over high concentration (Salleh et al. 1993). Based on the results, 1.2% (w/v) peptone concentration was considered as optimum amount in the medium.

4.8.5. Validation of the model

The accuracy of the model was validated with triplicate experiments for lipase activity. The mean value of the lipase activity was 2.89 ± 0.6 U/mL whereas, the predicted value was 3.11 U/mL, indicating that the generated model was an adequate prediction of the enzyme activity.

4.9. Time dependent study of transesterification reaction in bioreactor using immobilized whole cells as catalysts

The transesterification reaction was carried out with optimized conditions identified through RSM, (pH 7.1, temp. 31°C, peptone 1.2% (w/v) and immobilized pieces 10 in 100 mL (150 pieces in 1.5 L)) in 5 L bioreactor, by using immobilized whole cells as catalysts. The reaction resulted in complete hydrolysis of acid oil to FFA over 6 h. Esterification was carried out with addition of ethanol through pulse feeding (1:4 molar ratio of oil to alcohol) at the rate of 2.4 mL/min by peristaltic pump, followed by 1 h incubation, resulting in generation of ethyl esters

with corresponding decrease in FFA content. At regular time interval of 1 h, samples were withdrawn from the reactor up to 12 h. The conversion of oil to FFA and formation of ethyl ester in samples were analyzed through ^{1}H NMR. The whole cell catalyzed transesterification of acid oil is assumed to follow an acyl-enzyme-intermediate mechanism. The transesterification reaction is expected to proceed as follows: the enzyme (E) reacting with triacylglycerol (TAG) to form the first complex (E-TAG), after which an acylated enzyme fatty acid complex is formed (E-Ac-F). Subsequently, in esterification, oxygen atom of alcohol molecule is linked to the carbonyl group of the acyl enzyme intermediate to form an acylated enzyme-alcohol complex (E-Ac-A). At the end of transesterification, alkyl ester was generated and the enzyme (E) remains in medium in free form (Al-Zuhair et al. 2007; Azócar et al. 2014). This mechanism could be described as Michealis-Menten mechanism based on the concentration of substrate (FFA) and formation of product (ethyl ester). Therefore, expectedly lipase first binds to FFA and the acyl-enzyme intermediate is formed; followed by the reaction with alcohol resulting generation of ester. As FFA are the only species present in significant amounts, it is predicted that the reaction proceeds only in the forward direction. The K_M value and V_{max} were determined by measuring the initial rate of esterification by using concentration of substrate. The K_M and V_{max} at each concentration of FFA were obtained from the initial rate data directly fitted to the Michealis-Menten equation (eqn. 3). It was found that the maximum velocity (V_{max}) the Michealis constant (K_m) of reaction was 78 g L^{-1} h^{-1} and 75 g L^{-1} respectively.

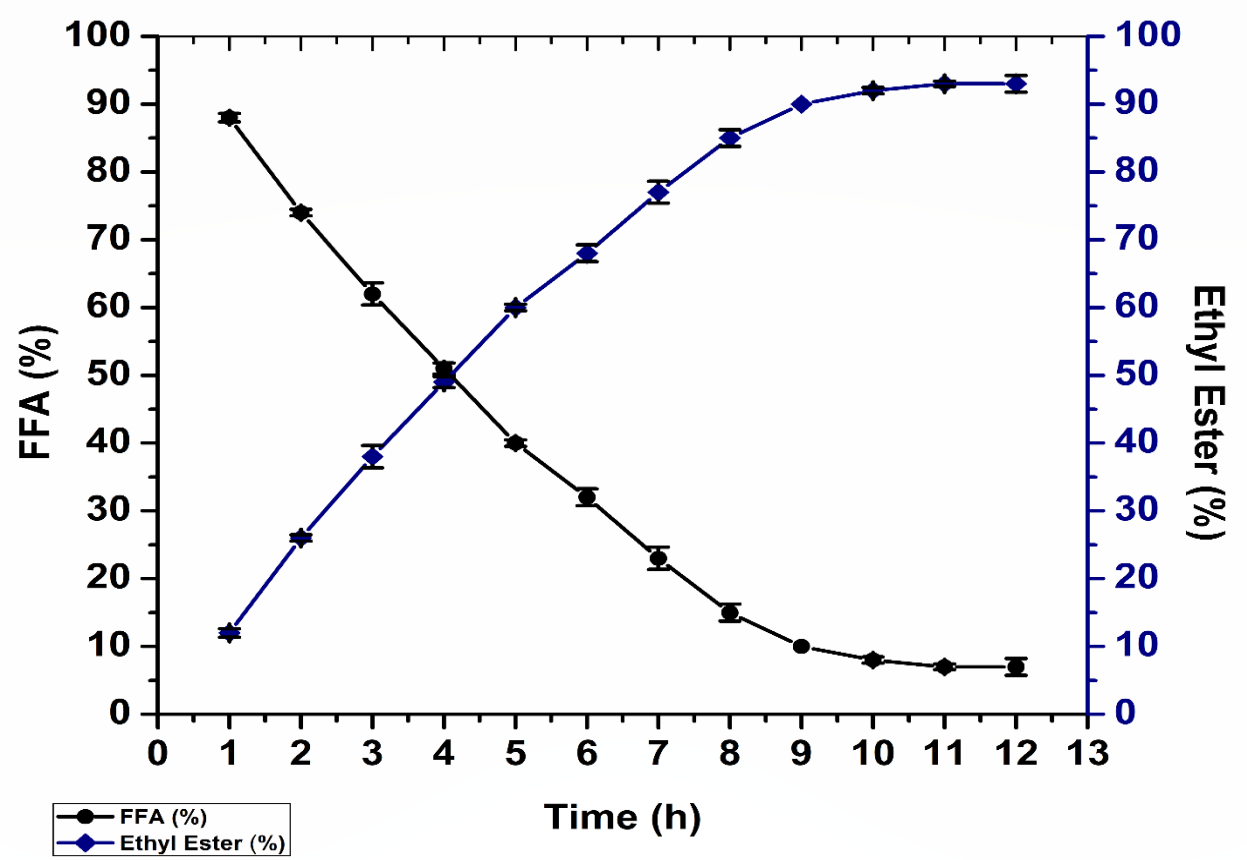


Figure 4.17. Kinetics of transesterification reaction; product formation and substrate consumption with respect to time

Figure 4.17 shows the results in terms of initial reaction rate of fatty acid ethyl ester (FAEE) generation at regular time interval. The initial reaction rate increased with increasing time, this was apparently due to presence of high substrate concentration at initial stage of the reaction. At this stage, all the enzyme molecules are bound to the substrate molecules, the acylated enzyme-alcohol complex (E-Ac-A) convert FFA to FAEE. After 10 h, the product formation stabilized, due to low substrate concentration in the reaction mixture.

The present study is significant in term of the process, wherein the hydrolysis and esterification steps were carried out with immobilized whole cells of A. flavus in a bioreactor resulting in conversion of acid oil to ethyl ester. During the esterification reaction, the ester

yield was 12 % in first hour, which increased gradually to 60% in 5 h and 90% in 9 h with corresponding decrease in FFA. The yield of biodiesel reached to 93% with 7% FFA in 12 h and the maximum yield of biodiesel attained 95.4% with 4.6% FFA within 24 h [Annexure IV; Fig: 4.9].

To the best of our knowledge, there are limited reports on generation of ethyl ester from acid oil specially by using fungal whole cells as catalyst in a bioreactor. Li et al. (2007) reported the use of immobilized whole cells of *Rhizopus oryzae* within the biomass support particles for production of biodiesel from acidified, refined and crude rapeseed oil in tert-butanol system, where tert-butanol was used as media component to enhance the stability of *R. oryzae* whole cells. The authors obtained maximum 70% yield of methyl ester when acidified rapeseed oil was used as a feedstock in place of refined and crude rapeseed oil. Zhou et al. (2015) used a two step biocatalytic process using lipase and whole cell catalysts for biodiesel production from unrefined jatropha oil. The hydrolysis reaction was carried out by using the commercial enzyme *C. rugosa* lipase followed by esterification reaction by *R. oryzae* IFO4697 cells immobilized within biomass support particles. A maximum yield of 88.6% fatty acid methyl ester was achieved at 35°C in 42 h. The immobilized lipase Lipozyme TL IM was also employed for the transesterification reaction of rice bran acid oil in packed-bed reactor resulting a maximum yield of 92 % over 4 h duration (Choi et al. 2016). Aguieiras et al. (2014) developed a process of hydroesterification for biodiesel production from acid oil of *Acrocomia aculeate,* with ethyl alcohol using lipase from *Rhizomucor miehei* to catalyze the esterification reaction. The authors achieved a maximum yield of 92.2% ethyl ester in 72 h by solid state fermentation. Further, one stage lipase (Novozym 435) catalyzed methanolysis was employed for generation of biodiesel from soapstock oil, wherein, a maximum FAME yield of 95.2% was obtained in 10 h with 4% (w/w) of lipase and methanol/oil molar ratio

of 5:1 at 45°C with tert-pentanol as lipase inducer (Su et al. 2014). Soares et al. (2013) reported the biodiesel production from soybean soapstock acid oil using hydroesterification process and *Burkholderia cepacia* LTEB 11 lipase as catalyst. In this particular study, complete hydrolysis of feedstock to FFA was obtained in subcritical water followed by 92% esterification in packed-batch reactor containing fermented solid over 31 h at 50°C.

The re-usability of immobilized whole cells was examined by carried out esterification reaction of acid oil, over 5 cycles. Figure 4.18 shows that there was no obvious loss in ester yield even after cells were repeatedly used for 5 cycles. The catalytic potential of immobilized whole cells resulted in only 5% decrease from 1^{st} cycle to 5^{th} cycle. It could be due to glutaraldehyde cross-linking treatment of cells, which provided the operational stability to whole cells and prevented the leakage of lipase from the cells (Sun et al. 2010). Ban et al. (2001) also observed the effect of glutaraldehyde cross-linking on membrane bound lipase and reported that the enzyme maintained high lipolytic activity over several cycles after glutaraldehyde treatment.

Thus, the use of immobilized whole cells system for generation of biodiesel from acid oil can provides an alternative and cost effective process to enzymatic catalysis resulting in better yield of biodiesel in a bioreactor in shorter time duration and solvent free medium.

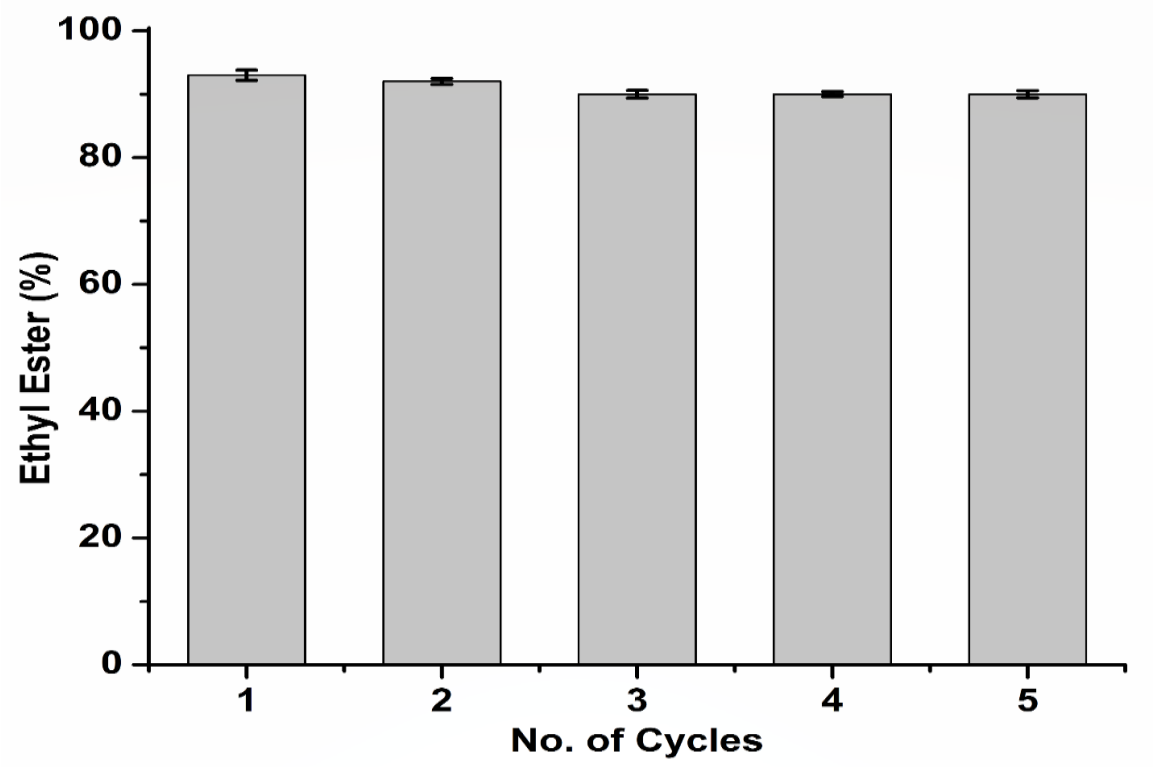


Figure 4.18 Reusability of Immobilized whole cells

4.10. Fatty acids composition of alkyl ester

The fatty acid profiles are the major indicators of the properties of any alkyl ester. The fatty acid composition of alkyl ester obtained from acid oil was analyzed by gas chromatograph. The composition of the fatty acids affect various properties of biodiesel, such as viscosity, cold flow properties and density. Table 4.10 indicates that the fatty acids of alkyl esters are all long chain carbon compounds that contain number of saturated and unsaturated double bonds. The acid oil used in the present study, comprised approximately 70% fatty acids having double bond at position Δ9. ***The composition of acid oil showed the dominant esters to be ethyl oleate, ethyl linoleate, ethyl palmitate and ethyl stearate.*** Lipase also discriminates between fatty acids with different double bonds positions wherein, the reaction of fatty acids with Δ9 double bonds position is significantly faster than the Δ4, Δ5 and Δ6 when catalyzed by enzyme (Shimada et al. 1998). ***The***

lipase present in the A. flavus carried out complete conversion of fatty acids having double bonds at Δ9 positions, which may depict the specificity of lipase enzyme for double bonds at Δ9 positions of fatty acids. The distinctive ester composition of biodiesel, due to combination of these compounds, has a strong influence on its fuel properties, because of presence of saturated and unsaturated fatty acids. The generated fuel had a significant degree of unsaturation due to ethyl oleate and ethyl linoleate. The unsaturation inhibits crystallization of fuel, and reduces cloud point and pour point levels of the fuel. The alkyl ester of acid oil obtained from transesterification was examined for fuel properties with reference to standards of biodiesel [Annexure V; Fig: 4.10].

Table 4.10. Fatty acids composition of fatty acid ethyl ester (FAEE)

Fatty acids	FAEE (wt %)
Palmitic acid (C16:0)	18.64
Stearic acid (C18:0)	2.46
cis 9 Oleic acid (C18:1)	37
Linoleic acid (C18:2)	35.41

4.11. Fuel properties of biodiesel samples

Typical fuel properties of obtained alkyl ester (B100), its blend (B20) and petroleum diesel (PD) were compared with ASTM standard in Table 4.11. ***The ester generated from acid oil closely met the standard specifications and was distinctly different from those of diesel fuel.*** The properties of blend varied with the composition as well as properties of neat biodiesel and diesel fuel used. Most of the properties matched with standards confirming the potential use of these ester as biodiesel.

Table 4.11. Fuel properties of B100 and B20 compared with PD and ASTM standard

S.No.	Parameters	ASTM Standard (D6751)	PD	B100	B20
1.	Viscosity@40˚C (cSt)	1.9 – 6.0	4.7	6.8	4.8
2.	Pour point (˚C)	NA	-30	-26	-29
3.	Density@15˚C (Kg/m^3)	878	822	876	842
4.	Flash point (˚C)	130	58	180	120
5.	Calorific value (MJ/kg)	NA	43.34	39.92	46.32
6.	Sulphur content (%)	0.05	<0.1	<0.1	<0.1
7.	Ash (%)	NA	0.011	0.96	0.26

4.11.1. Pour point

The pour point is a characteristic property of biodiesel, and is the lowest temperature at which the fuel can continue to flow. Cloud point and pour point are used to measure the cold temperature usability of a fuel. The cloud point of the biodiesel samples (B100 & B20) could not be observed due to dark colour of the samples. The B100 had a notable pour point (-26ºC) which is significantly lower than reported. Pour point of the reference petroleum diesel fuel and B20 were (-30ºC) and (-29ºC) respectively, which were lower than those of B100 (Table 4.11 & Figure 4.19 (a)). Blending 80 vol.% diesel with 20 vol.% biodiesel (B20) significantly decreased the pour point of biodiesel. These values of pour points are perceived very low particularly for application in cold climatic conditions. This is due to high degree of unsaturation (monounsaturated and polyunsaturated) as depicted by composition of biodiesel. Transesterification process does not change the fatty acid composition of feedstocks, thus, biodiesel generated from feedstocks which contains saturated fatty acids in higher concentrations, tends to have comparatively poor cold flow properties (Dunn et al. 2005). Whereas, high unsaturation in fatty acids favours appropriate cold flow properties (Ramos et al. 2009). In the present study, the feedstock acid oil had approximately

70% unsaturated fatty acids, and therefore showed low pour point. From these results, it is clear that biodiesel generated from acid oil would be a suitable candidate as a diesel fuel substitute in countries with colder climate. Cloud point and pour point are related properties in ASTM D6751, but limits have not been clearly defined till date (Campus 2011).

4.11.2. Viscosity

Viscosity is an important fuel property that measures the internal fluid friction between molecules of fuel to flow, having a tendency to oppose any dynamic change in the fluid motion. That was the main purpose why neat oils were transesterified to alkyl esters (or biodiesel). High viscosity gives rise to incomplete combustion, carbon deposition on the injectors and poor fuel atomization (Alptekin et al. 2008; Knothe et al. 2007). High viscosity also causes difficulties in cold weather, because this property increases with decrease in temperature (Joshi et al. 2007). On the other hand, the fuel having low viscosity value, may not provide appropriate lubrication for the precision fit of fuel injection pumps, resulting in leakage (Knothe et al. 2005). Viscosity of biodiesel increases with number of carbon atoms and degree of saturation present in the sample wherein, unsaturation influences viscosity. This also holds good for the alcohol moiety as the viscosity of ethyl esters is somewhat higher than the viscosity of methyl esters (Knothe et al. 2005). This confirms that the viscosity is directly related to the composition of fatty acids in feedstock as well as ethyl ester. In the present study, the biodiesel (B100) produced from the acid oil, had marginally higher kinematic viscosity 6.8 cSt to the specified ASTM standard (1.9 to 6.0 cSt) when compared to B20 and PD having kinematic viscosities of 4.8 cSt and 4.7 cSt respectively that were within the range specified (Table 4.11 & Figure 4.19 (b)). Due to presence of large number of hydrogen bonds, viscosity value of obtained fuel was higher than ASTM specified. Lin et al. (2012) reported the generation of biodiesel from soybean soapstock acid oil with minimum viscosity of

5.2 cSt when using 42 molar ratio of supercritical-methanol. Similarly, Kulkarni et al. (2008) reported 5.2 cSt viscosity of biodiesel, generated from acid oil.

4.11.3. Density

Density is an important property that directly affects the engine performance characteristics, as well as mass of fuel injected into the combustion chamber. As the fuel injection pumps measure fuel by volume not by mass, a denser fuel will comprise a greater mass in the same volume. Thus, the alterations in the fuel density will effect engine output power due to a different mass of fuel injected (Alptekin et al. 2008). Table 4.11 and Figure 4.19 (c) shows that the density of B100 (876 Kg/m^3) was nearly similar to ASTM standard (878 Kg/m^3). The densities of B20 (842 Kg/m^3) and PD (822 Kg/m^3) had no significant difference between each other. Ong et al. (2014) reported the density of *Calophyllum inophyllum* methyl ester as 877.6 kg/m^3 using alkali catalyst for generation of biodiesel. Aguieiras et al. (2014) reported the use of enzyme/enzyme hydroesterification for production of biodiesel from *Acrocomia aculeata* acid oil and reported the density of 872.2 Kg/m^3. Generally, the density of biodiesel is higher than that of petroleum diesel, however, the energy content is low in volume and mass basis. (Canakci et al. 2001). The density of biodiesel depends on its fatty acid composition and increases with decrease in chain length vis-a-vis increase in number of double bonds in ester. In the present study, the density of B20 was lower than the specified ASTM standard but nearly closer to the petroleum diesel. On the other hand the density of B100 was similar with standard specified.

