Fungus Mediated Transesterification of Triglycerides

A thesis submitted in fulfillment of the

Requirement for the award of the degree of

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in

School of Chemistry and Biochemistry

by

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Certificate

Certified that the thesis entitled "**Fungus mediated transesterification of triglycerides**" which is submitted by Mr. Satnam Singh Aulakh in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy in the School of Chemistry and Biochemsitry, Thapar University, 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 "**Fungus mediated transesterification of triglycerides**" in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy, School of Chemistry and Biochemsitry , Thapar University, Patiala, is an authentic record of my own work carried out under the supervision of Dr. Ranjana Prakash, Associate Professor, School of Chemistry & Biochemistry, Thapar University, Patiala. India and Dr. N. Tejo Prakash, Associate Professor, Department of Biotechnology and Environmental Science, 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: 22nd June 2012.

Satnam Singh Aulakh

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B100	Biodiesel (100% + 0% diesel)
B20	Biodiesel (20% + 80% Diesel)
BHB	Bushnell hass broth
BSPs	Biomass support particles
CFS	Cell free supernatant
CTAB	Cetyl trimethyl ammonium bromide
$CDCl_3$	Deuterated chloroform
dATP	Di adenosine tri phosphate
dCTP	Di cytosine tri phosphate
DG	Diglycerides
dGTP	Di guanidine tri phosphate
dTTP	Di thiamine tri phosphate
EE	Ethyl ester
FAAE	Fatty acid alkyl ester
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GL	Glycerol
ITS	Intenal transcribed spacer
MAG	Mono acyl glyceride
MAG	Mono acyl glycerides
ME	Methyl ester
ME	Methyl ester
MG	Monoglycerides
MHz	Mega hertz
MTCC	Microbial type culture collection
^1H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
PDA	Potato dextrose agar
PDB	Potato dextrose broth
rRNA	Ribosomal ribo nucleic acid
TAG	Triacylglycerol
TBE	Tris Borate EDTA
TE	Tris EDTA buffer
UCO	Used cooking oil
UV	Ultra violet
WCO	waste cooking oil

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1.0 Introduction

The vegetable oils and their derivatives (especially alkyl esters), alias "biodiesel," are not new to the world of fuels. In present scenario, they are pleasingly accepted as distinguished alternatives to diesel fuel. Gone are the times when they were exclusively dealt with as experimental fuels. Today they have evolved to the preciously achieved stage of commercialisation for being technically competitive and offering technical advantage over conventional diesel fuel. Though in terms of qualitative engine performance and fuel economy lines, biodiesel and conventional fuels have equanimity, biodiesel is not only a renewable and domestic resource but also reduces most emissions (Knothe et al. 1997).

As on date, no unambiguous definition of biodiesel is available; although, it stands for vegetable oils and specifically their methyl esters being used as diesel fuels. However, commutatively it also constitutes under its name animal fat and blends of conventional fuel with vegetable oils or methyl esters. Attributed to the emphasis on the use of esters as diesel fuels, the term 'biodiesel' however increasingly refers to alkyl esters of vegetable oils and animal fats and not the oils and fat themselves (Knothe et al. 1997). According to international standard for biodiesel properties, outlined in ASTM D 6751, 'Biodiesel' is the term for fuel comprised of mono-alkyl esters of long chain fatty acids derived either from vegetable oils or from animal fats, designated as B100. On the other hand the blend of 20% biodiesel with 80% petrodiesel; by volume is termed B20 (Gerpen et al. 2004).

Mono-alkyl esters are produced on the reaction of a straight chain alcohol (ethanol or methanol) with a fat or oil to form glycerol and the esters of long chain fatty acids.

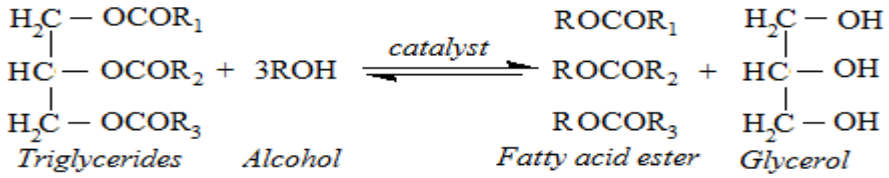
Biodiesel fuels have the following salient features (Sheehan et al. 1998; Schafer, 1998; Syassen, 1998; Sams, 1998; Yamane et al. 2001):

1) Derived from plant, its combustion does not affect to increase net current level of CO_2 in the atmosphere. Moreover, Its combustion products as compared to conventional fuels, have relatively decreased level of particulates carbon monoxide, SO_X and, under some conditions, nitrogen oxides;
2) Domestically producible, it signifies towards economic support to nations petroleum imports; and

3) It is biodegradable.