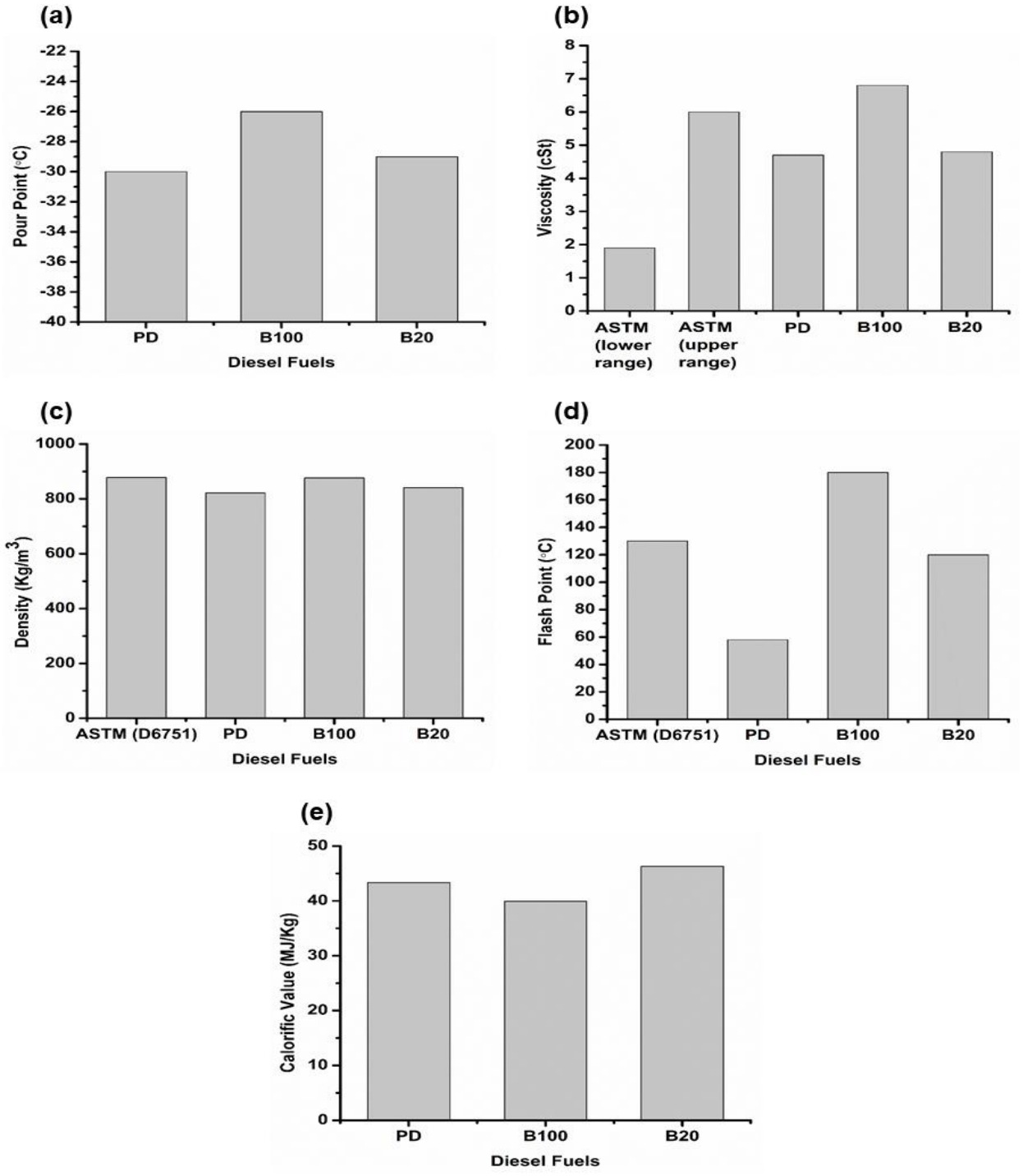


Figure 4.19. Fuel properties (a) Pour point; (b) Viscosity; (c) Density; (d) Flash point; and (e) Calorific value – (B100, B20 and PD)

4.11.4. Flash point

The flash point is a fuel property that regulates the safety of a fuel during its usage and storage. It measures the tendency of a fuel to form inflammable mixtures with air, under controlled conditions. This test, in part, is a measurement of residual alcohol in biodiesel that determines the flammability classification of the fuel. Table 4.11 and Figure 4.19 (d) show that the flash points of B100 (180ºC) and B20 (120ºC) are higher than that of petroleum diesel (58ºC). B100 had high flash point than ASTM specified (130ºC) whereas, B20 (120ºC) was marginally lower. This may be due to the presence of largely saturated esters. Biodiesel has considerably higher flash point than diesel fuel, which means that the fire hazard related with utilization of biodiesel, storage and transportation is much less than that of petroleum diesel (Ahmad et al. 2009; Barua 2011). Lin et al. (2012) reported the flash point 118ºC of biodiesel produced from soybean soapstock. Kulkarni et al. (2008) also reported generation of biodiesel from acid oil having flash point of 102ºC. High flash point (151ºC) was obtained by Aguieiras et al. (2014) through hydro-production process for generation of biodiesel from *Acrocomia aculeata* acid oil. Further, Ong et al. (2014) reported 79.5ºC flash point of *Calophyllum inophyllum* B20 blend whereas, Keskin et al. (2008) obtained 73ºC flash point of cottonseed soapstock biodiesel blend. Our findings differ from the observations of other reports and is assumed that the obtained biodiesel had no residual amount of alcohol and therefore does not require additional precautions during handling, transportation and utilization processes.

4.11.5. Calorific value

Calorific value is another important fuel property that determines the energy content and its suitability as transport fuel. The results presented in Table 4.11 and Figure 4.19 (e) show that the calorific values of B100 (39.92 MJ/Kg) was lower than that of petroleum diesel. However, B20

(46.32 MJ/Kg) had high calorific value compared to that of petroleum diesel (43.34 MJ/Kg). Ong et al. (2014) reported the calorific value of high fatty acid *Calophyllum inophyllum* biodiesel and its B20 blend to be 41.44 MJ/Kg and 41.52 MJ/Kg respectively. Similarly, Kulkarni et al. (2008) reported calorific value of biodiesel of 40.6 MJ/Kg obtained from acid oil. In the present study, the lower calorific value of biodiesel (B100) is expectedly due to higher oxygen content, as the oxygen improves emissions and combustion properties, but reduces the calorific value (Yamane et al. 2001). In addition, McCormick et al. (2005) reported that the biodiesel does not contain any aromatic components however it contains alkyl esters with different levels of saturation and unsaturation. Unsaturated esters have lower energy content, but have more energy per unit volume owing to their higher density.

- ***In summarizing the work carried out on the whole cell catalyzed transesterification of acid oil using selected fungi; the study demonstrates some salient features which hitherto have been either known to a limited extent or not reported till date. The study resulted characterization of five fungi exhibiting potential to grow and utilize acid oil as carbon source to the extent of upto 70% (v/v) supplementation in growth medium. One of the strains, Aspergillus flavus, was observed to catalyze transesterification of acid oils to alkyl esters, to the extent of ≥ 98% at 70% (v/v) acid oil supplementation. The optimized conditions required for maximum lipase activity of the said strain with acid oil have been identified. Stepwise addition of alcohol resulted in better conversion of acid oil to ethyl ester. In addition, the dried biomass could effectively enhanced the transesterification reaction in shorter duration. Scale-up production of biodiesel from acid oil using immobilized whole cells catalyst with the use of bioreactor reached upto 95.4% within 24 h reaction. The resultant alkyl ester was noted to meet the specifications of major international biodiesel standards to a significant extent. The present study is of importance in identifying the potential use of whole-cell fungi as biocatalyst for generation of alkyl esters including biodiesel from acid oil is useful for commercial applications.***

Conclusion

Six different fungal strains namely *Aspergillus flavus* (MTCC 5436), *Aspergillus sydowii* (MTCC 10397), *A. aculeatus, Curvularia pallescens* (MTCC 10390), *and Periconia sp* (MTCC 10391) (isolated from contaminated butter) and *Rhizopus oryzae* (obtained from Bhabha atomic research centre, Mumbai) were observed to be acid oil tolerant in the growth medium. Among all the strains *A. flavus,* showed maximum oil tolerance upto 70% (v/v) acid oil in the growth medium. This strain exhibited maximum hydrolytic activity (100% FFA) with 70% (v/v) acid oil supplementation in the growth media followed by *R. oryzae* which also resulted in 100% FFA with 50% (v/v) acid oil.

A. flavus was subjected to strain improvement through induced mutation using gamma radiation with an aim to reduce the time period for lipase production and transesterification reaction. Different doses (i.e. 50Gy, 100Gy, 200Gy, 300Gy, 400Gy) of gamma radiation were applied to induce mutation in the cells of wild type fungal strain. It was observed from the screening studies, that the wild type, *A. flavus,* was a better strain for lipase production and hydrolysis of acid oil over other variants obtained through gamma irradiation as radiation did not significantly improve lipase production/activity. *A. flavus* exhibited maximum lipase activity of 2.46 U/mL at 70% (v/v) acid oil as main carbon source peptone and bi-ammonium hydrogen ortho-phosphate (0.5% w/v) as nitrogen sources. The optimum pH and temperature for enzymatic activity were observed to be 7.5 and 30°C, respectively.

Four types of acid oils were used viz; cottonseed, soybean, sunflower and rice bran, which contained common saturated (palmitic acid and stearic acid) and unsaturated fatty acids (oleic acid and linoleic acid). *A. flavus* was observed to facilitate transesterification in acid oil: minimal medium with the ratio of 70:30 resulting in near complete conversion of acid oil to ethyl esters

within 48 h at 30°C and 120 rpm. Further stepwise addition of ethanol, at an interval of 12 h, facilitated near complete conversion of acid oil to ester.

A. flavus was also used for transesterification reaction of virgin oil and acid oil by using biomass suspension and dried biomass of *A. flavus* with short and medium chain alcohols (methanol to decanol) as acyl acceptors. The dried biomass of *A. flavus* could effectively enhanced the transesterification reaction in shorter duration. The acyl acceptors (propanol, butanol and pentanol) had less negative effects on lipase activity and the alkyl esters thus obtained had improved cold flow properties of fuel. Thus, the acid oil was noted an effective feedstock for alkyl ester generation, over virgin oil.

Further, the transesterification by *A. flavus* was studied by immobilizing the biomass on polyurethane foam. Optimization for maximum lipase activity was carried out by response surface methodology. The maximum lipase activity of 3.10 U/mL was achieved with 70% (v/v) acid oil, peptone (1.2% w/v) and immobilized pieces (10) in 100 mL growth medium. The optimum pH and temperature for enzymatic activity were 7.1 and 31°C, respectively. Scale-up production of biodiesel from acid oil was carried out in 5L lab-scale bioreactor using immobilized whole cells catalyst. The maximum yield reached upto 95.4% within 24 h of reaction. The catalytic potential of immobilized whole cells resulted in only 5% decrease from 1st cycle to 5th cycle.

The resulted biodiesel was noted to meet the specifications of major international biodiesel standards such as ASTM D6751 to a significant extent. The B100 and B20 fuel had good pour point and density along with other properties that were also comparable with petroleum diesel.

The work, thus carried out on various aspects of whole cell catalysis and use of acid oil as feedstock for generation of alkyl esters, observed to be a potential means of producing these products for diverse variety of industrial/commercial use such as biodiesel.

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Fig: 4.5.2 (I) Gas Chromatograph of FAME Mix C_8-C_{24}

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Method File: .MET
Detector: FID.                                          System: GC
Date:  4 Jul 2016                                       Time:  18:45:46
Run: ch1:  0
Type of Analysis: Percent On Area
Report printed on: 11/1/2018 at: 12:21:44
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Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
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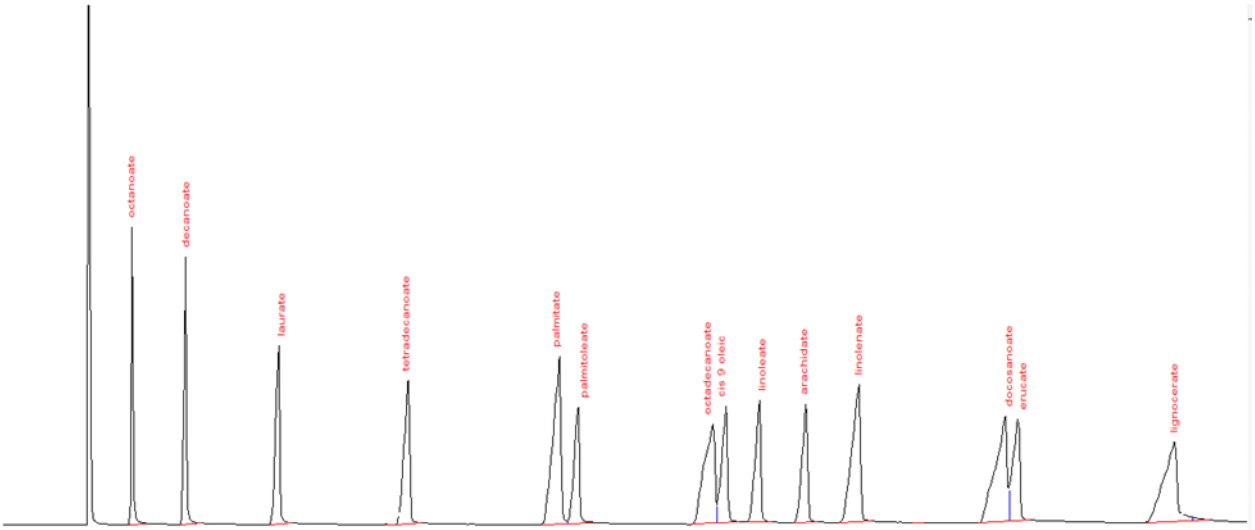

No.	R.T.	Area	Area %	Pk Ty	Comp Name
1	3.15	1347269	5.1728	BB	octanoate
2	4.43	1506472	5.7840	BB	decanoate
3	6.67	1633160	6.2704	BB	laurate
4	9.38	3262	0.0125	S	
5	9.79	1781976	6.8418	PB	tetradecanoate
6	14.13	2833660	10.8797	BV	palmitate
7	14.57	1368331	5.2536	VB	palmitoleate
8	17.62	2249952	8.6386	BV	octadecanoate
9	18.03	1502733	5.7697	VB	cis 9 oleic
10	18.93	1449602	5.5657	BB	linoleate
11	19.36	1431436	5.4959	BB	arachidate
12	20.63	2470902	9.4869	BB	linolenate

13	21.99	8180	0.0314	BB	
14	24.14	2565892	9.8516	BV	docosanoate
15	24.45	1529988	5.8743	VB	erucate
16	28.21	2316322	8.8934	BV	lignocerate
17	28.66	46260	0.1776	S	
		26045397	100.0000		

Fig: 4.5.2 (II) Gas Chromatograph of Cottonseed acid oil

Sample Name: CSAO CAT
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Method File: fame mix 050716.MET
Detector: FID. System: GC
Date: 5 Apr 2017 Time: 10:59:2
Run: ch1: 0
Type of Analysis: Percent On Area

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
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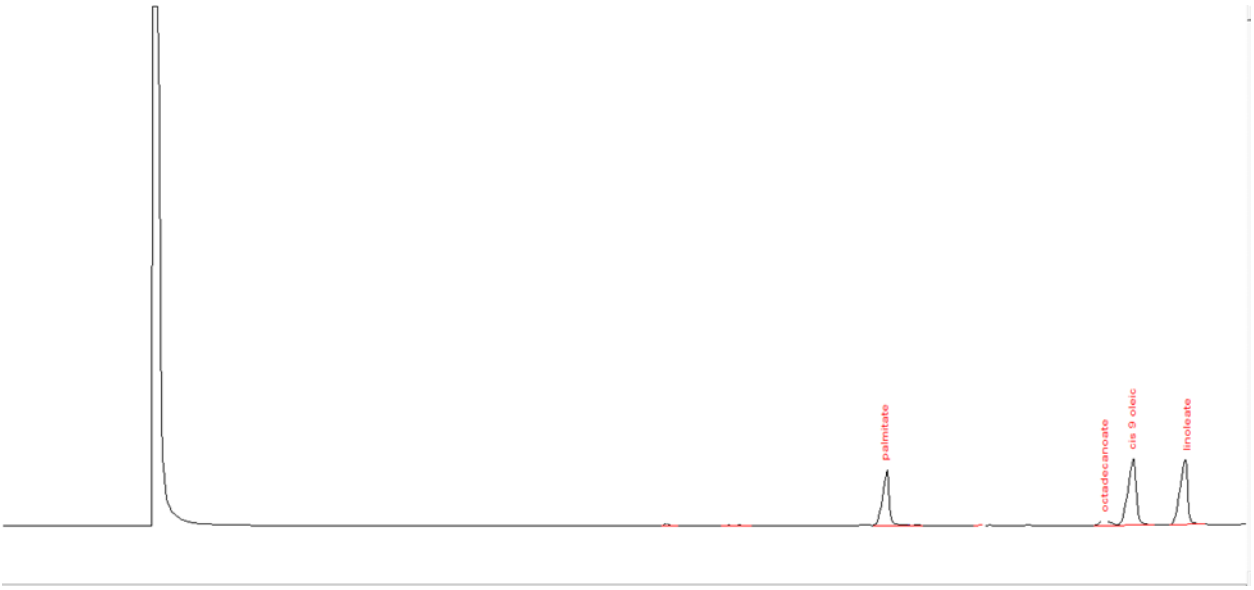

No.	R.T.	Area	Area %	Pk Ty	Comp Name
1	14.21	530757	24.2405	BV	palmitate
2	15.69	2636	0.1204	BB	
3	17.68	72604	3.3160	BV	octadecanoate
4	18.07	805521	36.7894	VB	cis 9 oleic
5	18.90	733256	35.4284	BB	linoleate
6	19.67	2310	0.1053	BB	
		2147084	100.0000		

Fig: 4.5.2 (III) Gas Chromatograph of Sunflower acid oil

```
Sample Name: Sunflower 140917
Data File: D:\AKS\Sunflower 140917.DAT
Method File: .MET
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Date:  14 Sep 2017                                      Time:  11:31:36
Run: ch1:  0
Type of Analysis: Percent On Area
Report printed on: 11/1/2018 at: 12:59:33
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Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
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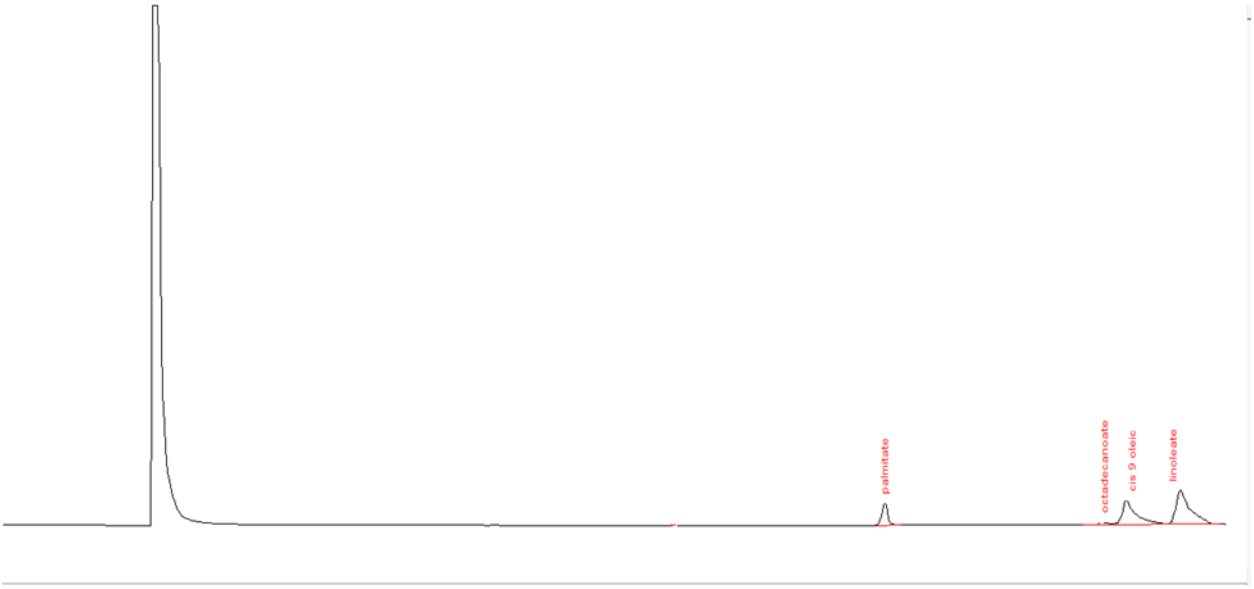

No.	R.T.	Area	Area %	Pk Ty	Comp Name
1	10.79	1256	0.0889	BB	
2	14.18	187301	13.2532	BB	palmitate
3	17.40	2979	0.2108	S	
4	17.65	40113	2.8383	BV	octadecanoate
5	18.05	486615	34.4323	VV	cis 9 oleic
6	18.91	694989	49.1765	VB	linoleate
		1413253	100.0000		

Fig: 4.5.2 (IV) Gas Chromatograph of Rice bran acid oil

Sample Name: RB 220917
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Method File: .MET
Detector: FID. System: GC
Date: 22 Sep 2017 Time: 18:35:48
Run: ch1: 0
Type of Analysis: Percent On Area
Report printed on: 11/1/2018 at: 12:39:49

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
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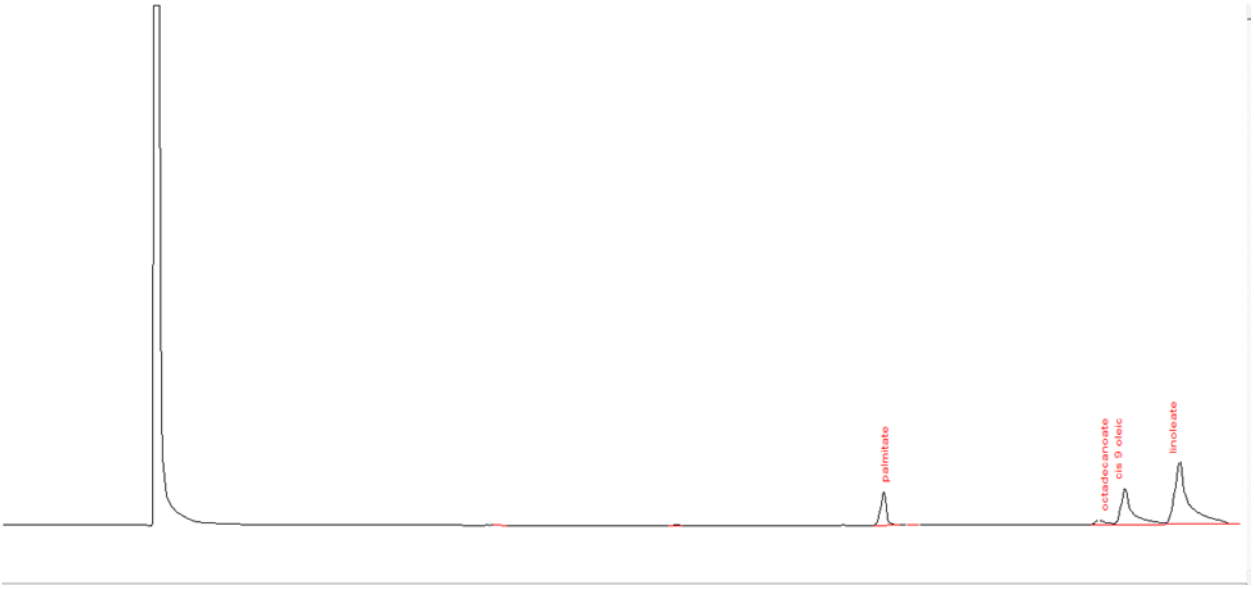

No.	R.T.	Area	Area %	Pk Ty	Comp Name
1	7.96	4835	0.2190	BB	
2	10.78	6204	0.2810	BB	
3	14.16	273641	12.3950	BB	palmitate
4	14.59	2543	0.1152	BB	
5	17.62	67492	3.0572	BV	octadecanoate
6	18.03	595297	26.9649	VV	cis 9 oleic
7	18.91	1257660	56.9677	VB	linoleate
		2207673	100.0000		

Fig: 4.5.2 (V) Gas Chromatograph of Soybean acid oil

Sample Name: Soybean 260917
Data File: D:\AKS\Soybean260917.DAT
Method File: .MET
Detector: FID. System: GC
Date: 26 Sep 2017 Time: 11:8:56
Run: ch1: 0
Type of Analysis: Percent On Area
Report printed on: 11/1/2018 at: 12:36:00

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
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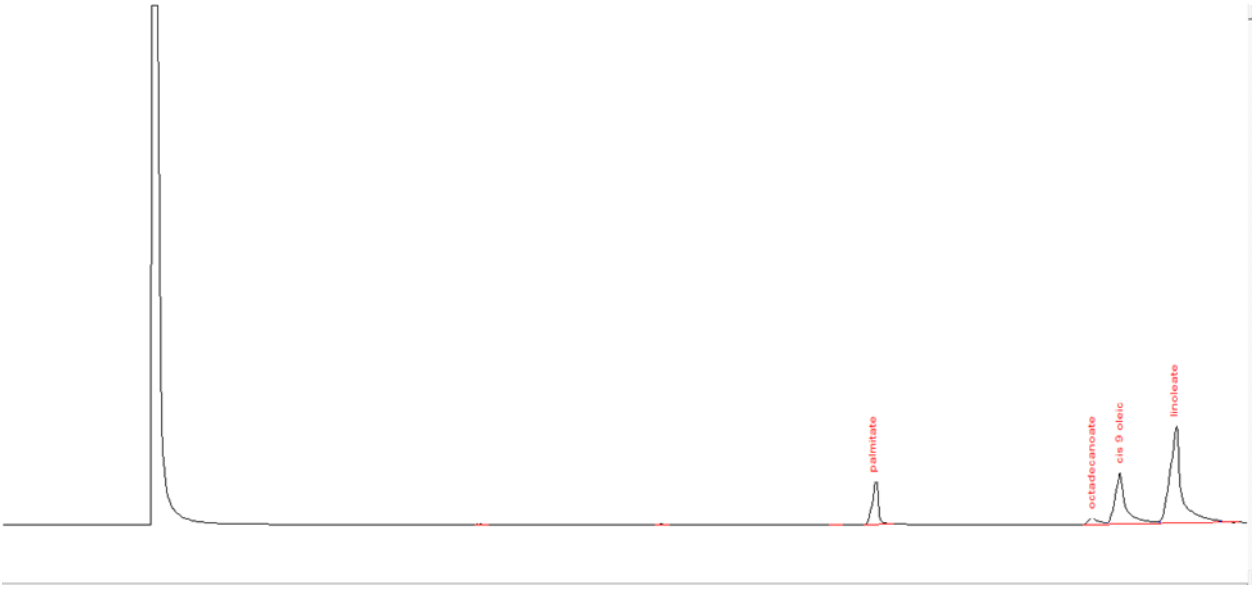

No.	R.T.	Area	Area %	Pk Ty	Comp Name
1	7.66	8205	0.2793	BB	
2	10.56	8375	0.2851	BB	
3	13.35	4097	0.1395	BB	
4	14.12	380742	12.9919	BB	palmitate
5	17.58	94331	3.2114	BV	octadecanoate
6	18.03	793184	27.5429	VV	cis 9 oleic
7	18.94	1632439	55.5743	VV	linoleate
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Fig: 4.6.2. ^{1}H NMR spectra of ethyl ester generation over time profile using whole cell suspension

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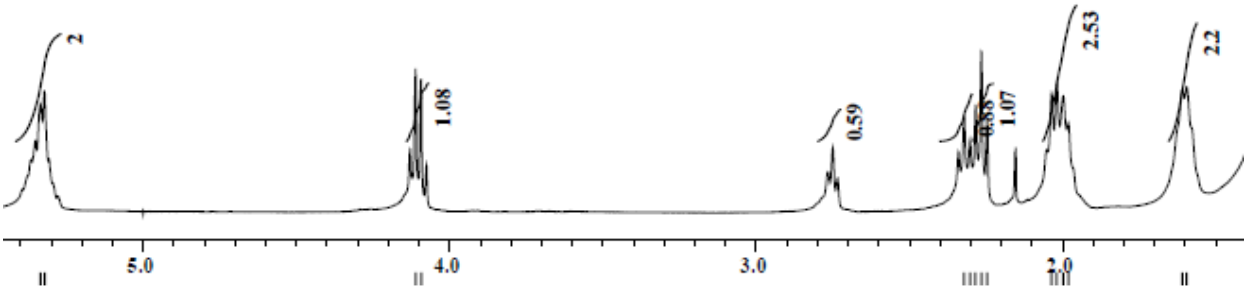

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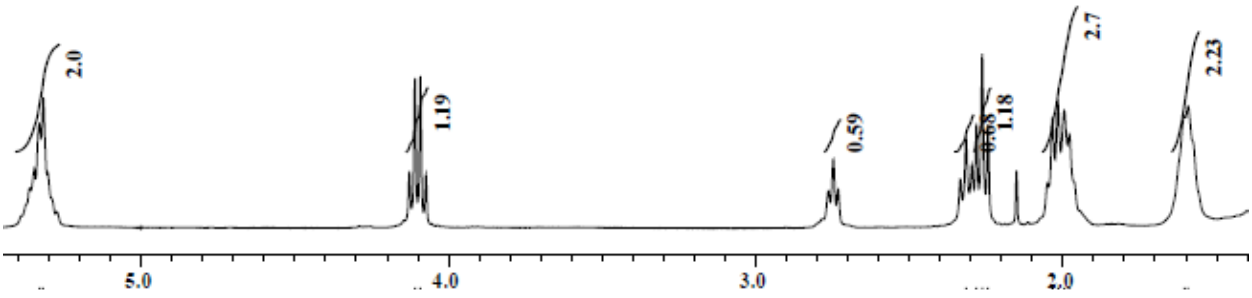

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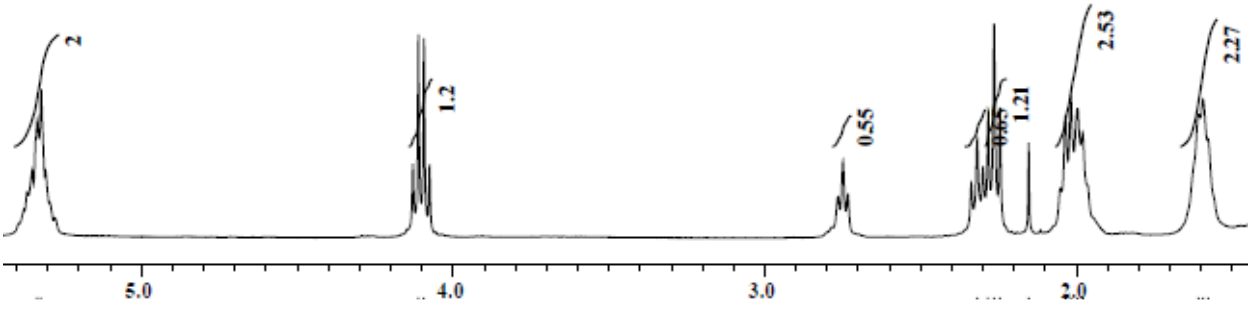

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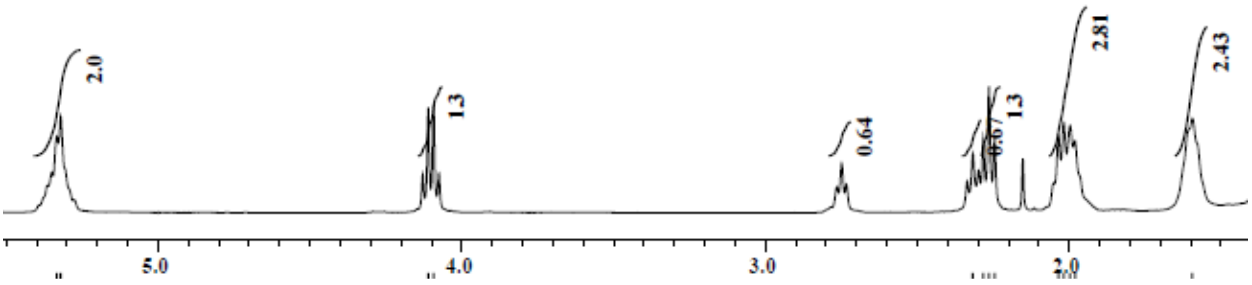
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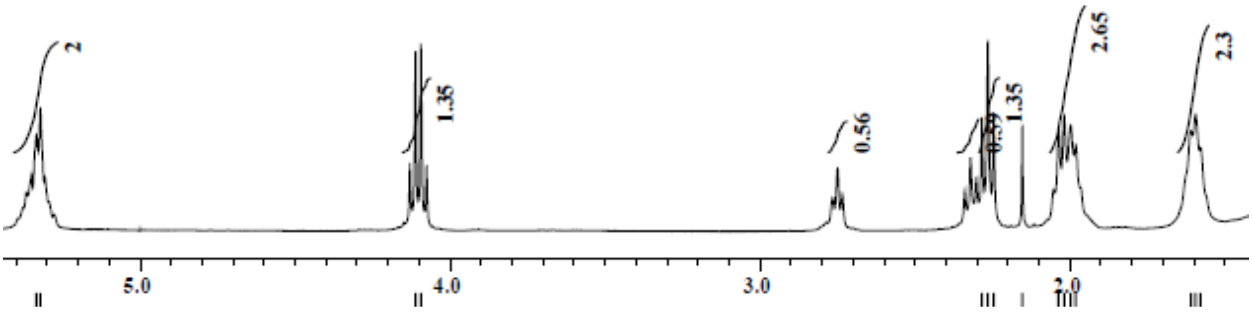
2.0

16h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4.0
3.0
2.0

24h

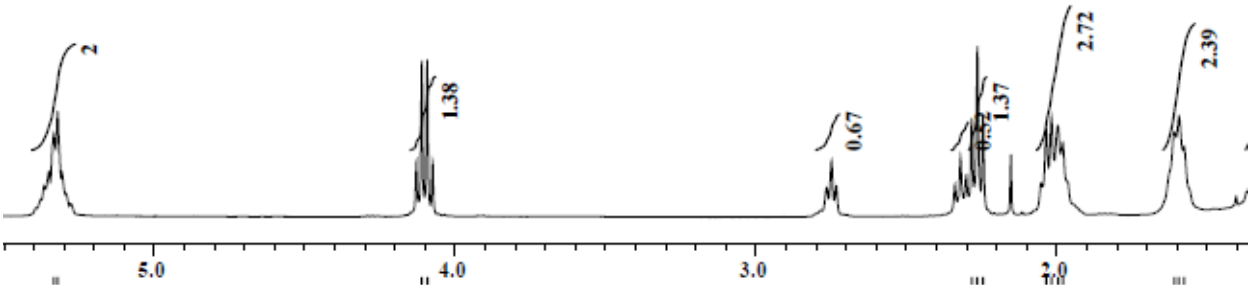
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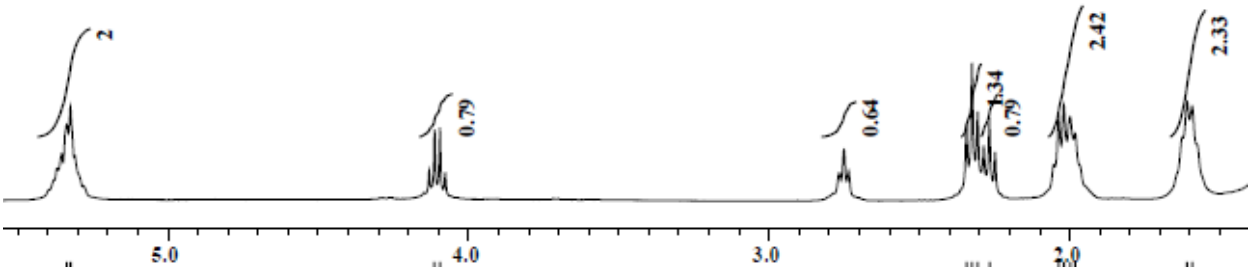
Fig: 4.8.3. [1]H NMR spectra of ethyl ester generation over time profile using immobilized culture

1h



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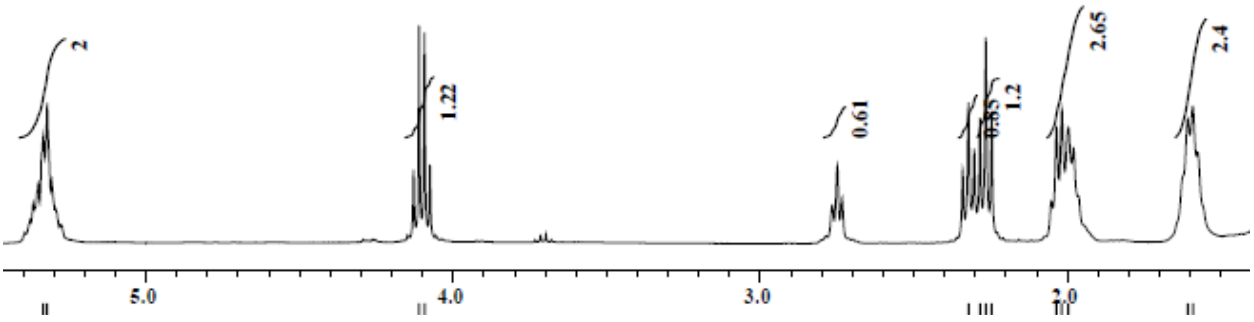

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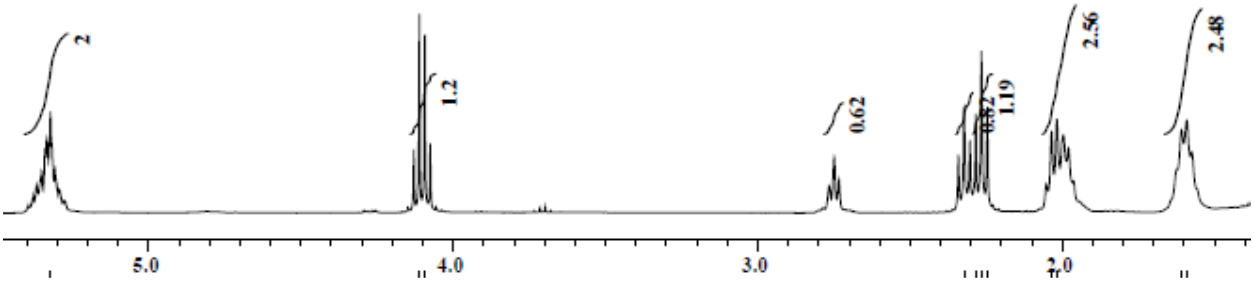

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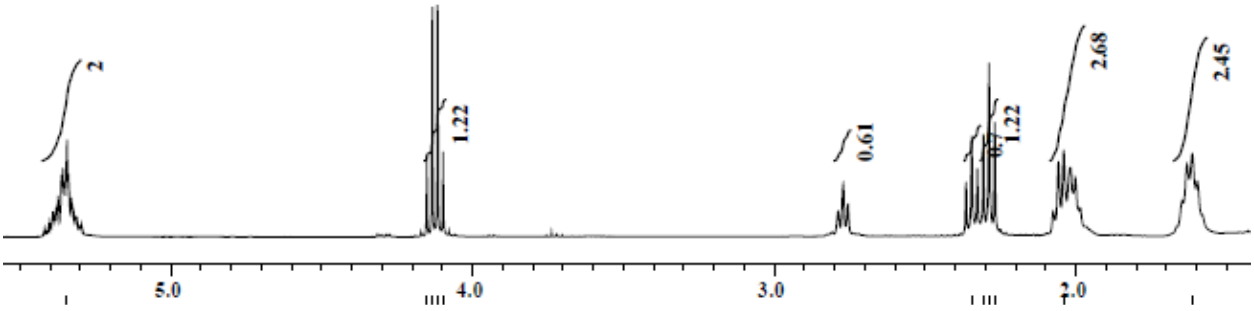
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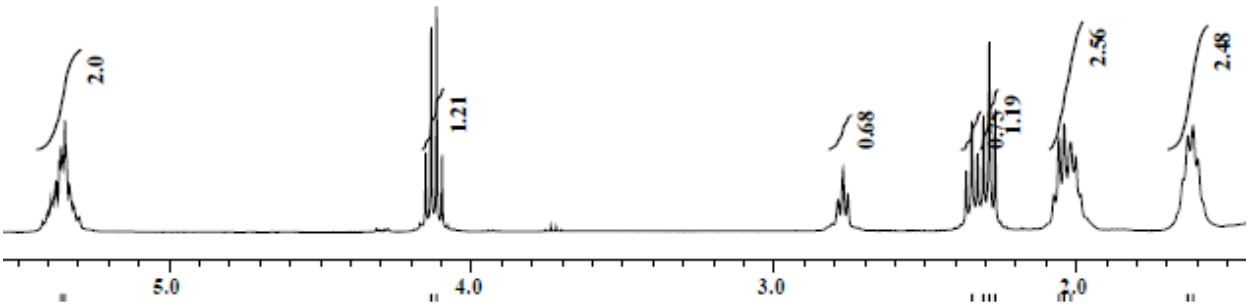
2.0

16h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2.0

24h

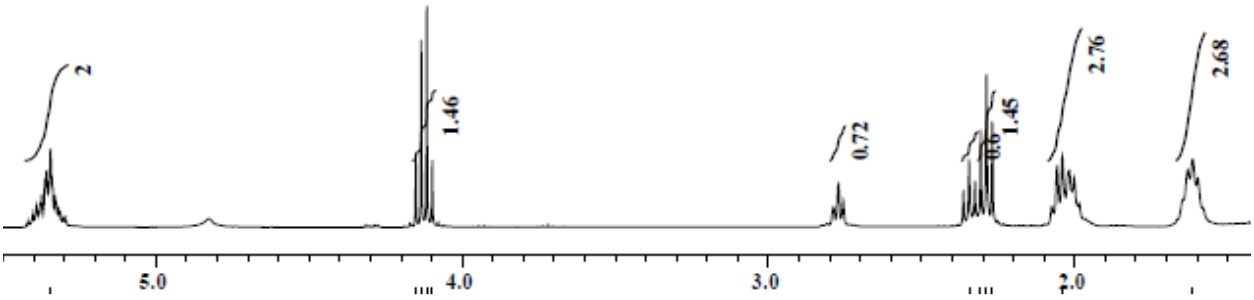
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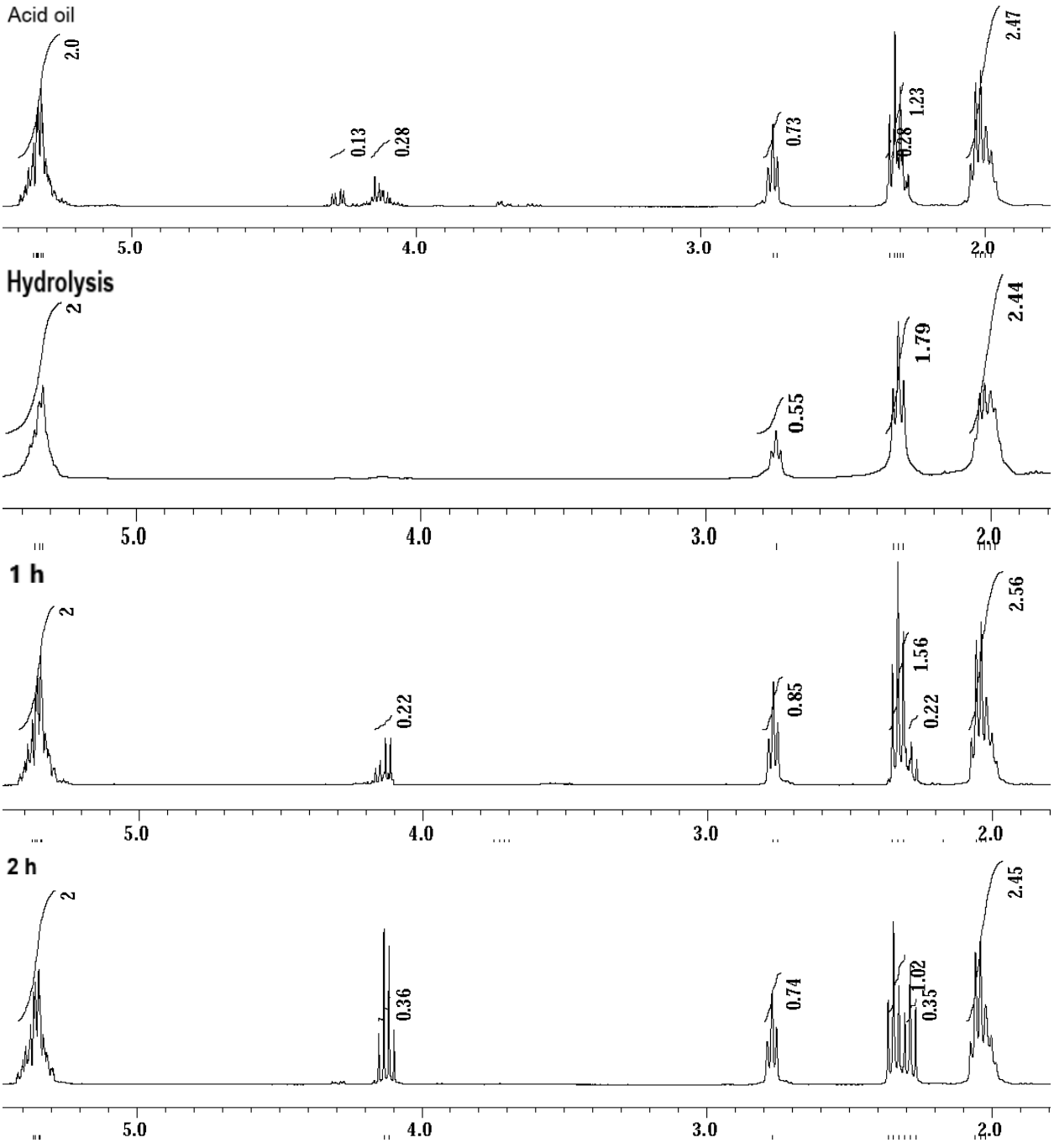
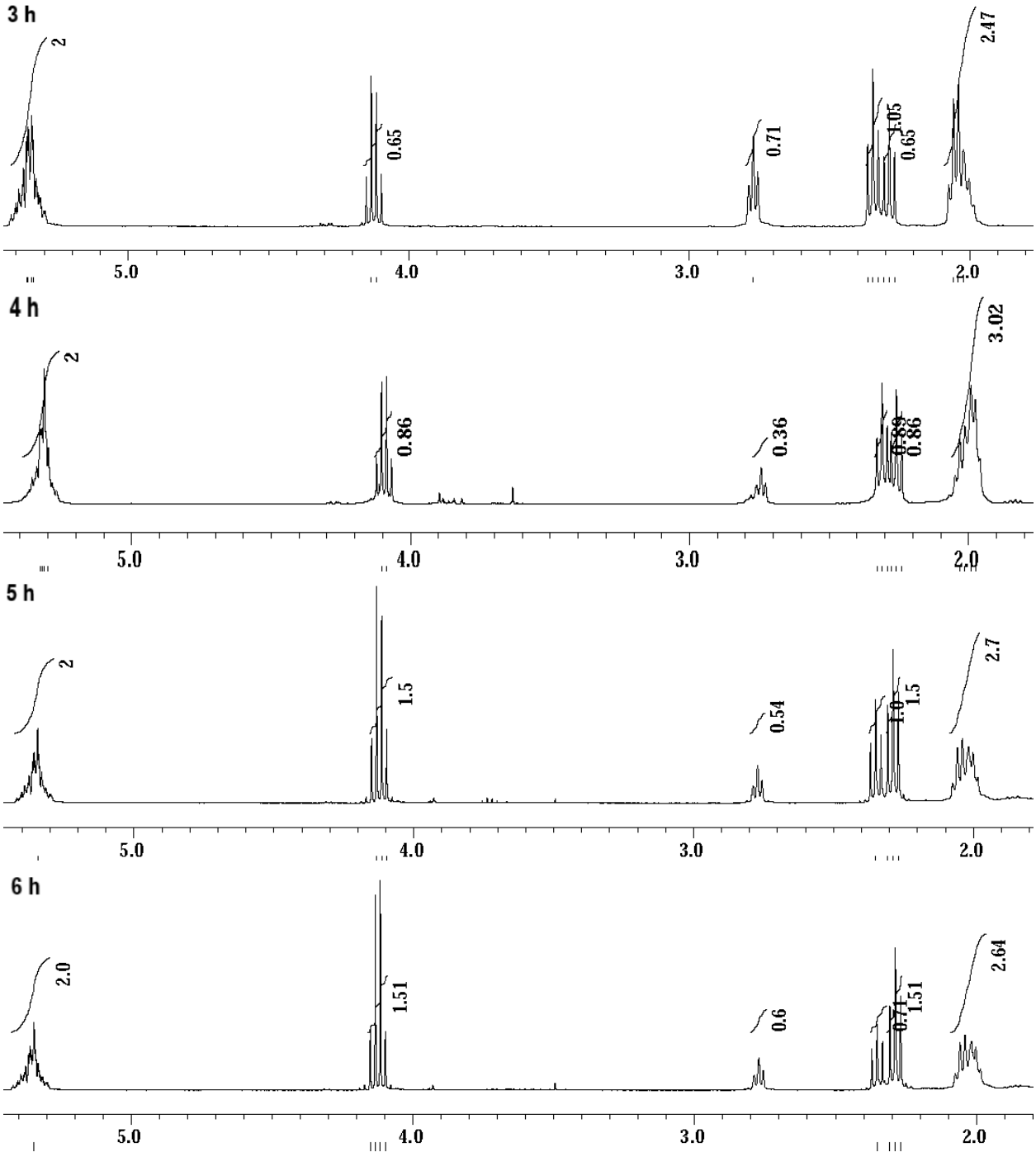
Fig: 4.9. [1]H NMR spectra of ethyl ester generation over time profile using bioreactor

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0.36
0.89
0.86
3.02
5.0
4.0
3.0
2.0
5 h
2
1.5
0.54
1.0
1.5
2.7
5.0
4.0
3.0
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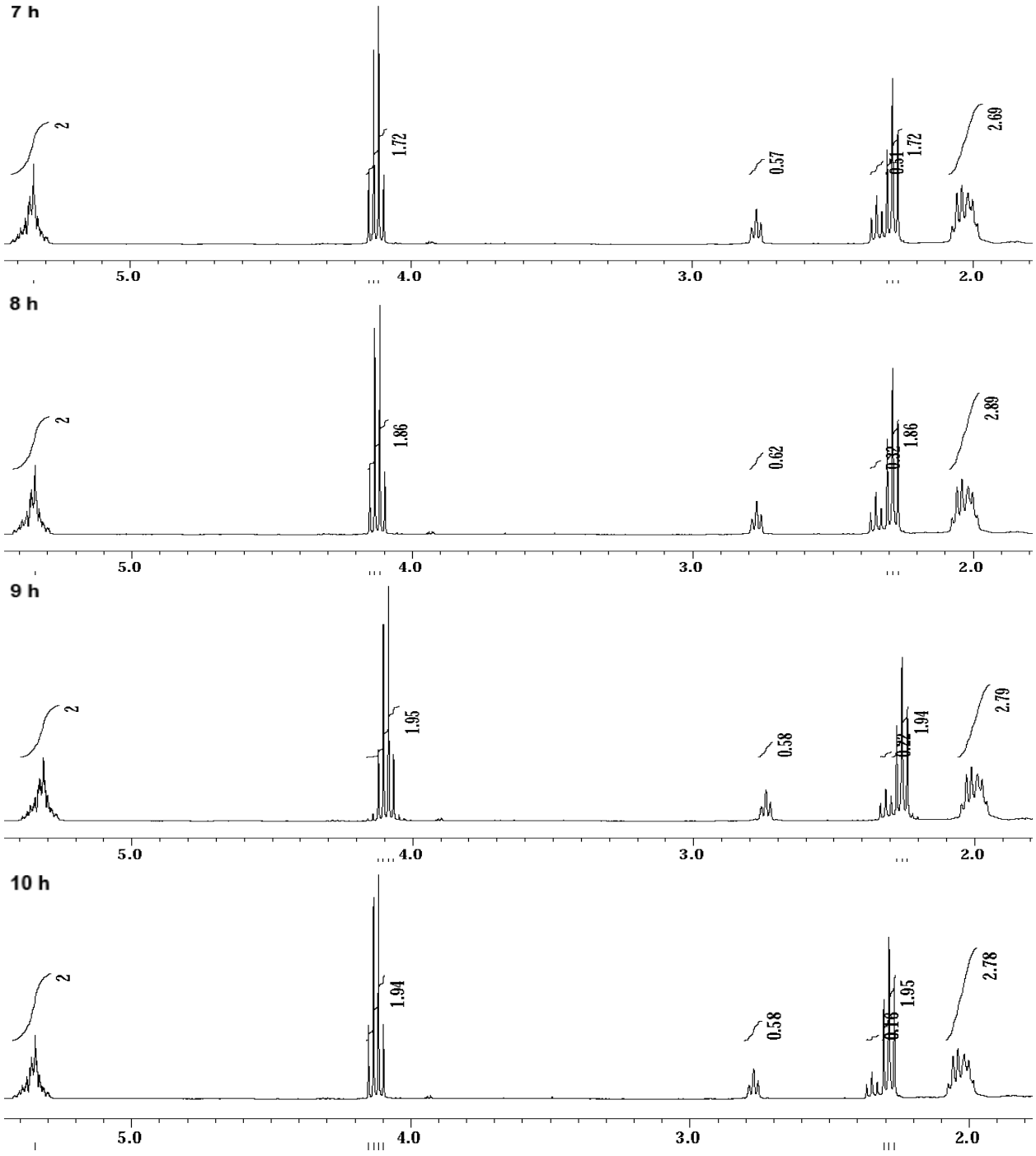
2.0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2.89
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4.0
3.0
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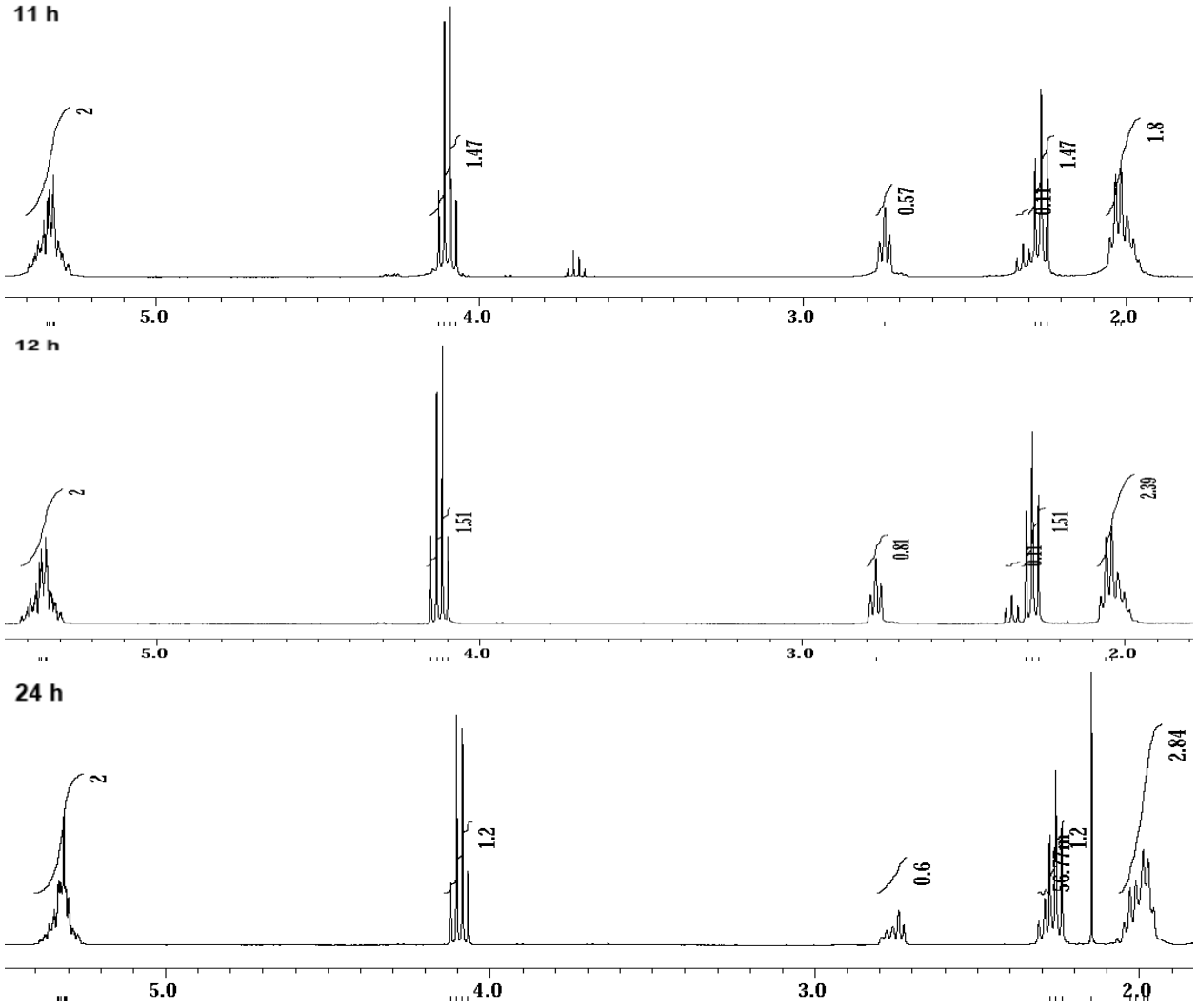
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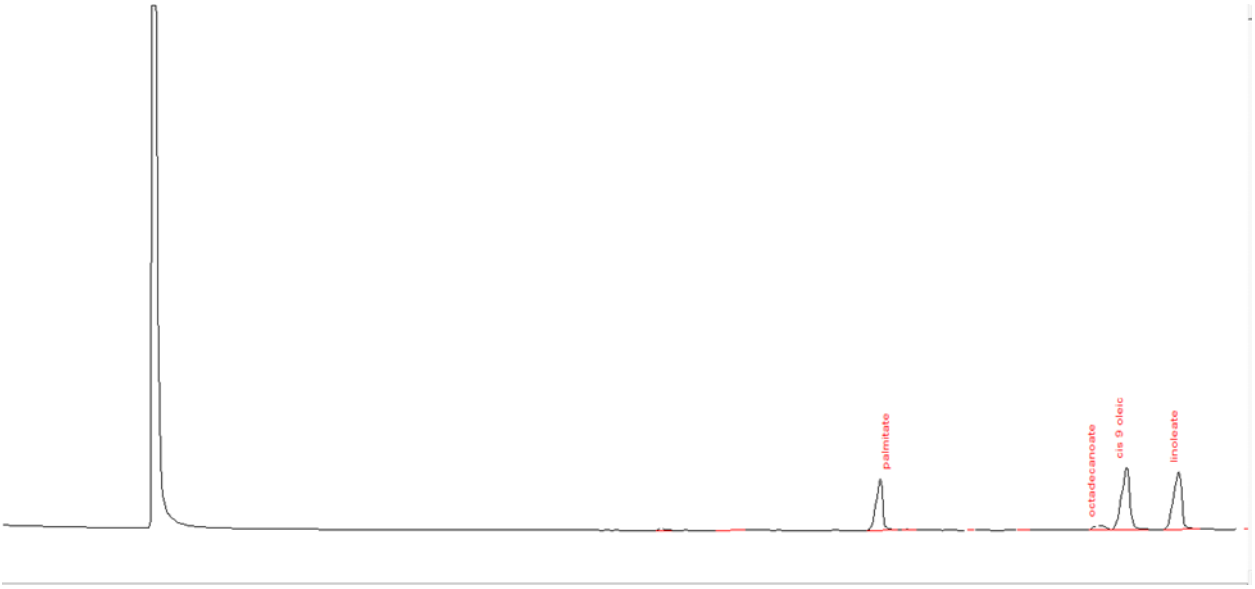
0.57
1.47
1.8
5.0
4.0
3.0
2.0
12 h
2
1.51
0.81
1.51
2.39
5.0
4.0
3.0
2.0
24 h
2
1.2
0.6
1.2
2.84
5.0
4.0
3.0
2.0

Fig: 4.10. Gas chromatograph of obtained ethyl ester

Sample Name: CSAO BD 050417
Data File: D:\AKS\CSAO BD 050417.DAT
Method File: fame mix 050716.MET
Detector: FID. System: GC
Date: 5 Apr 2017 Time: 10:29:36
Run: ch1: 0
Type of Analysis: Percent On Area

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	10	5	4	20.0



No.	R.T.	Area	Area %	Pk Ty	Comp Name
1	10.58	15354	1.4438	BB	
2	11.54	5431	0.8667	BV	
3	11.79	4280	0.9102	VB	
4	14.19	481362	18.6479	BB	palmitate
5	14.50	5924	0.7909	BB	
6	15.54	1414	0.9694	BB	
7	16.35	4492	0.8206	BB	
8	17.59	70660	2.4600	BV	octadecanoate
9	18.04	755624	37.0058	VB	cis 9 oleic
10	18.88	688453	35.4173	BB	linoleate
11	19.97	3407	0.6673	BB	
		2036401	100.0000		

Publications

1) Anirudh Sharma, Atish Verma, Vijay Luxami, Jose Savio Melo, Stanislaus Francis D'Souza, N.Tejo Prakash, Ranjana Prakash. New proton nuclear magnetic resonance-based derivation for quantification of alkyl esters generated using biocatalysis. *Energy & Fuels*. 27, (2013) 2660-2664.
2) Anirudh Sharma, N.Tejo Prakash, Ranjana Prakash. Fungus-mediated generation of ethyl ester using acid oil as substrate. *Environmental Progress & Sustainable Energy.* 36, (2017) 1840-1846.
3) Anirudh Sharma**,** Jose Savio Melo**,** N. Tejo Prakash, R. Prakash. Fuel properties of blend and biodiesel generated from acid oil using whole cell biocatalyst. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects.*40, (2017) 148-154.
4) Anirudh Sharma, Jose Savio Melo, N. Tejo Prakash, R. Prakash. Effect of feedstocks and chain length of alcohols on whole-cell catalyzed generation of alkyl esters. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects.* 40, (2018) 2612-2619.
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Article

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New Proton Nuclear Magnetic Resonance-Based Derivation for Quantification of Alkyl Esters Generated Using Biocatalysis

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Supporting Information

ABSTRACT: Monoalkyl esters of fatty acids commonly known as biodiesel are synthesized from triglycerides by the transesterification reaction with monohydric alcohol, usually methanol or ethanol. Biodiesel is an attractive alternative fuel for diesel engines because of its renewability, biodegradability, and nontoxicity. Several methods/approaches have been developed for analyzing the fuel quality of biodiesel. Mainly chromatographic techniques [e.g., gas chromatography (GC), high-performance liquid chromatography, etc.] are being used for the analysis of biodiesel. The equation for quantification of the transesterification reaction using proton nuclear magnetic resonance (^{1}H NMR) is available in the literature, wherein methanol/ethanol are being used as an acyl acceptor. In the present work, we report the equation based on ^{1}H NMR, which can be used for the quantification of the transesterification reaction, using other primary alcohols as an acyl acceptor. Simultaneously, we have also studied the effect of the chain length of alcohols on the extent of transesterification using whole cell catalysts. Transesterification was enhanced using butanol (67%) and pentanol (76%), followed by a decrease with hexanol (66%) and octanol (56%). The correlation coefficient (R^2) between GC and ^{1}H NMR methods was 0.97. The results obtained by the new ^{1}H NMR equation proposed in this work were well-correlated with GC analysis of the same samples.

1. INTRODUCTION

A high energy demand and increased environmental pollution-related problems because of the use of fossil fuels have necessitated the development and adaptation to renewable and ecofriendly fuels. Making use of biodiesel is one such initiative that has been projected as a renewable alternative to diesel fuel. Its reduced engine emission profiles and direct usability with existing diesel engines have attracted the world's attention to a large extent.[1]

Biodiesel consists of a long chain of fatty acid esters produced by the transesterification reaction of vegetable oils with alcohols using a suitable catalyst, viz., chemicals or enzymes. Chemical catalysts include various acids (e.g., H_2SO_4) and alkalies (e.g., NaOH). Transeterification by acid catalysis is much slower and more suitable for oils and fats with relatively high free fatty acid (FFA) and water contents. An acid-catalyzed reaction also commonly requires a high temperature. For alkali-catalyzed transesterification, the starting materials (oil or fats) must be devoid of moisture and FFA. The presence of a minor amount of FFA and moisture in the reaction mixture produces soap, which interferes in the process of transesterification and, thus, lowers the yield of esters.

In comparison to the chemical approach, the use of lipase (extracellular and intracellular) as the biocatalyst has promising potential because it eliminates obvious disadvantages of the chemical process. In addition, biocatalysis facilitates the yield of a high-purity product with less or no intensive downstream operation associated with the recovery of glycerol or catalyst.[2] Microbial lipase technology has shown enormous potential for making ester derivatives for various specific applications.[3,4] Despite numerous advantages, the enzymatic process has some drawbacks, such as a low reaction rate, low enzyme stability in the presence of excess methanol, and high cost of pure lipase. A higher level of oil supplementation can inhibit the activity of pure lipase during the transesterification reaction. As an effective alternative, whole cells that are capable of producing lipase in specific culture conditions have been shown to effectively catalyze the transesterification reaction, even in high oil supplementations.[5−7]

Methanol has been the most commonly used alcohol in the production of biodiesel. The degree of deactivation is estimated to be inversely proportional to the number of carbon atoms in the alcohol, which means that methanol is the most deactivating alcohol.[8,9] Other alcohols used include ethanol, propanol, isopropanol, butanol, and pentanol for the conversion of oil to alkyl esters.[10−17] It is also thought that the rate of the transesterification reaction using lipase increases with the length of the carbon chain of the alcohol, implying that the use of ethanol over the use of methanol increases the rate of the transesterification reaction.[18]

Besides, several studies have focused on the development and improvement of analytical methods for monitoring the yield of alkyl esters and determining their fuel quality.[19−24] Among the various approaches, the NMR method can be used for quantitative analysis based on the fact that the amplitude of a proton nuclear magnetic resonance (^{1}H NMR) signal is proportional to the number of hydrogen nuclei contained in the molecule.[20,23] Although gas chromatography (GC) and

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high-performance liquid chromatography (HPLC) are more sensitive techniques than NMR, the latter is a more rapid and easier method to use than the former. The area of the NMR peak mainly depends upon the number of protons and not the response factor, as obtained through chromatographic technique(s). Each component of alkyl esters in the sample has a different response factor, which needs to be predetermined and used in the quantitative determination by the chromatographic techniques. Such laborious processes can be eliminated in the case of NMR detection. However, equations based on ^{1}H NMR are reported for the quantification of methyl and ethyl esters[23,25] but not for the quantification of alkyl esters generated from other alcohols to the best of our knowledge.

In the present work, alkyl asters were generated using different alcohols (methanol to decanol and 2-methyl-propane-1-ol) as acyl acceptors using whole cell biocatalysts and an equation based on ^{1}H NMR was derived, which can be applied for the quantification of alkyl esters having primary alcohols other than methanol and ethanol as acyl acceptors. The yield of alkyl esters, as obtained from the derived equation, was further cross-validated with the yield quantified using GC. In addition, the study also demonstrates the use of dry biomass for biocatalysis and waste cooking oil (rice bran) as a substrate for whole-cell-catalyzed hydrolysis.