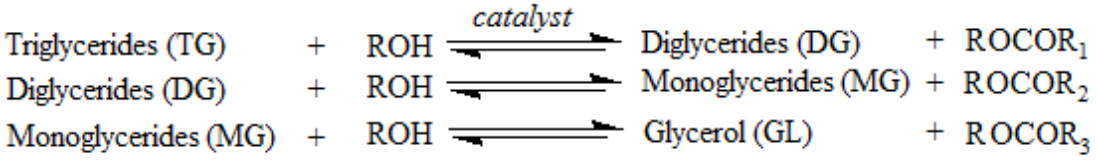
Two approaches have been explored extensively for transesterification of vegetable oils towards production of biodiesel (Haas et al. 2002). The first being a chemical approach in which alcoholysis of oil is carried out with methyl or ethyl alcohol in the presence of strong acid or base to produce biodiesel and glycerol. The second approach is through enzymatic catalysis, in which lipase catalyzed transesterification is carried out in aqueous and non-aqueous environment (Fukuda et al. 2001).

Transesterification, is also called alcoholysis, as it marks the displacement of alcohol from an ester by another alcohol in a process similar to hydrolysis.



Alcohols such as methanol, ethanol, propanol, butanol and amyl-alcohol are suitable for transesterification. Amongst all of these, methanol and ethanol are frequently utilized, with methanol being more favourable because of its low cost and favourable physical and chemical properties. This process has been widely used to reduce the viscosity of triglcerides, thereby enhancing the physical properties (Clark et al. 1984).

The chemically catalyzed transesterification reaction with alcohol takes place in series of steps. The first step is the conversion of triglycerides to diglycerides, which is followed by the conversion of diglycerides to monoglycerides and of monoglycerides to glycerol, yielding one methyl ester molecule from each glycerides at each step (Freedman et al. 1986; Noureddini and Zhu, 1997).



Transesterification reaction can be catalyzed chemically either by acid or alkalies (Freedman et al. 1986; Noureddini and Zhu, 1997).

Acid catalysis facilitates very high yield of esters using a variety of acids including sulfuric, phosphoric, hydrochloric and organic sulfonic acids, however the reaction is very slow (Ma et al. 1999; Srivastava and Prasad, 2000; Freedman et al. 1984; Marcetti et al. 2007). Although better triglycerides conversion can be obtained by excess of alcohol, glycerol recovery becomes more difficult. In addition to this, serious environment deterioration and corrosion related problems mask their use to produce biodiesel at the industrial scale (Canakci and Gerpen, 1999; Li et al. 2008).

Most commonly used alkali in the process of transesterification includes sodium hydroxide, potassium hydroxide, carbonates, and alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide and sodium butoxide. Particularly anhydrous glycerides are required for alkali transesterification because water if present causes saponification (Wright et al. 1944) thereby reducing the catalytic efficiency and increasing the viscosity by forming gels, consequently making the separation of glycerol a difficult process (Fukuda et al. 2001). Moreover alkali catalyzed reaction needs very small free fatty acid content in the oil (≤0.5%) (Ma et al. 1998). It is observed that if the reactants do not satisfy the requirements, ester yield reduced significantly (Freedman et al. 1984).

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. 2005); alkyl guanidines heterogenized on organic polymers (Schuchardt et al. 1996); immobilized enzymes (Nelson et al. 1996; Watanabe et al. 2000; Shimada et al. 2002; Dossat et al. 2002), calcium carbonate (Suppes et al. 2001) in addition to other complexes. However, transesterification reactions catalyzed by heterogeneous catalysts require high temperature and pressure with longer reaction period and higher energy consumption (Meher et al. 2006).