2. EXPERIMENTAL SECTION

2.1. Materials. The refined rice bran oil and cotton seed oil were procured from a retail market. Culture media, viz., Bushnall Hass Broth (BHB) and potato dextrose broth (PDB), and mycological peptone were purchased from Hi-Media, India. Other chemicals, such as ethanol (and other alcohols), hexane, ethyl acetate, silica gel (G) for thin-layer chromatography (TLC), biammonium hydrogen *ortho*-phosphate [$(NH_4)_2HPO_4$], potassium hydroxide (KOH), hydrochloric acid (HCl), sodium thiosulphate ($Na_2S_2O_3$), starch, potassium iodide (KI), and phenolphthalein, were purchased from SD Fine-Chem Limited, India. All of the reagents used were of analytical grade.

2.2. Preparation of Used Frying Oil. Used rice bran oil was generated following deep frying for 5 h. The FFA value of virgin and fried oil samples was determined using a standard method outlined by AOCS Ca5a-40.

2.3. Preparation of Biomass. The spores of *Aspergillus* sp. (MTCC 5436) isolated from biocontaminated clarified butter were inoculated aseptically in a 500 mL Erlenmeyer flask containing 200 mL of sterile PDB and incubated at 30 °C and 120 rpm for 3 days.[7] The active culture obtained from PDB was further used for experimentation. The minimal medium BHB containing $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.02 g/L), KH_2PO_4 (1.0 g/L), K_2HPO_4 (1.0 g/L), and $FeCl_3$ (0.05 g/L), supplemented with mycological peptone (0.5%, w/v), $(NH_4)_2HPO_4$ (0.5%, w/v), and virgin cotton seed oil (30%, v/v), was used as a growth medium. Mycological peptone and $(NH_4)_2HPO_4$ were used to supplement nitrogen, and cotton seed oil was used as a main carbon source for fungal growth. The culture flask was incubated at 30 °C and 120 rpm for 5 days. Fungal biomass was separated by filtering through Whatman filter paper, washed with hexane to remove the excess oil, and dried with blotting paper. The partially dried biomass was crushed in liquid nitrogen to make homogeneous powder using a pestle and mortar.

2.4. Transesterification Reaction. A total of 1.0 g of dried powdered biomass was taken in a round-bottomed flask containing 10 mL of used frying oil with FFA at 0.93 ± 0.05%. A total of 3.0 mL of alcohol (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol, or 2-methyl-propane-1-ol) was added, and the mixture was stirred for 36 h on a magnetic stirrer at 30 °C. The reaction mixture along with biomass was washed 3 times with 10 mL of hexane to separate the product. The progress of the reaction was checked regularly by TLC.

2.5. Identification and Quantification of Alkyl Esters. The product (ester) obtained was analyzed using TLC with silica gel G as the stationary phase and hexane/ethyl acetate (9:1) as the mobile phase. The chromatogram was developed in the iodine chamber. Further, the product was quantified by GC using methyl heptadecanoate as a standard. The percentage of alkyl ester of fatty acid present in the sample was determined according to EN ISO 5508 with internal calibration (10 mg/mL methyl heptadecanoate). The sample was prepared by weighing 250 mg of alkyl ester in a 10 mL vial, followed by the addition of 5 mL of methyl heptadecanoate (10 mg/mL). A total of 1.0 μL of sample was injected into GC-5765 (Nucon, India) equipped with a flame ionization detector. A fused silica capillary column (0.25 mm internal diameter, 30 m length, and 0.25 μm film thickness, wall coated with EC wax/polythene glycol) was used to separate alkyl ester. The flow rates of nitrogen as a carrier gas and hydrogen were 30 mL/min, while that of zero air was 300 mL/min. The injector and detector temperatures were maintained at 230 and 240 °C, respectively. The oven initial temperature (160 °C) hold time was 1 min, and the final oven temperature was 240 °C. The rate of increase in the temperature was 4 °C/min, and complete program duration was 45 min. A split injection ratio of 1:30 and a split flow rate of 30 mL/min were maintained. The ester content C, expressed as a mass fraction in percent, was calculated using the following formula:

$$C = \frac{(\sum A) - A_{EI}}{A_{EI}} \frac{C_{EI} V_{EI}}{m} \times 100$$

where $\sum A$ is the total peak area from the alkyl esters of oil, A_{EI} is the peak area corresponding to methyl heptadecanoate, C_{EI} is the concentration in milligrams per milliliter of the methyl heptadecanoate solution, V_{EI} is the volume in milliliters of methyl heptadecanoate solution being used, and m is the mass in milligrams of the sample.

The alkyl esters were analyzed further, using ^{1}H NMR (Bruker-Advance II-400 with 5 mm BBO probes) with $CDCl_3$ as a solvent, and chemical shifts were expressed in parts per million with tetramethylsilane (TMS) as an internal standard. A duplicate analysis was carried out using JEOL (400 MHz, ^{1}H NMR). Table 1 presents the mean and standard deviation (SD) between the two analytical values. Methyl and ethyl ester contents in the reaction mixture were quantified using the equation proposed by Gelbard et al.[23] and Ghesti et al.,[25] respectively.

Table 1. Effect of Different Alcohols on the Extent of Transesterification[a]

	percent conversion	
acyl acceptor	GC	^{1}H NMR
methanol	8.13 ± 0.06	9.47 ± 0.49
ethanol	7.90 ± 0.58	12.76 ± 0.00
propanol	3.84 ± 0.26	4.88 ± 0.38
butanol	73.79 ± 4.27	67.15 ± 2.89
pentanol	75.55 ± 2.30	76.35 ± 1.24
hexanol	59.96 ± 1.40	66.25 ± 1.87
heptanol	75.41 ± 2.49	73.40 ± 0.96
octanol	54.91 ± 4.79	55.69 ± 2.53
nonanol	50.62 ± 1.08	46.40 ± 2.06
decanol	40.27 ± 1.63	47.92 ± 1.39
2-methyl-propane-1-ol	67.26 ± 2.72	65.94

[a]Data are the mean ± SD (n = 3) in the case of GC.

The alkyl esters produced by the reaction with various other primary alcohols, viz., propanol to decanol and 2-methyl-propane-1-ol, were quantified by deriving a modified equation proposed by Gelbard et al.[23] for methyl esters given below, wherein the triplet at 4.00−4.10 ppm of methylenic protons indicates alkyl ester ($-CO_2\mathbf{CH_2}(CH_2)_xCH_3$) formation. The signals at 2.30 ppm result from the protons on the CH_2 groups adjacent to the alkyl or glyceryl ester moieties ($-\mathbf{CH_2}CO_2CH_2(CH_2)_xCH_3$ for alkyl esters)

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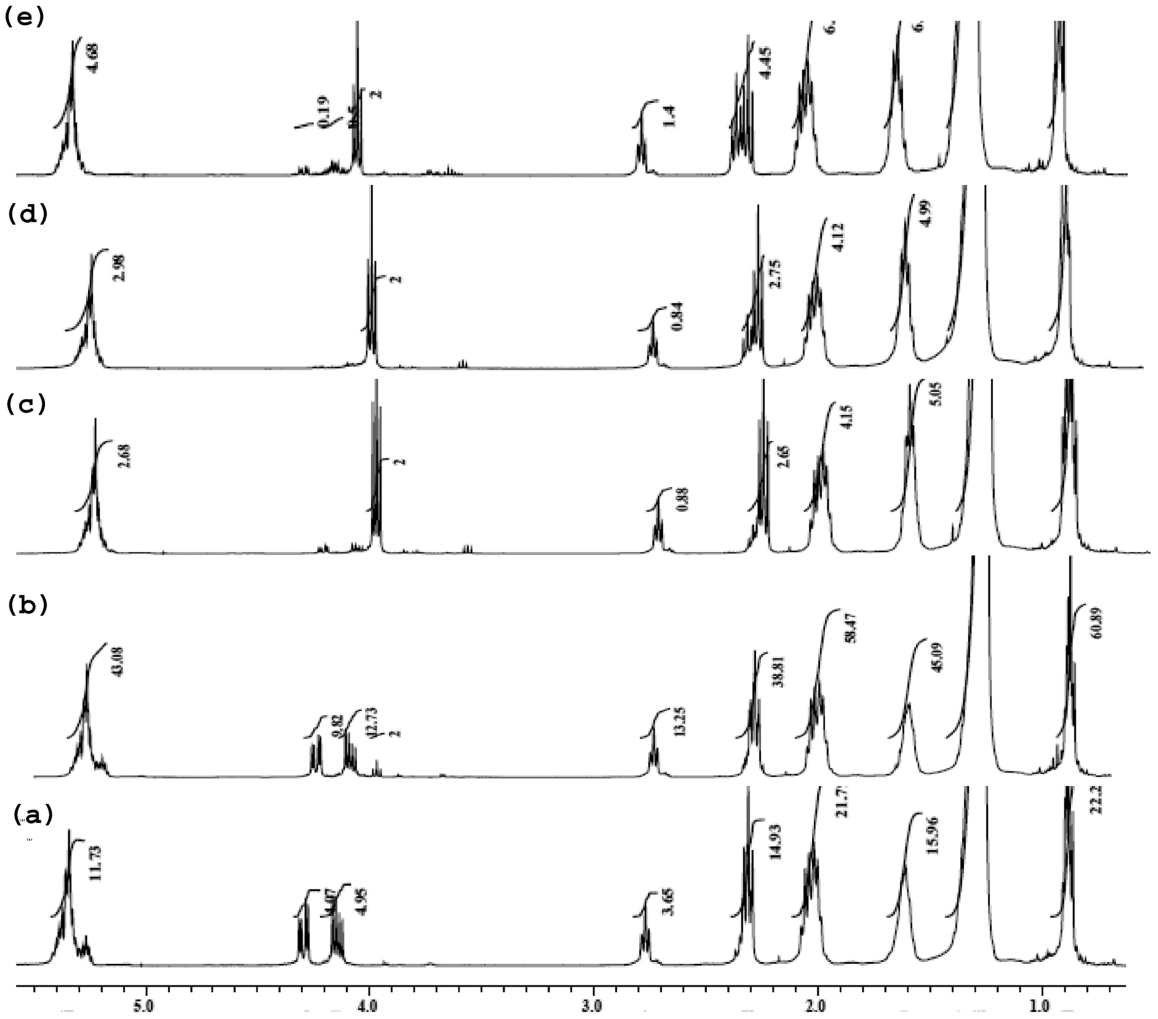

Figure 1. ^{1}H NMR spectra for (a) pure oil, (b) alkyl ester of propanol, (c) pentanol, (d) heptanol, and (e) nonanol.

$$C = 100\left(\frac{AE_{\alpha\text{-}CH_2}}{A_{\alpha\text{-}CH_2}}\right)$$

where C is the conversion of triacylglycerol of the feedstock (vegetable oil) to the corresponding alkyl ester, $AE_{\alpha\text{-}CH_2}$ is the integration value of the methylene protons of the alkyl esters (the triplet peak), and $A_{\alpha\text{-}CH_2}$ is the integration value of the methylene protons.

3. RESULTS AND DISCUSSION

3.1. GC and ^{1}H NMR Analyses. The present work is based on the whole-cell-catalyzed transesterification reaction of used rice bran oil in the presence of alcohols of different chain lengths. Preliminary examination by TLC indicates the formation of ester in each case. Table 1 presents the results obtained from GC analysis, showing a noticeable influence of the chain length of alcohol on the extent of transesterification.

The ^{1}H NMR results further confirmed the formation of alkyl esters. The ^{1}H NMR of methyl ester indicated a singlet in the region of 3.60 ppm because of the proton of methyl ester (see Supplementary Figure 1 of the Supporting Information). In the case of ethyl ester, the appearance of a quartet of $-OCH_2$ at 4.10−4.20 ppm confirmed the formation of the ester (see Supplementary Figure 2 of the Supporting Information). With reference to other alkyl esters, ^{1}H NMR of alkyl ester obtained after transesterification indicated the appearance of a triplet at an integration value around 4.10 ppm. The integration value of this triplet was used in the modified equation for the quantification of different alkyl esters. The small triplet at 3.60 ppm (panels c and d of Figure 1) is due to methylene protons of unreacted alcohols.

To the best of our knowledge, there is no equation known in the literature to quantify alkyl ester based on ^{1}H NMR for acyl acceptors other than methanol and ethanol. The new derivation can be applied for the quantification of any alkyl ester generated from primary alcohols other than ethanol and methanol, whereas the derivation given by Gelbard et al.[23] can only be applied for quantification of methyl esters. The objective behind proposing this equation was to exploit the efficacy of the NMR technique because of obvious advantages, such as faster and easier adaptable analysis, non-destructive measurements, and ease with smaller amounts of samples.[24,25] In the present study, the percent conversion obtained by GC significantly correlated (R^2 = 0.98) with the percent conversion obtained by ^{1}H NMR (Figure 2).

The extent of transesterification enhanced from butanol (≅70%) to pentanol (≅76%) and decreased in hexanol (≅63%) and octanol (≅55%). These observations differ from those reported by Romero et al.,[26] wherein there was no noticeable influence on the esterification effect when propanol, butanol, hexanol, and octanol were used as acyl acceptors.

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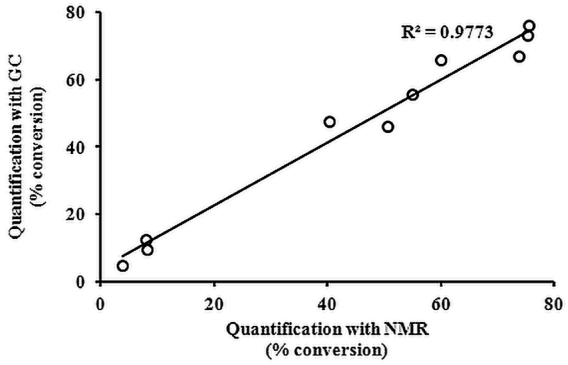

Figure 2. Correlation between the alkyl ester yields quantified using GC and NMR.

In the case of the transesterification reaction employing methanol, methanolysis takes place between two immiscible liquids.[27,28] Upon formation of diacyl and monoacyl glyceridic intermediates in sufficient quantities, they serve as surfactants that improve mass transfer of triacylglycerides into the methanol phase.[29] Ethanol is of particular interest primarily because it is less expensive than methanol in some regions of the world (such as Brazil). However, the ethanolysis reaction proceeds at a slower rate than methanolysis because of the higher reactivity of the methoxide anion in comparison to ethoxide. As the length of the carbon chain of the alkoxide anion increases, a corresponding decrease in nucleophilicity occurs, resulting in reduced reactivity of ethoxide in comparison to methoxide.[30] The observations obtained in the case of chemical catalysis by other researchers also support our results.[31] However, there are limited reports on the use of other alcohols as acyl acceptors in whole-cell-catalyzed transesterification. Long-chain alcohols, 2-propanol and *n*-butanol, have a less negative effect on lipase stability, and they also improve low-temperature properties of the fuel.[32] However, excess alcohol leads to inactivation of the enzyme, and glycerol, a major byproduct, can block the immobilized enzyme, resulting in low enzymatic activity.[33] Butanol may also be obtained from biological materials, thus yielding completely bio-based biodiesel as well.[34,35] Butanol is completely miscible with vegetable oils and animal fats because it is significantly less polar than methanol and ethanol.[36] Consequently, transesterification reactions employing butanol are monophasic throughout.[37,38] The monophasic nature of the butanolysis reaction further enhances the rate and extent of the reaction.

4. CONCLUSION

The present study thus proposes an easier method for quantification of alkyl esters with data obtained through ^{1}H NMR spectroscopic analysis with primary alcohols as acyl acceptors. The study also outlines the application of whole cell catalysis for the transesterification of waste edible oils, which can be an alternative approach for the generation of alkyl-ester-based biodiesel in place of chemical catalysis.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra used in the study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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AIChE

Fungus-Mediated Generation of Ethyl Ester Using Acid Oil as Substrate

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Transesterification reaction of soybean acid oil was carried out by whole cell biocatalysis. The lipase production by Aspergillus sp. (RBD01) was optimized by modulating sources of nitrogen, pH of growth medium, and percentage of carbon source (acid oil). Soybean acid oil containing significant amount of free fatty acids (FFA) was hydrolyzed and transesterified using cell suspension and immobilized culture of Aspergillus sp. (RBD01) as catalysts. Complete hydrolysis of acid oil to free fatty acids was achieved by both forms (suspension and immobilized) of catalysts, within 12 h of the reaction time. Further, addition of ethanol resulted in conversion of FFA to ethyl ester to the extent of up to 72% in case of both immobilized and cell suspension. FFA and ethyl ester were quantified using ^{1}H NMR. The process of generation of ethyl esters through biocatalyzed transesterification of acid oils is thus observed to be a potential means of producing these products for diverse variety of industrial/commercial use such as biodiesel. © 2017 American Institute of Chemical Engineers Environ Prog, 36: 1840–1846, 2017

Keywords: acid oil, transesterification, whole cell catalysis, immobilization, ethyl ester

INTRODUCTION

Currently, the predominant feedstocks for biodiesel production are refined and edible-grade vegetable oils [1]. The cost of the feedstock comprises a very substantial portion of the overall cost of biodiesel. These considerations have led to efforts toward identifying less expensive lipid-bearing substrates that could serve as feedstocks for biodiesel production. As an effective alternative, feedstocks like acid oils are being extensively evaluated as possible substitutes [2]. Acid oil comprises of 40–80% long chain free fatty acids mixture, 1–2% mineral acids, 5–8% free moisture, 8–10% phospholipids and sterols, and 20–50% neutral glycerides and unsaponifiable constituents of the oils and other impurities [3,4]. All these impart a characteristic pungent odor and dark brown color to the acid oil. Acid oil's fuel properties are different from diesel fuel due to its oxygenated nature and presence of chain type of configurational compounds; in addition, the heating value of acid oil's fuel is slightly lower and the viscosity vis-à-vis the ignition values are higher than diesel fuel [3].

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Acid oils contain high percentage of free fatty acids and therefore are not amenable to conventional alkali catalysis due to saponification [5], whereas the biocatalysts effectively act on the functional groups of fatty acids like epoxy and hydroxyl groups which are not possible with chemical catalysts [6]. In addition, disadvantages in use of chemical catalysts, such as difficulty in recovery of glycerol, the energy intensive nature of the processes, need for the removal of catalysts from the product, and the interference of the reaction by free fatty acids and water, also contribute to the limited use of chemical catalysts [7,8]. Biocatalysts such as enzymes or whole cells are therefore envisaged as excellent alternatives due to the obvious advantages such as mild reaction conditions, insensitive to high FFA, regiospecificity,; immobilization possibilities, acceptability for wide variety of substrates, and better thermo-stability [9–13]. Enzymatic methods overcome the limitations of chemical approach but have not been exploited at industrial scale due to cost-intensive nature. This has resulted into exploring the potential use of whole cell systems such as bacteria, yeast, and filamentous fungi that produce lipase use as whole cell catalysts for transesterification reactions.

Among the established whole-cell biocatalyst systems, filamentous fungi have proven to be robust organisms for industrial applications [14]. The use of *Rhizopus oryzae*, *Rhizopus chinensis*, recombinant *Saccharomyces cerevisiae*, and most recently *Aspergillus niger* as whole cell biocatalysts have been studied and reviewed by different research groups [12,15,16]. Our group had earlier reported the conversion of used oil to alkyl esters using *Aspergillus* sp. as a whole cell catalyst [17]. This work addresses the use of whole cell of *Aspergillus* sp. immobilized on polyurethane foam. The distinct advantages of immobilized form of cell are reusability and higher stability. In this view, we have used polyurethane foam as immobilization support for cells in the present study as it is much cheaper, higher surface area, and easily available [18,19]. To the best of our knowledge, there are limited reports available on the use of whole cells as catalysts, especially with fungal systems, for generation of alkyl esters using acid oil as a substrate. In the present study, we demonstrate the use of whole cell as well as immobilized whole cell of *Aspergillus* sp. (RBD01) for catalyzing transesterification reaction using soybean acid oil as feedstock.

MATERIALS AND METHODS

Reagents and Chemicals

Soybean acid oil was arranged from an edible oil processing industry. Culture media viz., mycological peptone, Bushnall-Hass

Table 1. Experimental layout for lipase production.

	Constant parameter	Variable parameter	Range
Step 1	Minimal salt medium + 10% acid oil as main carbon source + pH 7.5 ± 0.2 + growth temperature 30°C	Nitrogen source: peptone; biammonium hydrogen orthophosphate (BAHP); urea; or sodium nitrate	0.5–2.5%
Step 2	Minimal salt medium + 10% oil as carbon source + 0.5% peptone + growth temperature 30°C	pH	5.0–8.5
Step 3	Minimal salt medium + 0.5% peptone + pH 7.5 + growth temperature 30°C	Percentage of acid oil as main carbon source in the growth medium	40–90%

broth (BHB), and potato dextrose broth (PDB) was purchased from HiMedia, India. Other chemicals such as ethanol, hexane, ethyl acetate, silica gel (G), bi-ammonium hydrogen orthophosphate ($(NH_4)_2HPO_4$), sodium nitrate ($NaNO_3$), urea, sodium hydroxide (NaOH), ethyl alcohol, isopropyl alcohol, toluene, sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), and phenolphthalein were purchased from SD Fine-Chem Ltd., India. $CDCl_3$ (deuterated solvent) from Sigma, India. All the reagents used were analytical grade.

Experimental Method

FFA Determination of Soybean Acid Oil

FFA was determined by the standard method outlined in AOCS Ca5a-40 [20] (AOCS, 1989). Acid oil (2.0 g) was dissolved in 1:1 iso-propanol and toluene and titrated against standardized 0.1M NaOH rather than KOH solution using phenolphthalein as indicator. The results are presented as percent FFA expressed as oleic acid, where the molecular weight of oleic acid (282) is divided by sample weight.

$$\%\text{FFA} = \text{alkali volume (mL)} \times \frac{\text{Alkali normality} \times 28.2}{\text{Sample weight (g)}}.$$

Optimization of Culture Conditions for Lipase Production

The *Aspergillus* sp. RBD01 (MTCC5436), used in the present study, was obtained from bio-contaminated butter and reported earlier to exhibit significant oil tolerance and transesterification potential. The spores of RBD01 were inoculated aseptically in 500 mL Erlenmeyer flask containing 200 mL of sterile PDB and incubated at 30°C, 120 rpm for 72 h. The culture obtained from PDB was used for further experimentation. Mineral salt medium containing magnesium sulfate (0.20 g/L), calcium chloride (0.02 g/L), monopotassium phosphate (1.0 g/L), dipotassium phosphate (1.00 g/L), and ferric chloride (0.05 g/L) was used for the cultivation of culture. The medium was supplemented with acid oil as carbon source as well as lipase inducer. Various step-wise modifications were carried out in growth conditions (Table 1) so as to standardize parameters for obtaining optimum lipase activity. The enzyme activity was determined after 72 h in all the steps outlined. In the step 1, *Aspergillus* sp. (RBD-01) was grown in mineral salt medium along with 10% acid oil by varying percentage of different organic (peptone and urea) and inorganic ($NaNO_3$, $(NH_4)_2HPO_4$) nitrogen sources (0.5–2.5%) maintaining pH 7.5 ± 0.2 at 30°C. In step 2, pH of the growth medium was modulated from 5.0 to 8.5 by maintaining acid oil supplementation at 10% and using peptone as nitrogen source (0.5% w/v) with growth conditions set at 30°C. In step 3, modulation was carried out in the percent supplementation of acid oil (40–90%) to growth medium that contained mineral salt medium and peptone (0.5% w/v) in growth condition set at 30°C.

Lipase activity was determined by the separation of biomass from the growth medium [21]. The cell-free supernatant (CFS) was further separated from acid oil using a separating funnel. The CFS was centrifuged to remove the debris and used for estimating lipase activity. The enzyme activity was determined by adding 0.1 mL of CFS to a reaction mixture containing 0.8 mL of 0.05 M phosphate buffer (pH 7.0) and 0.1 mL of 0.05 M pNP (p-nitrophenol laurate) in ethanol. The mixture was incubated at 30°C for 30 min, followed by addition of 0.25 mL of ethanol on cooling to stop the reaction. The activity was determined at 420 nm. One unit of lipase activity is defined as the amount of enzyme that liberates 1 μg p-nitrophenol with pNP-laurate as substrate under standard assay conditions in 30 min. The calculations of lipase units were carried out by preparing a standard curve using commercial lipase enzyme (Sigma–Aldrich L1754-25G).

Cultivation of Culture

The minimal media (BHB) was supplemented with mycological peptone (0.5% w/v), $(NH_4)_2HPO_4$ (0.5% w/v), and virgin cottonseed oil (30% v/v) and was used as growth medium. Mycological peptone and $(NH_4)_2HPO_4$ were used to supplement nitrogen.

Immobilization of Culture on Polyurethane Foam

Polyurethane foam was cut into pieces of 1 cm^3 and taken in separate 500 mL Erlenmeyer flasks containing 70 mL mineral medium and 30 mL cottonseed oil (as main carbon source). The medium was further supplemented with mycological peptone (0.5% w/v) and diammonium hydrogen ortho phosphate (0.5% w/v) as nitrogen source followed by sterilization at 120°C, 15 psi for 15 min. On cooling, the freshly grown biomass of RBD01 was inoculated and incubated for 72 h at 30°C and 120 rpm. On colonization of the strain over polyurethane foam, the biomass filled matrices were removed from the culture flasks and used for transesterification reaction.

Hydrolysis of Acid Oil to Fatty Acids

The culture of RBD01 (as whole cell suspension) and 15 pieces of immobilized matrices (as immobilized biomass) were inoculated separately in BHB supplemented with soybean acid oil (70% oil and 30% media) and incubated at 30°C at 120 rpm for 12 h for carrying out hydrolytic reaction. Through this period of incubation, samples were collected at periodic intervals of 6 h and subjected to thin layer chromatography (TLC) and further analyzed by ^1H NMR.

Transesterification

Subsequent to the hydrolytic reaction over 12 h, ethanol was added at 1:4 molar ratio of oil to alcohol in each (cell

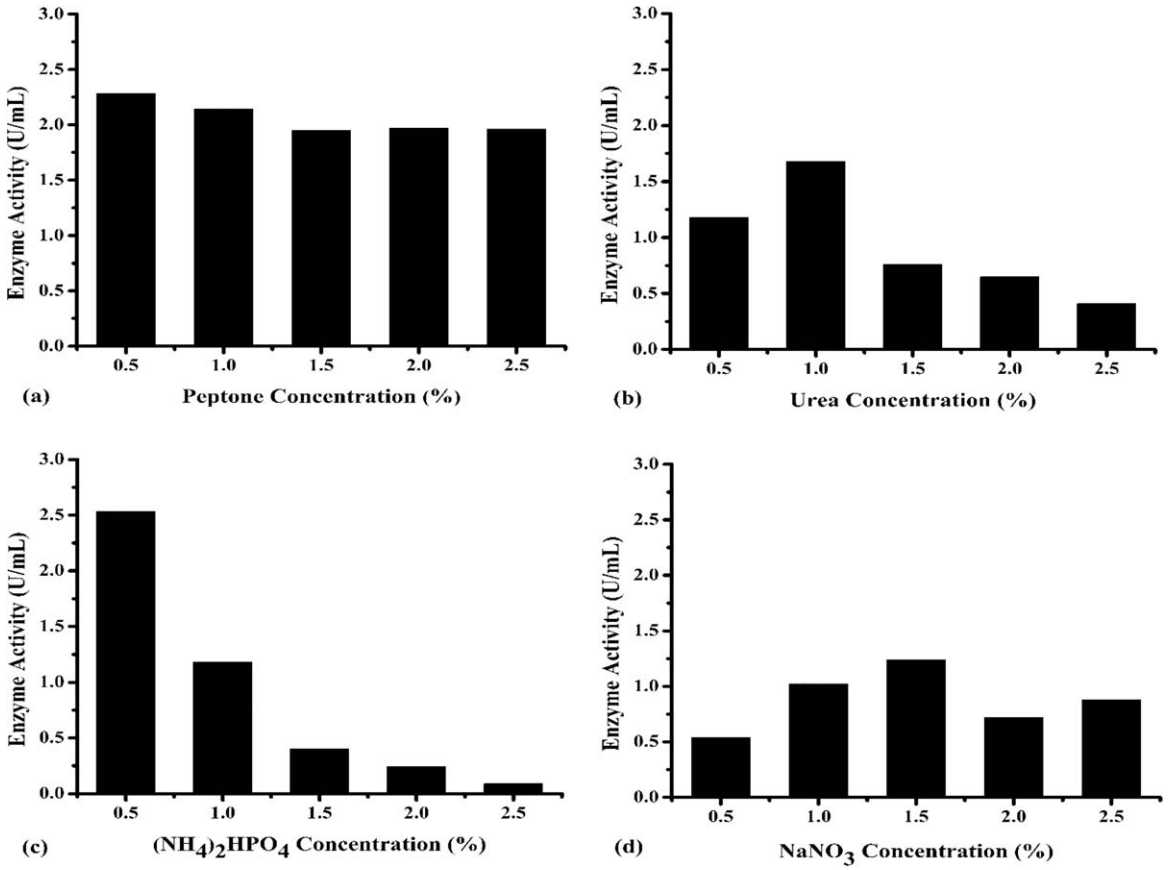

Figure 1. Effect of concentration of different nitrogen sources on lipase activity. (a) Peptone, (b) urea, (c) BAHP, and (d) sodium nitrate.

suspension/immobilized) of the reactions. Followed by alcohol addition, samples were collected at an interval of 1 h up to 24 h and washed thrice with hexane to remove excess of oil from ester. Hexane was evaporated out on water bath and the esters, thus obtained, were confirmed using TLC with silica gel-G as stationary phase and hexane:ethyl acetate (9:1) as a mobile phase. The chromatogram was developed in the iodine chamber [22]. The alkyl esters were then quantified using ^{1}H NMR (400 MHz; JEOL JNM-ECS 400). $CDCl_3$ was used as solvent and tetra methyl silane (TMS) as internal standard. ^{1}H NMR spectra were recorded with pulse duration of 2.18 s and relaxation delay of 4 s and 16 scans. Following derivation was used to determine the FFA yield through hydrolysis [23].

$$\%FFA = (\text{area of triplet of } \alpha\text{CH2 of FFA} \times 100)/(\text{total area of } \alpha\text{CH2 of FFA and ester}).$$

αCH2 is α-acyl methylenic hydrogens in oil and FFA at 2.20–2.40 ppm. The product formed from the transesterification reaction was quantified by using the equation proposed by Sharma et al. [24].

$$C = \frac{(AE_{\propto-CH2})}{(A_{\propto-CH2})} \times 100,$$

where C is the conversion of TAG to the alkyl ester, $AE_{\alpha CH2}$ is the integration value of the methylene protons of the alkyl esters, and $A_{\alpha CH2}$ is the integration value of the methylene protons.

The ethyl ester obtained from reactions (cell suspension and immobilized culture) was washed with 28 vol % batches of 5% (w/v) NaCl in tap water followed by 20 vol % of 11% (w/v) $NaHCO_3$ in tap water to remove residual unreacted FFA [25]. The ethyl ester was recovered by centrifugation (20 min, 4600*g*) and quantified using ^{1}H NMR.

RESULTS AND DISCUSSION

The present study was focused on generation of ethyl ester (EE) by using fungus (RBD01) as a whole cell catalyst through transesterification of soybean acid oil containing 42% FFA and optimization of process parameters and reaction condition with using different nitrogen sources, different pH and different acid oil concentration. The ethyl ester generated from the reaction was quantified by using ^{1}H NMR.

Effect of Different Concentration of Nitrogen Sources