Biocatalysis participates in the 'green chemistry' concept and its effect on sustainability is now established beyond question (Alcalde et al. 2006). Biocatalysts, using pure enzymes or whole cell catalysts, allow synthesis of specific alkyl esters, easy recovery of glycerol, and transesterification of glycerides with high free fatty acid content (Nelson et al. 1996; Fukuda et al. 2001). Extensive studies have been carried out on the lipase-catalyzed transesterification of triglycerides (Shieh et al. 2003). Enzyme catalyzed procedures using lipase as catalyst do not produce side

reactions (Iso et al. 2001), but use of lipases at the industrial scale is cost-intensive. The cost is further added due to involvement of a three-step process for 95% conversion of oil (Watanabe et al. 2000). However, the advantages of using enzyme as a catalyst in these reaction include (a) synthesis of specific alkyl esters and transesterification of triglycerides with high free fatty acid content (Nelson et al. 1996); (b) Prevention of glycerol contamination and ease in separation of product (Fukuda et al. 2001); and (c) transesterification in normal temperature (30-40°C) and reaction conditions (Fukuda et al. 2001). Most importantly process is possible with crude enzyme formulation. Akoh et al. (2007) reviewed extensive studies that has been carried out by various researchers on use of lipase in free or by immobilized forms.

In addition to cost intensive nature of pure enzyme application, other hindrance of the use of pure lipases in transesterification reaction includes loss of activity due to volume of oil to be used and lack of uniformity in performance of enzyme support system available till date (Perez et al. 2003).

An effective alternative to the use of pure lipase is the application of microbial strains exhibiting good potential of producing lipases at specific reaction conditions. These strains can be effectively exploited as whole cell catalysts for transesterification reaction. Several studies have reported the utilization of microorganisms such as bacteria, yeast and fungi as whole-cell biocatalysts to improve the cost effectiveness of the bio-conversion processes (Fukuda et al. 2008). Among the established whole-cell biocatalyst systems, filamentous fungi have proven to be the most robust biosystems for industrial applications. The use of *Rhizopus oryzae, R. chinensis,* recombinant *Saccaromyces cerevisiae* and most recently *Aspergillus niger* as whole-cell biocatalysts, have been studied and reviewed by different research groups (Rottig et al. 2010; Fukuda et al. 2008; Hama et al. 2008; Xiao et al. 2009). These studies have however reported transesterification to a maximum extent of upto 90% (yield of FAME). Limited reports are available on bio-catalyzed transesterification and alcoholysis especially using fungi. The available reports are mostly associated with *Rhizopus* sp. which indicated generation up to 90% of methyl ester in the presence of 10-20% water. In addition, there are scarce reports on the use of cell-suspension in these studies. This lacuna necessitates a focused exploration on the use of whole cell biocatalysts with lipase producing organisms for transesterification reaction.

A major barrier in the commercialization of biodiesel production from vegetable oil is its high manufacturing cost, which is due to the cost of virgin vegetable oil. The cost of vegetable oil has a crucial role in the economics of the biodiesel. The distribution of the cost of biodiesel production indicated that oil feedstock incurs the major cost of biodiesel production accounting over 70 % of the total (Nelson et al. 1996). Alternatively, the economics of biodiesel can be significantly improved by the use of the waste vegetable oil as biodiesel feedstock. Even though some of this waste cooking oil is used for soap production, a major part of it is discharged into the environment (Chhetri et al. 2008). Restaurant waste oils and rendered animal fats are less expensive than food-grade canola and soybean oil (Canackci et al. 2007). The use of waste cooking oil as biodiesel feedstock reduces the cost of biodiesel production (Canackci, 2007) since the feedstock costs constitutes approximately 70-95% of the overall cost of biodiesel production (Connemann and Fischer, 1998; Kulkarni and Dalai, 2006). The use of waste cooking oil proves economically sound and significant as feedstock cost comprises nearly 90% of the total production cost (Connemann and Fischer, 1998; Canackci, 2007). However, hitherto, quite limited reports indicate the use of such oil sources for transesterification reaction.

India, being a net importer of edible oils, poses a host of non-edible oils, which are not only underutilized but also can be effectively used in the production of biodiesel. A few names in this class non-edible include trees like Sal, Neem, Mahua, Karanja, and castor, Jatropha, Honge and Rubber-seed etc. which not only can tolerate long drought and dry period conditions but also grow on waste land (Agarwal, 2007). India reports availability of nearly 450 such non-edible oils from jatropha, karanja and nahar dominating over others