The fungal strain RBD01 was grown in increasing concentration of various organic and inorganic nitrogen sources, with 10% acid oil as carbon source to evaluate the effect of nitrogen concentration on the enzyme activity. Nitrogen sources, including organic nitrogen (peptone and urea) and inorganic nitrogen (BAHP and sodium nitrate) sources, play an important role in the synthesis of the enzyme. The influence of organic nitrogen sources on enzyme activity is shown in (Figure 1). The supplementation was most effective in case of peptone with significant lipolytic activity (2.28 U/mL) obtained after 72 h at 0.5% of peptone. Further, increase peptone concentration to 1.0%, the enzyme activity marginally decreased and remained constant (Figure 1a). Among the inorganic nitrogen sources, BAHP was most effective for lipase production. Maximum lipolytic activity (2.53 U/mL) was observed in the presence of 0.5% BAHP with activity significantly higher than from other inorganic sources (Figure

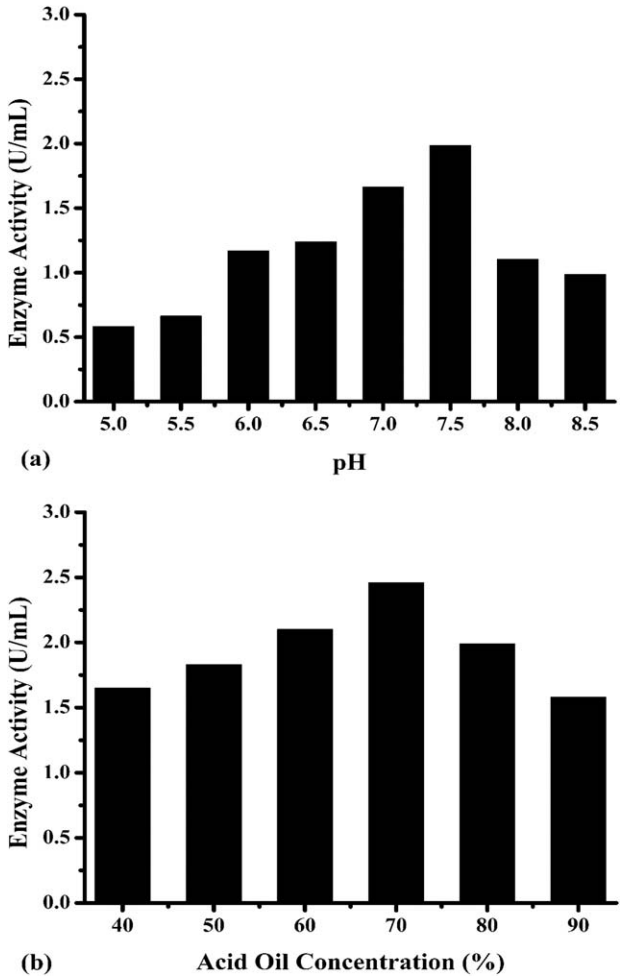

Figure 2. (a) Effect of pH, (b) effect of different concentration of acid oil as main carbon source; on lipase activity.

1b). However, in presence of inorganic ($NaNO_3$) as well as organic (urea) nitrogen sources beyond 1.5 and 1.0%, respectively, showed sharp decrease in enzyme activity (Figure 1c,d). Aulakh et al. [17] and Chander et al. [26] also reported that peptone was highly efficient nitrogen sources than other nitrogen sources. They also state that the lipase activity is dependent upon the source of nitrogen and not the nitrogen content of the medium.

Effect of pH

To observe the effect of pH on the lipase production by *Aspergillus* sp., the culture was grown in conditions as outlined in step 2 (Table 1). Lipase production increased from 0.58 to 1.98 U/mL with corresponding increase in pH from 5.0 to 7.5 (Figure 2a). Maximum lipase activity of 1.986 U/mL was obtained at pH 7.5. Further increase in pH from 7.5 to 8.5 resulted in reduction of activity, i.e., from 1.104 to 0.987 U/mL. Corzo and Revah [27] reported maximum lipase activity at pH 6–7 with activity decreasing significantly beyond pH 8. The optimum pH for lipase activity of *Geotrichum*-like R59 was observed to be near neutral with reduction in activity at pH above 8.0 [28]. Thus pH 7.5 was selected for further studies.

Effect of Different Acid Oil Concentration on Lipase Activity

The type and concentration of carbon source also influenced the lipase production and hence lipolytic activity. In the present study, different levels of acid oil supplementation (40–90%) were taken as carbon source, other parameters remaining unaltered. Maximum of lipase activity 2.46 U/mL was obtained by supplementation of 70% oil in the growth medium (Figure 2b). Elibol and Özer [29] indicated that the synthesis of lipase was inhibited by the higher concentration of olive oil in the medium, it could be due to poorer oxygen transfer in the medium. Low oxygen supplies can alter fungal metabolism, and consequently, the production of lipases. The favorable effect of olive oil and oleic acid on lipase production has been observed by various researcher groups [30–32]. Fatty acids such as oleic acid, linoleic acid, and linolenic acid and triglycerides such as olive, groundnut, and cotton seed oils stimulated lipase production by *P. mephitica* [33]. Majority of the reports, to-date, have only used virgin oil as an inducer for microbial synthesis of lipases, with limited reports available on use of acid oil as the main carbon source. The extent of acid oil supplementation as the main carbon source, i.e., 70% of the growth medium is significantly higher than the observations reported till date.

Transesterification Reaction

Whole cell (cell suspension/immobilized) catalyzed hydrolysis of soybean acid oil containing initial FFA 42% showed gradual increase in FFA through conversion of oil to FFA in 12 h. The trend was similar in whole cell as well as immobilized culture. The ^{1}H NMR of hydrolyzed acid oil at 4.25–4.35 ppm (Figure 3a) indicated the disappearance of glyceryl methylenic hydrogen peak (Figure 3b) confirming the complete conversion of oil to FFA. Esterification was carried out with addition of alcohol, followed by 1 h incubation, resulting in the generation of alkyl esters with corresponding decrease in FFA content (Supporting Information Figure 1A). The ethyl ester formation was evident with appearance of quartet of ethoxy hydrogens ($-OCH_2CH_3$) of ester at 4.10–4.20 ppm. Further, in case of cell suspension, the trend in esterification of FFA to ethyl esters is shown in Figure 4a, corresponding to the FFA content. The trend depicts the formation of ethyl esters with decrease in FFA content from 100 to 45% within 1 h of alcohol addition leading to ester yield of 55%. The yield of ester further increased to 66 and 70% over duration of 4 and 16 h, respectively. The reaction which was continued up to 24 h resulted in maximum ester yield of 72% with 28% corresponding FFA (Supporting Information Figure 1A).

The esterification of soybean acid oil carried out by immobilized culture, resulted in the decrease of FFA content from 100% to 63% within 1 h of alcohol addition and ester yield obtained was 37% which increased gradually to 59% in 2 h and 64% in 4 h with corresponding decrease in FFA (Supporting Information Figure 1B). There is slight decrease in ester content, i.e., 62% in 16 h which can be attributed to reversibility of the reaction [34]. The reaction continued up to 24 h and the maximum yield of ester reached 72% with 28% of corresponding FFA (Figure 4b).

To the best of our knowledge, there are limited reports on generation of ethyl ester from acid oil specially by using fungal whole cells as biocatalysts. Wei et al. [35] reported the use of whole cells of *Rhizopus oryzae* immobilized within the biomass support particles as a catalysts for production of biodiesel from acidified, refined and crude rapeseed oil in tert-butanol system, where tert-butanol was used component to enhance the stability of *R. oryzae* whole cells.

However, transesterification of acid oil to alkyl esters has been attempted with pure enzymes by some research groups. The complete hydrolysis of feedstock to fatty acids was carried out in subcritical water followed by the use of a packed-bed reactor that contained fermented solids with *Burkholderia cepacia* LTEB11 enzyme. The maximum conversion of fatty

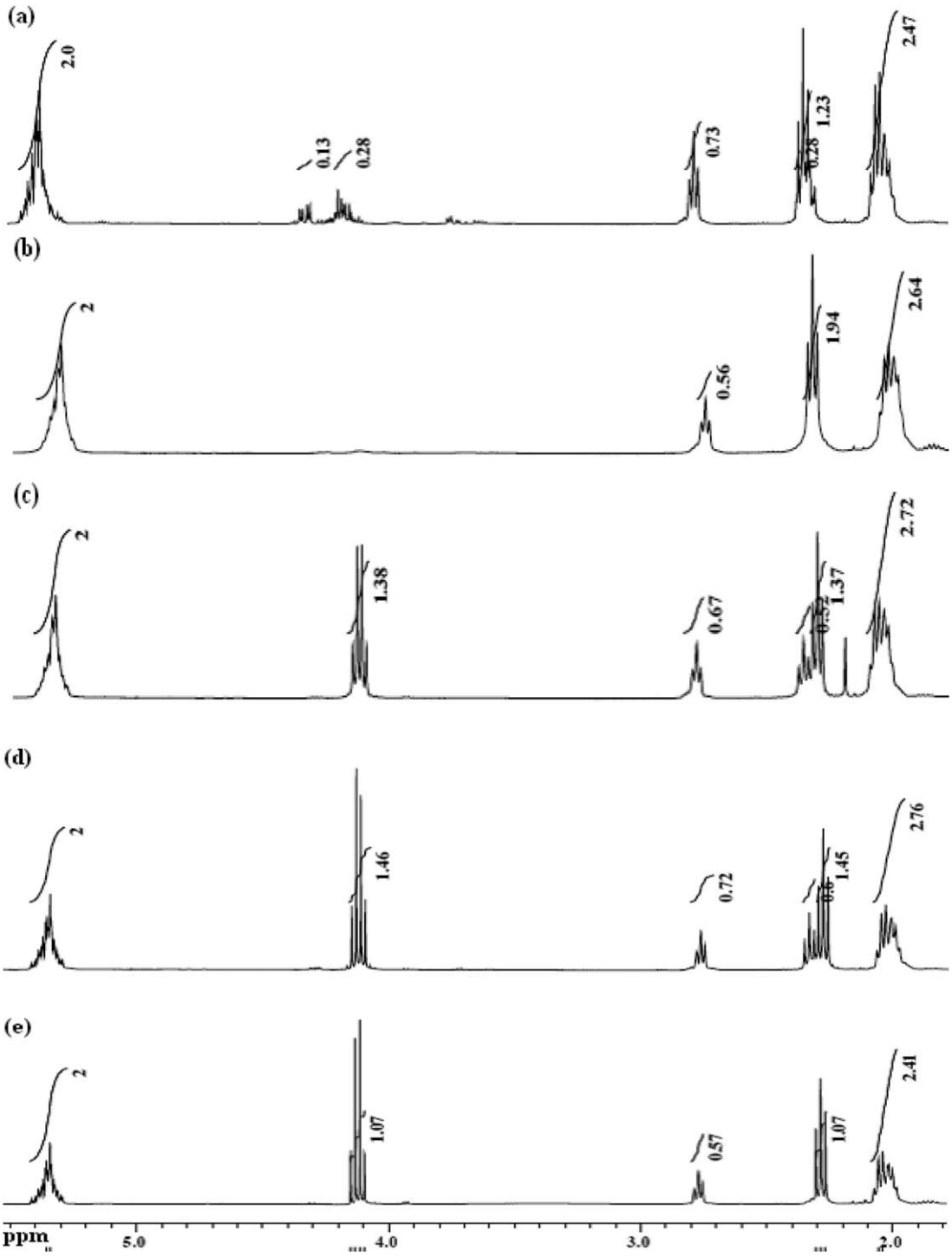

Figure 3. ^{1}H NMR spectra for (a) soybean acid oil, (b) conversion of acid oil to FFA after 12 h incubation, (c, d) ethyl ester generated by using whole cell and immobilized culture, and (e) ethyl ester obtained after washing.

acids to alkyl esters, in this study, was 92% in 31 h [36]. Ghosh and Bhattacharya [6] reported the transesterification reaction of different types of acid oils such as coconut, soybean, mustard, sunflower, and rice bran with *Candida cylindracea* lipase as a catalyst for hydrolysis followed by esterification or alcoholysis reaction with *Mucor miehei* lipase. The acid oils were hydrolyzed almost completely within 48 h and the fatty acids were converted into fatty acid esters of short and long chain alcohols. The use of *Candida antarctica* (Novozym 435) lipase B for esterification of corn and sunflower acid oils using straight and branched chain alcohols resulted in only 50 and 70% conversion to fatty acid methyl esters when reaction was carried out with corn acid oil and sunflower acid oil, respectively [4]. Further, Haas et al. [37] reported that 81% conversion of the fatty acids in soapstock to simple alkyl esters by two step process, in which, second step of the process involved use of lipase enzyme instead of KOH as a catalyst. Thus, the use of whole cell systems, as reported in the present study, provide an alternative to enzymatic catalysis resulting in similar or better yield of alkyl esters.

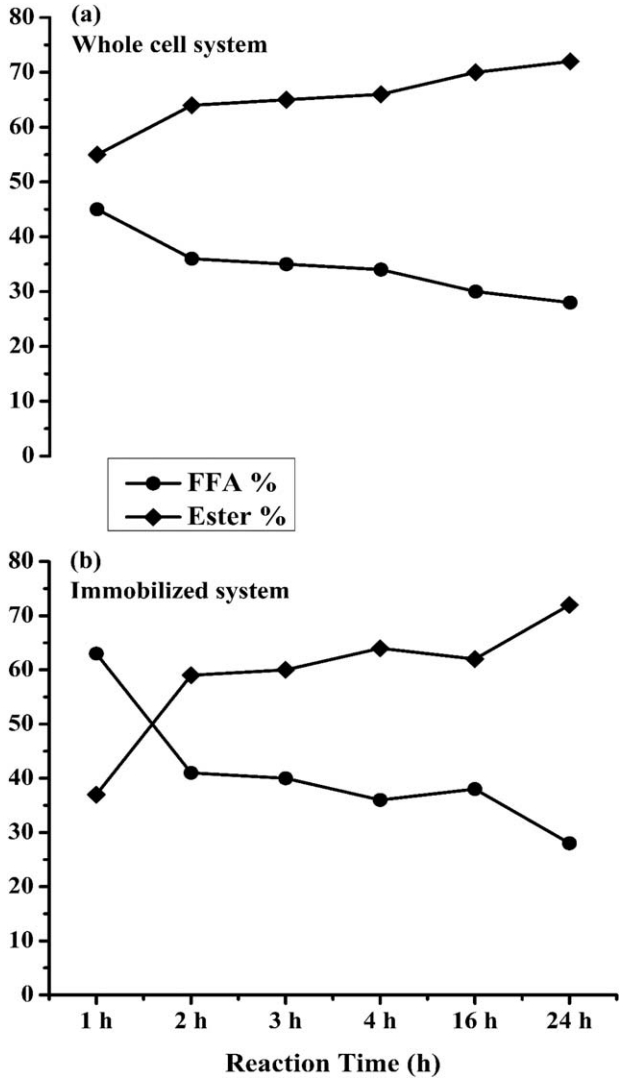

Figure 4. Profile of ethyl ester generation over time by (a) whole cell system and (b) immobilized culture.

In contrast, the present study is significant in term of the process, wherein the hydrolysis and esterification steps were carried out with whole cells of RBD01 through a continuous process resulting in near conversion of oil to ester.

A comparison was drawn between the cell suspension and the immobilized systems keeping in view the obvious advantages offered by immobilization technique over those with free cell suspension in terms of industrial applications such as avoiding the step of preproduction of cells, large mass transfer rate of substrate and production within biomass support particles, enhanced durability, reusability of the particles, and reduced production costs.

The ^{1}H NMR of ethyl ester obtained after transesterification indicating the peak due to $–OCH_2$ of ethoxy hydrogens of ester at 4.10 ppm, are shown in Figure 3c,d for cell-free suspension and immobilized culture reactions, respectively. The ethyl ester, generated from both reactions (cell suspension and immobilized culture) after 24 h, on subjected to washing with NaCl-$NaHCO_3$ resulted in complete removal of FFA (Figure 3e). Further, the derivation proposed by our group [24] was used for quantification of alkyl esters. Wherein the triplet at 4.00–4.10 ppm of methylenic protons indicates alkyl ester ($–CO_2$ $\mathbf{CH_2}$ $(CH_2)x$ CH_3) formation. The signals at 2.30 ppm result from the protons on the CH_2 groups adjacent to the alkyl or glyceryl ester moieties ($–\mathbf{CH_2}CO_2$ $CH_2(CH_2)x$ CH_3 for alkyl esters).

The study, thus, demonstrates the use of whole cell and immobilized whole cell as catalyst for hydrolysis and esterification of a cost effective lipid source such as acid oil for generation of ethyl ester having diverse variety of industrial applications along with its use as biodiesel.

CONCLUSION

Acid oil, the by-product of edible oil processing, can be an effective alternative as feed stock for generation of alkyl esters through transesterification process. As this material is not amenable to alkali catalysis, due to high percentage of free fatty acids, biocatalytic approach presents a better potential in catalyzing the reaction. The present study with fungus strain, *Aspergillus* sp., in the form of immobilized system as well as free cell suspension, demonstrates the use of whole cell catalyst for hydrolysis and esterification of as acid oil for generation of ethyl ester having diverse variety of industrial applications and its possible use as biodiesel.

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Fuel properties of blend and biodiesel generated from acid oil using whole cell biocatalyst

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ABSTRACT

Biodiesel was generated through whole cell catalyzed transesterification of acid oil, to the extent of up to 92%. The fuel properties of biodiesel (B100) and its blend (B20) were determined and compared with standard biodiesel as per American Society for Testing and Materials (ASTM) standard (ASTM D6751). B100 and B20 showed good pour point of −26°C and −29°C, respectively, indicating their operation viability in colder environment. Other properties of biodiesel are quite similar to petroleum diesel and ASTM standard. The results of this study reveal the potential use of acid oil as feedstock for generation of fuel grade biodiesel through biocatalyzed transesterification.

Keywords
Acid oil; biodiesel; fuel properties; transesterification; whole cell biocatalysis

Introduction

The cost of the feedstock comprises a very substantial portion for the overall cost of biodiesel production (Marchetti and Errazu 2008). Acid oil, a byproduct generated during acidulation process of edible oil refining, is one of the alternative lipid-bearing, nonedible feed stock material that is cost-effective for use in biodiesel production (Watanabe et al. 2005). It contains a mixture of long chain free fatty acids and small amounts of phospholipids and sterols, mineral acids, and glycerides (Kulkarni, Pujar, and Shanmukhappa 2008; Wang et al. 2007). The conventional alkali catalysts are not suitable to transesterify acid oil, as the process results in soap formation owing to high free fatty acid content (40–80%) (Fukuda, Kondo, and Noda 2001). The use of whole cell biocatalyst effectively overcomes these limitations of chemical catalysts. Among the established whole-cell biocatalyst systems, filamentous fungi have proven to be robust for industrial applications (Aulakh and Prakash 2010; Fukuda et al. 2008). Biodiesel is generally characterized according to its fuel properties that include kinematic viscosity, density, pour point, calorific value, flash point, ash content, and sulfur content. The primary objective of the present study was to determine the fuel characteristics of feedstock (acid oil): whole cell catalyzed biodiesel generation and comparison of fuel properties with that of its blend and conventional petroleum diesel.