Several studies have shown that there exists an immense potential for the production of plant-based oil to produce biodiesel. Azam et al. (2005) studied the prospects of fatty acid methyl esters (FAME) of some 26 non-traditional plant seed oils including Jatropha to use as potential biodiesel in India. Among them, *Azadirachta indica*, *Calophyllum inophyllum*, *Jatropha curcas* and *Pongamia pinnata* were found most suitable for use as biodiesel and they meet the major specification of biodiesel for use in diesel engine. Subramanian et al. (2005) reported that there are over 300 different species of trees, which produce oil-bearing seeds. Fatty acid methyl

ester from cold pressed soapnut seed oil was envisaged as biodiesel source for first time by Chhetri et al. (2008). Recent reports on transesterification of non-edible oils using different catalytic routes have demonstrated the feasibility of using such raw material sources (Karmee and Chadda, 2004). Meher et al. (2005) examined transesterification of karanj oil with methanol, using alkali catalyst; under optimal conditions the yield of methyl ester from karanja oil was obtained to be 97-89%.

Lipase catalyzed transesterification, hitherto has been reported only to limited extent with respect to the literature examined till date. To the best of our knowledge, very less information on the whole cell catalyzed transesterification of edible and non-edible oils has been observed in the literature examined, which formed the basis of this study.

2.0 Literature Review

Bio-'Diesel' world encompasses within itself the pioneering work of Rudolf Diesel (1858-1913) and his ever fruitful contribution. The esteemed scientist had intense interest in the engines and the same bear his name as well 'The Diesel Engine'. As the work under presentation in a matter to this industry, it is therefore apt to begin this history with the impressions of Diesel himself. As written in his book *Die Entstehung des Dieselmotors* [*The Development* (or *Creation* or *Rise* or *Coming*) *of the Diesel Engine*] the idea of developing an engine germinated in his mind while studying at an institute *Polytechnikum* in Munich in 1878. He consistently kept giving a practical shape to his dream that finally emerged as the diesel engine. "Liquid Fuels," by Rudolf addresses vegetable oils as fuels and plant oils were actually successfully used as fuel in diesel engine by 1900. In the same year during the Paris exposition, a French Otto company demonstrated the use of arachide (peanut) oil in a small diesel engine at the request of the French Government. The result was quite smooth working of the engine which consequently is a part of knowledge on only a few people (Diesel, 1912; 1913).

Use of vegetable oils as emergency fuel during the World War II resulted in initiation of research and development on these oils as domestic fuels in India (Chowhury et al. 1942). However the work ceased as soon as petroleum based diesel became getting easily available at affordable cost (Amrute, 1947), in those times. Year after the World War II, the United States of America started showing concerns about the rising use of petroleum fuels and thereby, the possibility of resultant fuel shortages. This concern ignited a 'dual fuel' project at the Ohio State University (Columbus, OH). 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 under consistent investigations since the historic times.

Today once again, the importance of energy security and environmental concerns are acting significantly as driving forces for use of vegetable oil-based diesel fuels. Non-vegetable oil sources such as industrial tallow (Lugaro and Medina, 1944)

and fish oils (Kobayashi and Yamaguchi, 1921; Faragher et al. 1932; Lumet and Marcelet, 1927; Marcelet, 1927; Okamura, 1940) remained being investigated. In India, around eleven such vegetable oils namingly peanut, karanj, punnal, polang, castor, kapok, mahua, cottonseed, rapeseed, coconut, and sesame were investigated as fuels (Chowhury et al. 1942). A study made in Brazil also reported the use of fourteen vegetable oils (Borges, 1944) as fuel. Further, results on twenty vegetable oils, such as castor, grapeseed, maize, camelina, pumpkinseed, beechnut, rapeseed, lupin, pea, poppyseed, peanut, hemp, linseed, chestnut, sunflower seed, palm, olive, soybean, cottonseed, and shea butter, were summarized by Walton (1938). Satisfactory performance of vegetable oils as fuel and fuel sources has been discussed in many "historic" publications although its widespread use was not found viable due to cost intensiveness as compared to petroleum derived fuels and some physico-chemical properties.

"To get the utmost value from vegetable oils as fuel it is academically necessary to split off the triglycerides and to run on the residual fatty acid," said by Walton (1938) which later paved way for research and development towards what we today call 'biodiesel'. His words recommended the elimination of glycerol from fuel with no mention about esters. Some remarkable work has been performed in Belgium and Zaire (now the Belgian Congo) - its former colony in the field of biodiesel and deserves the much recognition than it has received. The first report constituted on Biodiesel, as it appears, by the Belgian Patent 422,877, granted on Aug 31, 1937 to G. Chavanne (University of Brussels, Belgium) (Chavanne,1937) describes about using the ethyl ester of palm oil as diesel fuel, however other oils and methyl ester have also been mentioned. Acid catalysed transesterification was used as means of obtaining esters. A related extensive report on the production and use of palm oil ethyl ester as fuel, published in 1942 is of particular interest (Abeele and de Palme, 1942).