Materials and methods

Physiochemical properties and fatty acid composition of acid oil

Physical and chemical analyses of the acid oil were conducted according to the Indian Standard (SP: 18 [P:13]-1984). The fatty acid composition of acid oil was determined by preparation of fatty acid methyl

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esters (FAME) of the acid oil through transesterification using hydrochloric acid. The FAME was analyzed by gas chromatograph GC-5765 (Nucon, India) equipped with a flame-ionization detector and a fused silica capillary column coated with polyethene glycol.

Transesterification of acid oil using whole cell biocatalyst

The active culture of *Aspergillus sp*. RBD01 (MTCC5436) was inoculated in Bushnell-Hass broth medium supplemented with acid oil (70% acid oil (v/v) and 30% (v/v) media) and incubated at 120 rpm and 30°C for conducting hydrolytic reaction (Sharma, Tejo Prakash, and Prakash 2017). Subsequent to the hydrolytic reaction, ethanol was added stepwise at an interval of 12 h at 1:4 molar ratio of oil to alcohol. Followed by addition of alcohol and further incubation for 12 h, the fatty acid ethyl ester (FAEE), thus obtained, was washed with 28 vol% batches of 5% (wt/vol) NaCl in tap water followed by 20 vol% of 11% (wt/vol) $NaHCO_3$ in tap water to remove the residual unreacted free fatty acids (FFA) (Haas et al. 2003). The product (FAEE) was analyzed by ^{1}H NMR (400 MHz; JEOL JNM-ECS 400) and quantified by the equation proposed by Sharma et al. (2013).

$$C = \frac{(AE_{\alpha-CH_2})}{(A_{\alpha-CH_2})} \times 100$$

wherein,

C—conversion of tri-acyl glycerol (TAG) to the corresponding FAEE.
$AE_{\alpha CH_2}$—Integration value of the methylene protons of the FAEE.
$A_{\alpha CH_2}$—Integration value of the methylene protons.

Fuel properties

Fuel properties of biodiesel (B100) and their blend (B20) with petroleum diesel were determined according to Indian standards (IS 15607:2005) and compared with those of commercial diesel/ petroleum diesel (PD) and American Society for Testing and Materials (ASTM) standards (D6751).

Results and discussion

Physical and chemical properties of acid oil

Table 1 presents the physical and chemical properties of acid oil. The acid oil constituting 55% FFA and 0.5% moisture are notably favorable for transesterification process with whole cell biocatalyst as the moisture content in the feed stock increases the reaction rate (Li, Du, and Liu 2007). Acid oil was extremely viscous (39.2 cSt viscosity), owing to high content of free fatty acids. From the viewpoint of the properties, acid oil can effectively be considered as feed stock for generation of biodiesel of good quality.

Table 1. Physiochemical properties and components of acid oil.

S.No.	Parameters	Acid oil
1.	Free fatty acids (%)	55
2.	Acidity (mg KOH/g oil)	109.6
3.	Density@15°C (kg/m^3)	926
4.	Viscosity@40°C (cSt)	39.2
5.	Saponification value	123
6.	Moisture (%)	0.50
7.	Iodine value	111
8.	Ash (%)	0.003
9.	(Kulkarni, Pujar, and Shanmukhappa 2008) Mineral acids (%)	1–2
10	(Kulkarni, Pujar, and Shanmukhappa 2008) Free moisture (%)	5–8
11	(Kulkarni, Pujar, and Shanmukhappa 2008) Phospholipids and sterols (%)	8–10
12	(Tüter et al. 2004) Neutral glycerides and unsaponifiable constituents (%)	20–50

Transesterification reaction

The hydrolytic reaction, carried out with whole cell biocatalysts resulted in gradual increase in FFA from initial level of 55% to 100% over 12 h of hydrolysis. The conversion of oil to FFA was confirmed through ^{1}H NMR wherein, the peak owing to glyceryl methylenic hydrogen at 4.25–4.35 ppm disappeared on the hydrolysis of oil to FFA (Figure 1(a,b)). Esterification was performed with the addition of ethanol, followed by 12 h incubation, resulting in the generation of ethyl esters with corresponding decrease in FFA content. The ethyl ester (EE) formation was evident with appearance of quartet of ethoxy hydrogens (–OC H_2CH_3) of ester at 4.10–4.20 ppm (Figure 1(c)). The average yield of EE obtained from four replicate reactions was 92% ± 0.8% with 8.0% ± 0.8% of corresponding FFA. Further washing of EE with NaCl–$NaHCO_3$ resulted in complete removal of FFA (Figure 1(d)). The use of whole cell systems of *Aspergillus* sp., as observed in the present study, provides an alternative to enzymatic catalysis resulting in similar or better yield of alkyl esters.

Fatty acids composition of acid oil and generated biodiesel

Table 2 presents the fatty acid composition of acid oil and FAEE. The acid oil used in the present study, comprises approximately 70% unsaturated fatty acids. Acid oil contains two most common saturated fatty acids, namely palmitic acid (16:0) and stearic acid (18:0). Similarly, oleic acid (18:1) and linoleic acid (18:2) were the most common monounsaturated and polyunsaturated fatty acids. The ester composition of biodiesel shows that the most plentiful compounds include ethyl oleate, ethyl linoleate, ethyl palmitate, and ethyl stearate. The distinctive ester composition of biodiesel, owing to the combination of these compounds, has a strong influence on its fuel properties (Ramos et al. 2009).

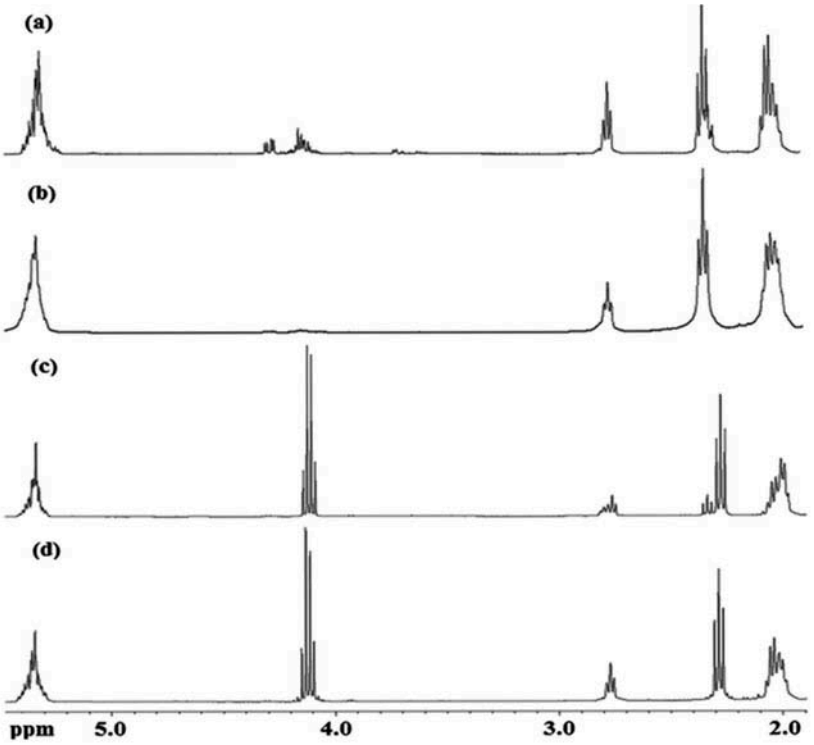

Figure 1. 1H NMR spectra. (a) Acid oil, (b) hydrolysis of acid oil to FFA, (c) FAEE, and (d) FAEE obtained after washing.

Table 2. Fatty acids composition of acid oil and FAEE.

Fatty acids	Acid oil (wt %)	FAEE (wt %)
Palmitic acid (C16:0)	24.24	18.64
Stearic acid (C18:0)	3.31	2.46
Cis-9-Oleic acid (C18:1)	36.78	37
Linoleic acid (C18:2)	35.42	35.41

The generated fuel, having a significant degree of unsaturation like ethyl oleate and ethyl linoleate, inhibits crystallization of fuel and reduces cloud point and pour point levels of fuel.

Fuel properties

In the present study, the B100 had a pour point (−26°C) that was significantly low. Pour point of the reference PD and B20 were −30°C and −29°C, respectively, which were lower than those of B100 (Table 3 and Figure 2(a)). Blending 80% (v/v) diesel with 20% (v/v) biodiesel (B20) significantly decreased the pour point of biodiesel. These values of pour points are perceived very low particularly for application in cold climatic conditions. This is due to high degree of unsaturation depicted by composition of biodiesel. Biodiesel generated from feedstocks, which contain saturated fatty acids in higher concentrations, tends to have comparatively poor cold flow properties (Dunn and Moser 2005); whereas, high unsaturation in fatty acids favors appropriate cold flow properties (Ramos et al. 2009). Cloud point and pour point are related properties in ASTM D6751, but limits have not been clearly defined till date (Campus 2011). The biodiesel (B100), which is produced from the acid oil, had marginally higher kinematic viscosity 6.8 cSt to the specified ASTM standard (1.9–6.0 cSt). The B20 and PD having the kinematic viscosity 4.8 cSt and 4.7 cSt, respectively, exist within the range specified (Table 3 and Figure 2(b)). Viscosity of obtained fuel was higher owing to the presence of large number of hydrogen bonds. It increases with number of carbon atoms and degree of saturation. Viscosity also depends on alcohol moiety as the viscosity of ethyl esters is somewhat higher than the viscosity of methyl esters (Knothe and Steidley 2005). The density of B100 (876 kg/m^3) was nearly similar to ASTM standard (878 kg/m^3). The densities of B20 (842 kg/m^3) and PD (822 kg/m^3) had no significant difference between each other (Table 3 & Figure 2(c)). Generally, the density of biodiesel is higher than that of petroleum diesel; however, the energy content is lower in both on a volume and a mass basis compared to diesel fuel (Canakci and Van Gerpen 2001). The density of biodiesel depends on its fatty acid composition and their purity, as it increases with decrease in chain length and increase in number of double bonds in ester. Table 3 and Figure 2(d) show that the biodiesel (B100) has considerably higher flash point (180°C) than B20 (120°C) and PD (58°C). This may be due to the presence of largely saturated esters. It is assumed that the obtained biodiesel had no residual amount of alcohol and therefore does not require additional cautions during handling, transportation, and utilization processes. In addition, the calorific values of B100 (39.92 MJ/kg) was lower than that of PD (43.34 MJ/kg) (Table 3 and Figure 2(e)). However, the B20 had high calorific value (46.32 MJ/kg) compared to that of

Table 3. Fuel properties of B100 and B20 compared with PD and ASTM standard.

S.No.	Parameters	ASTM Standard (D6751)	PD	B100	B20
1.	Viscosity@40°C (cSt)	1.9 – 6.0	4.7	6.8	4.8
2.	Pour point (°C)	NA	−30	−26	−29
3.	Density@15°C (kg/m^3)	878	822	876	842
4.	Flash point (°C)	130	58	180	120
5.	Calorific value (MJ/kg)	NA	43.34	39.92	46.32
6.	Sulfur content (%)	0.05	<0.1	<0.1	<0.1
7.	Ash (%)	NA	0.011	0.96	0.26

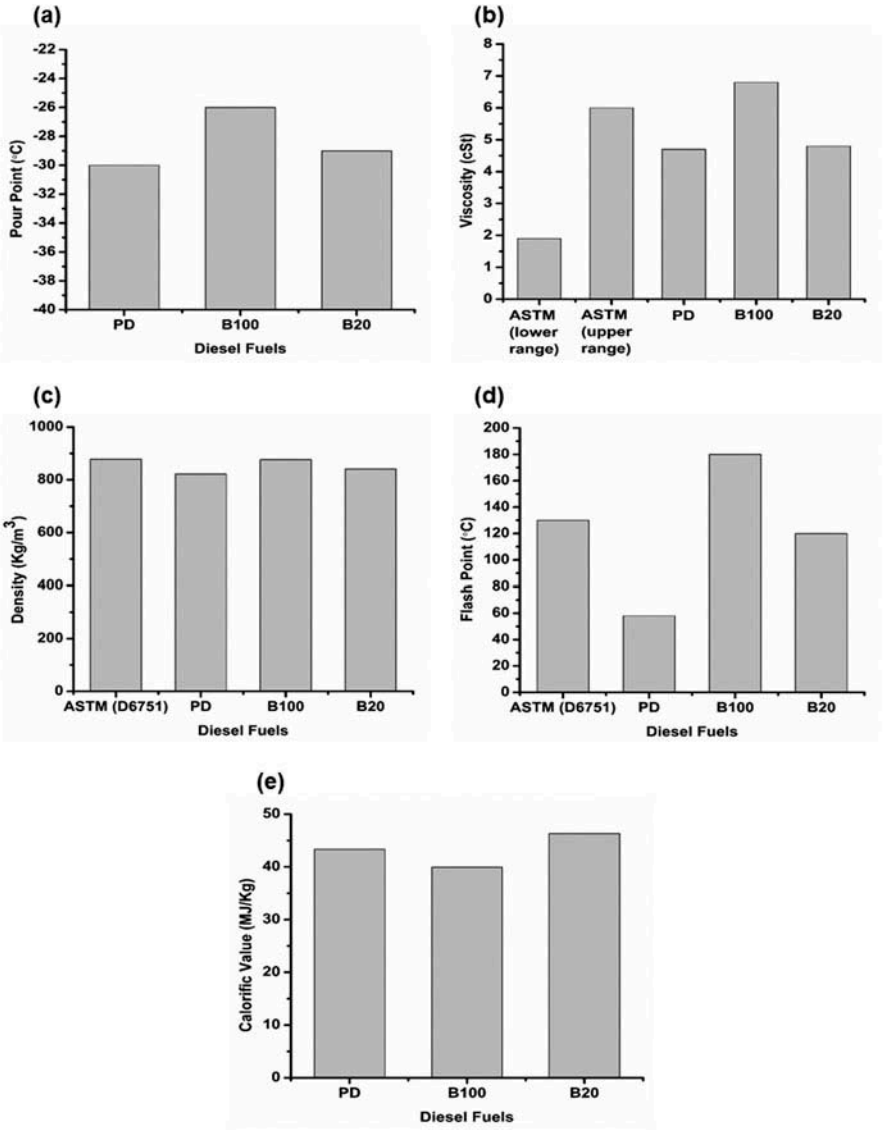

Figure 2. Fuel properties. (a) Pour point, (b) viscosity, (c) density, (d) flash point, and (e) calorific value—B100, B20, and PD.

PD. The lower calorific value of biodiesel (B100) may be due to higher oxygen content, as oxygen improves emission and combustion properties, but reduces the calorific value (Yamane, Ueta, and Shimamoto 2001). In addition, McCormick, Alleman, and Yanowitz (2005) reported that the biodiesel does not contain any aromatic components; however, it contains alkyl esters with different levels of saturation and unsaturation. Unsaturated esters have lower energy content, but have more energy per unit volume owing to their higher density.

Conclusions

The study demonstrates the whole cell catalyzed generation of biodiesel from acid oil containing 55% FFA, to the extent of 92% at 70% (v/v) acid oil supplementation in shorter time duration, to the best of our

knowledge, maximum yield obtained till date. In addition, the study demonstrates the use of acid oil as an alternative, low cost bearing and quality feedstock for biodiesel production. The resulted biodiesel meets the requirements of major international biodiesel standards such as ASTM D6751. The B100 and B20 fuel had good pour point and density along with other properties that were also comparable with petroleum diesel and ASTM standard.

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Effect of feedstocks and chain length of alcohols on whole-cell-catalyzed generation of alkyl esters

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ABSTRACT

The dried biomass of a whole-cell catalyst was used for the generation of alkyl esters from cottonseed oil and acid oil with different alcohols (methanol to decanol) as acyl acceptors. The conversion of alkyl esters increased from methanol (12%) to pentanol (96%), followed by decreased from hexanol (86%) to decanol (64%) in case of acid oil. The extent of transesterification was significantly higher ($P < 0.01$) in case of acid oil, with most of the acyl acceptors as compared to cottonseed oil. The study demonstrates the use of dried biomass of fungus as catalyst and an effective feedstock such as acid oil, for the generation of various industrially important alkyl esters.

KEYWORDS

Acid oil; alkyl esters; cottonseed oil; transesterification; whole-cell catalysis

Introduction

The feedstock used in biodiesel production processes are generally vegetable oils, waste edible oils, and animal fats. For example, *Cyprinus carpio* fish oil was used as a novel feedstock for biodiesel production using alkali-catalyzed one-step transesterification process (Al-Tikrity, Fadhil, and Albadree 2016). The currently used common feedstocks such as edible/nonedible oils, waste cooking oil, grease, tallow, and bitter almond seed oil have been reported extensively (Al-Tikrity et al. 2017; Haas 2005). However, such feedstocks are relatively expensive and correspond to 50–85% of total biodiesel production costs, making it necessary to search for low-cost substrates in order to increase the commercial competitiveness (Marchetti and Errazu 2008). As an effective alternative, feedstock like acid oils are being evaluated as possible substitutes as they comprise 40–80% long-chain free fatty acids mixture in addition to 1–2% mineral acids, 5–8% free moisture, 8–10% phospholipids and sterols, and variety of other constituents (Kulkarni, Pujar, and Shanmukhappa 2008; Tüter et al. 2004). Acid oil/soap stock is generated from crude vegetable oil at a rate of about 6% of the total volume. The application of whole-cell catalysis is another cost-saving approach in place of chemical processes to potentially enhance the productivity of the reactions associated with the generation of biodiesel (Fukuda et al. 2008; Shimada et al. 1999; Watanabe et al. 2000). The biocatalytic approach is more so amenable to acid oil, in place of chemical catalysts, as this feedstock has significantly high levels of fatty acids that hinder alkali catalysis, which is a conventional process for biodiesel generation. Here, we demonstrate the potential of the dried biomass of a whole-cell catalyst to esterify cottonseed oil and its acid oil to alkyl esters, with various acyl acceptors (methanol to decanol).