More systematic studies towards properties and application in the following decades, lead to culminating in the use of biodiesel as an alternative fuel in the present date. Though obvious advantages embellish its worth, however, direct use of vegetable oils in fuel engines is problem prone (Table 1) as well, for instance their high viscosity and low volatility doesn't allow them to undergo complete combustion in the fuel injector of diesel engine. Moreover, thermal decomposition of glycerol leads to generation of acrolein known for its toxicity (Gateau and Guibet, 1985;

Schwab et al. 1987; Schwab et al. 1988; Filho et al. 1993). In order to reduce the high viscosity of vegetable oils, different ways have been considered.

- Dilution of 25 parts of vegetable oil with 75 part of diesel fuel (Schwab et al. 1987)
- 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, (Filho et al. 1993) and
- Transesterification with ethanol or methanol (Freedman et al. 1986)

The process of transesterification, as mentioned in the above points, is relatively a simple process and physical characteristics of the fatty acid alkyl ester produced by this process resemble very close to those of diesel fuels (Schwab et al. 1987), due to which it is considered the best among all the alternatives mentioned above. In unmodified diesel engines, the methyl or ethyl esters of fatty acids can be burnt directly, that too with low deposit formation (Graille et al. 1985; Mittelbach and Tritthart, 1988; Zaher, 1990). This product is now particularly termed as biodiesel. It represents a fuel containing mono alkyl esters of long chain fatty acids derived from vegetable oil or animal fats (Gerpen et al. 2004).

An important section of organic reactions, used to transform an ester into another by interchanging the alkoxy moiety, is generally termed as transesterification. However, it has been reported by Jothiramalingam and Wang (2009) that the presence of catalyst could expedite and control the equilibrium in order to achieve high yield (Jothiramalingam and Wang, 2009). Triacylglycerols (TAG) consisting of long chain fatty acids which are chemically bound to a glycerol (1, 2, 3- propanetriol) is the principle component of vegetable oil and animal fats. TAG is chemically an ester and transesterification is the process of inter-conversion of one ester into another form involving reaction of TAG reaction with a short chain monohydric alcohol. This is generally carried out normally in the presence of a catalyst, at high temperature resulting in fatty acid alkyl ester (FAAE) and glycerol (Wright et al. 1944; Freedman et al. 1986).

Table 2.1. Known problems and probable cause of the use of vegetable oils as fuels (Fangrui et al. 1999)

Problem	Probable cause
Short-term Cold weather starting, Plugging and gumming of filters, lines and injectors	High viscosity, low cetane, and low flash point of vegetable oils, Natural gums (phosphatides) in vegetable oil. Other ash
Engine knocking	Very low cetane of some oils. Improper injection timing
Long-term Coking of injectors on Piston and head of engine	High viscosity of vegetable oil, incomplete combustion of fuel. Poor combustion at part load with vegetable oils
Carbon deposits on Piston and head of engine	High viscosity of vegetable oil, incomplete combustion of fuel. Poor combustion at part load with vegetable oils
Excessive engine wear	High viscosity of oil, incomplete combustion of fuel. Poor combustion at part load with vegetable oils. Possibly free fatty acids in vegetable oil. Dilution of engine lubricating oil due to blow-by of vegetable oil
Failure of engine lubricating oil due to Polymerization	Collection of polyunsaturated Vegetable oil blow-by in crankcase to the point where polymerization Occurs vegetable oil to an ester. Increase motor oil changes. Motor oil additives to inhibit oxidation

The stepwise process of conversion of TAG to biodiesel involves

- Initial reaction of alcohol with TAG as alkoxide anion to produce 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 combined FAAE, collectively is 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. Generally, transesterification reaction is either non-catalysed or catalysed, with latter being possible either with homogenous or heterogeneous catalyst with varying reaction conditions.

2.1 Non-catalytic transesterification

Supercritical fluid like methanol are used for the accomplishment of non-catalytic transesterification of biodiesel but pressure, temperature and alcohol amount needed for the reaction are significantly high i.e 45–65 bar, 350°C and 42:1 molar ratio (Demirbas, 2003; Kusdiana and Saka, 2004). There are many advantages of non-catalytic transesterification of biodiesel when compared to those of various catalytic methods. For instance, short reaction time (e.g, 4 min), in addition to simpler product purification due to absence of steps involving catalyst removal. However, the disadvantages of the process include batch wise processing, elevated energy and alcohol requirements during production, and increased capital, operational and maintenance costs (Demirbas, 2003; Kusdiana and Saka, 2004).