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Materials and methods

Materials

Acid oil was procured from an edible oil processing industry. Cooking cottonseed oil was purchased from the local market. All the reagents and chemicals used in the present study were of analytical grade and were supplied from Hi-media, SD Fine-Chem Limited, and Sigma, India.

Physiochemical properties of acid oil and cottonseed oil

Physical and chemical analysis of the acid oil and cottonseed oil were carried out according to the methods outlined in Indian standards SP: 18 [P:13]-1984.

Preparation of dried biomass

The *Aspergillus* sp

RBD01 (MTCC5436) used as the catalyst in the present study, was isolated from bio-contaminated butter, and was reported by our group to exhibit oil tolerance and transesterification potential (Aulakh and Prakash 2010; Prakash and Aulakh 2011). Active culture of RBD01 was inoculated in 100 mL of minimal medium (Bushnell-Hass Broth (BHB)) and supplemented with mycological peptone (0.5% w/v), di-ammonium hydrogen orthophosphate $(NH_4)_2HPO_4$ (0.5% w/v) as nitrogen sources and cottonseed oil (10% v/v) as main carbon source. The culture was incubated at 120 rpm and 30°C for 72 h. The culture was then separated by filtration through Whatman filter paper and washed with hexane to remove the excess of oil and free fatty acids (FFA) followed by overnight incubation at 30°C to completely dry the biomass. The dried biomass was then crushed in liquid nitrogen, using pestle and mortar, to prepare homogenous powder.

Estimation of enzyme activity

The enzyme activity of dried powdered biomass was determined by the method outlined by Sigurgísladóttir et al. (1993). Five milligrams of dried powdered biomass was added to the reaction mixture containing 0.9 mL of 50 mM phosphate buffer (pH 7.0) and 0.1 mL of 5.0 mM *p*NP (*p*-nitrophenol laurate). The mixture was incubated at 30°C for 30 min, followed by addition of ethanol to stop the reaction. The enzyme activity was determined at 420 nm. One unit of lipase activity is defined as the amount of enzyme that liberates 1 µg *p*-nitrophenol with *p*NP-laurate as the substrate under standard assay conditions in 30 min. The calculations of lipase units were carried out by preparing a standard curve using commercial lipase enzyme [Sigma-Aldrich L1754-25G].

Transesterification of acid oil using dried biomass as the catalyst

One gram of dried powdered biomass was inoculated in a round-bottom flask containing 10 mL of acid oil followed by addition of 1:4 molar ratio of acid oil to alcohol (viz.methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol or decanol) in each flask separately. The mixtures were stirred for 36 h at 30°C. The progress of the reactions was checked regularly by thin layer chromatography (Samukawa et al. 2000) (Figure 1). After 36 h, the reaction mixture was extracted with hexane to separate the product(s). Similar reactions were carried out with cottonseed oil and the mentioned acyl acceptors (methanol to decanol). All reactions were carried out in triplicates, and alkyl esters, thus obtained, were analyzed using ^{1}H NMR (400 MHz; JEOL JNM-ECS 400) and quantified by the equation proposed by Sharma et al. (2013).

$$C = \frac{(AE_{\alpha CH_2})}{(A_{\alpha CH_2})} \times 100$$

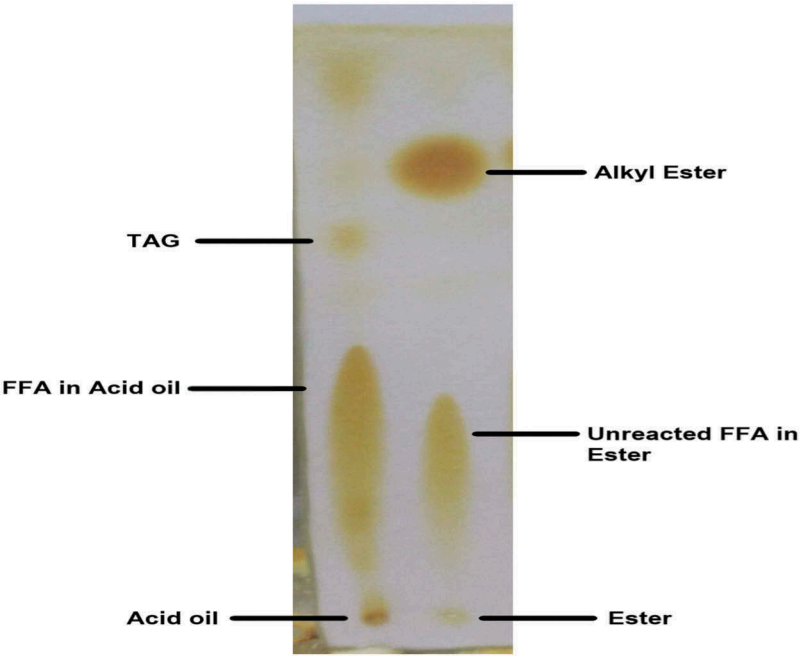

Figure 1. Thinlayer chromatography of acid oil and alkyl ester.

where

C is the conversion of tri-acyl glycerol to the corresponding FAAE.

$C = (AE_{\alpha CH_2})(A_{\alpha CH_2}) \times 100$ is the integration value of the methylene protons of the fatty acid alkyl ester (FAAE).

$A_{\alpha CH_2}$ is the integration value of the methylene protons.

All statistical (t) tests were carried out at $P < 0.05$ using Graphpad Prism v5.01 (GraphPad Software, La Jolla, CA, USA).

Results and discussion

Physical and chemical properties of acid oil and cottonseed oil

Table 1 presents the physical and chemical properties of acid oil as well as cottonseed oil. From the viewpoint of these properties, acid oil was considered as an effective feedstock for generation alkyl esters. Although alkali catalysis is the most conventional process being used for generation of biodiesel, raw materials/feedstocks such as acid oil are not amenable to this process due to high

Table 1. Physiochemical properties of acid oil and cottonseed oil.

S.No.	Parameters	Acid oil[a]	Cottonseed oil
1.	Free fatty acids (%)	55	0.04
2.	Acidity (mg KOH/gm oil)	109.6	0.09
3.	Density@15°C (Kg/m^3)	926	918
4.	Viscosity@40°C (cSt)	39.2	35
5.	Sponification value	123	192
6.	Moisture (%)	0.50	<0.05
7.	Iodine value	111	109
8.	Ash (%)	0.003	Nil

[a]Sharma et al. (2018).

FFA content as indicated by the acid value of acid oil and subsequent saponification. Therefore, the biocatalytic approach is obviously advantageous over alkali catalysis.

Transesterification reactions

The transesterification reaction was carried out using dried biomass having 280 U g^{-1} lipase activity, with short- and medium-chain alcohols (methanol to decanol).

The conversion of oil to alkyl esters was confirmed through ^{1}H NMR with disappearance the peak of glyceryl methylenic hydrogen of oil at 4.25–4.35 ppm (Figure 2 (a)). Esterification was confirmed with the appearance of singlet of methoxy hydrogen (-OCH_3) in the region of 3.60 ppm due to the proton of methyl ester (Figure 2 (b)). The ethyl ester formation was evident with appearance of quartet of ethoxy hydrogens (–OCH_2CH_3) of ester at 4.10– 4.20 ppm (Figure 2 (c)). With reference to other alkyl esters (propanol to decanol), the formation of ester was confirmed with the appearance of triplet of alkoxy hydrogens (–OCH_2CH_2–) at integration value at around 4.10 ppm (Figure 2 (d)).

The yield of methyl esters were somewhat similar ($P > 0.05$) in both the substrates (acid oil and cottonseed oil). The lower yield of the reaction with methanol could be due to the inhibition and inactivation of the lipase enzyme. The trend in the ester yield increased from methanol to pentanol (C1–C5) i.e., 12–96%, followed by decrease from hexanol to decanol (C6–C10) i.e., 86–64% in case of

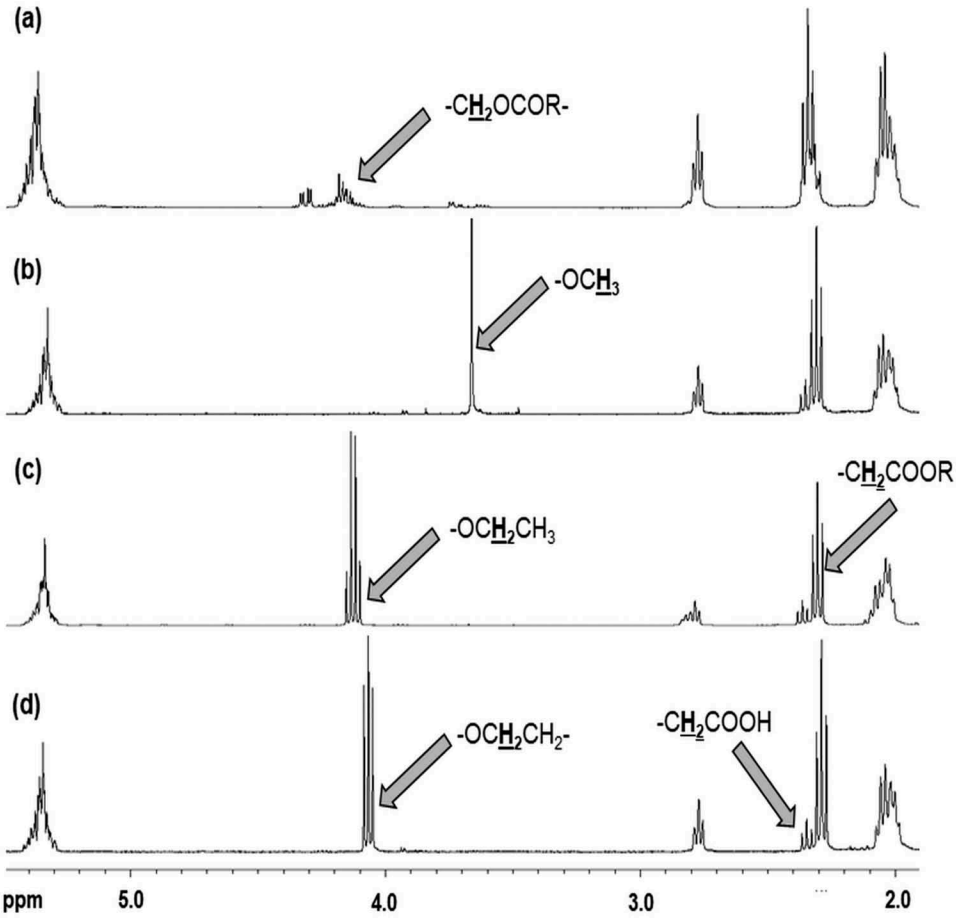

Figure 2. NMR spectra. (a) acid oil/cottonseed oil, (b) methyl ester, (c) ethyl ester, (d) other alkyl esters (propanol to decanol).

acid oil (Figure 3). Whereas, with cottonseed oil, the yield of esters marginally increased from 51 to 59% in the presence of hexanol to nonanol (C6–C9) with no significant conversion observed between methanol (5%) and propanol (5%). The variations in yield of ester with increasing chain length of alcohols from ethanol to decanol is expected to be due to the decrease in the polarity of alcohol, as esterification process occurs dominantly at the polar–nonpolar interfacial region (Aulakh et al. 2017; Sridharan and Mathai 1974). Observations reported by Gatfield (1986) and Romero et al. (2003) also indicated that the chain length of alcohol influences the ester yield only to a certain extent beyond which it is independent. Among the substrates, the extent of transesterification was significantly higher ($P < 0.01$) in case of acid oil as compared to ester yield from cottonseed oil. Thus, it is expected that the constituents such as FFA, moisture content, phospholipids, mineral acids, and sterols present in the acid oil may enhance the lipase enzyme activity, rate of reaction, and yield of esters during transesterification (Li, Du, and Liu 2007). Further, the increase in the yield of the reaction with propanol, butanol, and pentanol (C3–C5) is presumably due to the gradual increase in hydrophobicity of the alcohol resulting in lesser inhibitory and inactivating effect on the lipase (Shintre, Ghadge, and Sawant 2002). Blattner et al. (2006) reported the effect of chain length of alcohol for esterification of lauric acid with encapsulated *Candida antarctica* in $ScCO_2$. The study showed that, there is an increase of the rate of reaction when the chain length of the alcohol increases from ethanol to butanol followed by a sharp decrease toward the long chain alcohols. A similar observation was reported by Shintre, Ghadge, and Sawant (2002): the esterification of lauric acid with various aliphatic alcohols catalyzed by Lipolase in isooctane, the maximum reaction was observed with *n*-butyl alcohol. Yadav and Lathi (2004) reported the transesterification of methyl acetoacetate with different alcohols and Novozym 435 in toluene, and the conversion rate was decreased with increase in the chain length of primary alcohol. The enzymatic esterification of butyric acid was carried out with different primary and secondary alcohols;

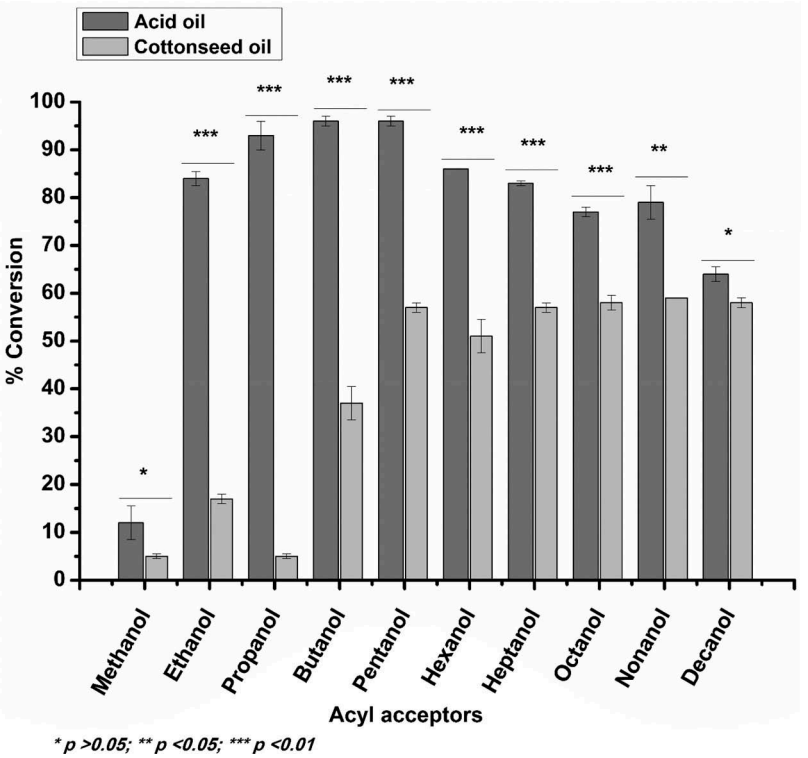

Figure 3. Extent of transesterification of acid oil and cottonseed oil with dried biomass as catalyst.

the results showed that the conversions with primary alcohols were higher than secondary alcohol. The conversion rate reduced with the chain length of alcohol for both primary and secondary alcohols (Molinari, Gandolfi, and Aragozzini 1996). Ethanol is of particular interest primarily as it is less expensive than methanol in some regions (such as Brazil) of the world. Butanol may also be obtained from biological materials thus yielding completely bio-based diesel (Qureshi et al. 2008). Butanol is completely miscible with vegetable oils and animal fats as it is less polar than methanol and ethanol (Boocock et al. 1996). Consequently, transesterification reactions employing butanol are monophasic throughout, enhancing the rate and extent of the reaction (Zhou and Boocock 2006). However, there are limited reports on the use of other alcohols as acyl acceptors in the whole-cell-catalyzed transesterification.

The variation of the conversion with the chain length of the acyl acceptors is due to the influence of various factors such as the miscibility of long-chain acyl acceptors in oil, molecular size of the acyl acceptors, interaction between the enzyme and acyl acceptors, and affinity of the lipase for the particular acyl acceptors. This differential affinity of lipase enzyme for different alcohols can possibly be explained in terms of the binding energy that is released when a substrate binds at the active site (Dixon and Webb 1966; Koshland 1959; Malcata et al. 1992). Substrates such as methanol, ethanol, propanol, which are relatively smaller in size, may not be able to release energy enough to facilitate change in conformation of the native lipase to the desired catalytically active form, resulting in reaction proceeding slowly. In case of butanol and pentanol, it is presumed that there is an optimal release of binding energy required for conformational change and subsequent catalytic activity (Malcata et al. 1992). Substrates that are longer (>C5) are also expected to release binding energy that ought to be sufficient to effect the desired conformational change. However, some of this energy may be required to change the conformation of the substrate so as to make it fit into the active site. Hence, only a small amount of the energy released by the binding process will actually be made available to derive the conformational change of the enzyme (Gandhi, Sawant, and Joshi 1995). Consequently, the optimum activity is not being achieved. Thus, the ester yield in our observations is presumed to be influenced by the above variations in binding energy, and the observations clearly indicated that acid oil is a better alternative to its corresponding virgin oil, as a substrate for alkyl ester generation.

Conclusion

Among the substrates, the extent of transesterification was significantly higher ($P < 0.01$) in case of acid oil with most of the acyl acceptors (ethanol to nonanol) as compared to ester yield from cottonseed oil. The maximum ester yield, obtained with pentanol was 96% over 36 h duration, in case of acid oil. The dried biomass can effectively enhance the transesterification reaction in shorter duration. The use of various alcohols, other than methanol and ethanol, as acyl acceptors has been less reported with acid oil and whole-cells as catalysts. In addition, these observations have potential value in the generation of various alkyl esters of industrial value.

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