2.2 Catalytic transesterification by using homogeneous and heterogeneous catalyst

Homogenous catalysis (homogenous process where catalyst(s) is/are in same phase of reactant) is done either with an acid or with a base. Sulphuric acid, hydrochloric acid and organo-sulphuric acids are usually preferred in acid catalysed transesterification. The biodiesel yield is increased through acid catalysed transesterification because acid catalyze the free fatty acids to esterify thereby yielding high amount of esters. However, the reaction is very slow (Marchetti et al. 2007). Though better conversion of triglycerides is possible by using excess of alcohol in the process, the glycerol recovery from the reaction becomes more difficult. On the other hand, serious environmental and corrosion related problems make their use non-practical for biodiesel production at the industrial scale (Canakci and Gerpen, 1999; Li et al. 2008).

On the other hand, for base catalysed transesterification, sodium alkoxides, sodium or potassium hydroxides as well as sodium or potassium carbonates are used. High conversion level of triglycerides (to corresponding methyl ester) is obtained, by using conventional alkali catalysed transesterification process that too in short times. However, their hygroscopic nature and presence of only hydroxide ion as impurity make it even difficult to manipulate. In addition, the reaction has several drawbacks viz. energy extensiveness, difficult glycerine recovery, removal of catalyst from the

reaction, treatment of alkaline waste water and interference of free fatty acids and water with reaction (Fukuda et al. 2001).

Only fewer articles are available till date over the development of new catalysts for homogenous process. The activity and efficiency of guanidine and eight substituted cyclic and acyclic guanidines has been reported as catalysts for regarding transesterification of rapeseed oil (Schuchardt et al. 1996). Production of 90% of methyl ester after 1 h from 1 mol% of 1, 5,7-triazabicyclo[4,4,0]dec-5-ene (TBD) was found be better over other forms like triamino(imino)phosphoranes (BEMP) (66%ME), tris(dimethylamino)methyliminophosphorane (Me7P) (63%ME), 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (MTBD) (47%ME), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (32%), 1,1,3,3-tetramethylguanidine(TMG) (18%) and 1,5-diazabicyclo[4.3.0]non-5-ene4 (DBN) (4.5%ME) (Schuchardt et al. 1996). Methanolysis of soybean oil under homogenous conditions was done by using metal complexes of the type M (3-hydroxy-2- methyl-4-pyrone) $2(H_2O)_2$, where M = Sn, Zn, Pb and Hg. Similar molar ratio of 400:100:1 (methanol:oil:catalyst) for comparison was used for all metal complexes, maximum of 93.8% ME was obtained from Sn complex after 5h of reaction time followed by Zn (37.9% ME in 6 h), Pb (24%ME in 10 h) and Hg (4.1% ME in 10 h) this showed that all complexes are active in this reaction, with the following decreasing activities: $Sn^{2+} >> Zn^{2+} > Pb^{2+} \approx Hg^{2+}$ (Abreu et al. 2003).

Heterogeneous (heterogeneous process where catalyst are in solid phase and reactant in liquid) catalysis has been introduced in alcoholysis of triglycerides, in order to overcome or minimize the problem of homogenous catalysis. A large number of heterogeneous catalysts including enzymes, zeolites, clay, guanidine, heterogenized or organic polymer, ion exchange resins or oxide etc. have been reported in the literature (Jothiramalingam and Wang, 2009; Schuchardt et al. 1996). Schuchardt et al. (1996) reviewed that the heterogeneous catalysts can completely substitute the homogenous catalysts to avoid environmental concerns (Schuchardt et al. 1996). Use of Polystyrene bound biguanidines as recyclable catalyst for the transesterification of various vegetable oils was demonstrated by Gelbard and Vielfaure-Joly (2000). Methanolysis of triglycerides have also been demonstrated using insoluble salt of amino acids as catalyst (Peter et al. 2002). Salts of variety of elements such as copper, zinc, cadmium, nickel, lanthanum, cobalt, calcium, magnesium, iron were tested as

their role as catalysts (Suppes et al. 2001). Soybean oil methanolysis has also been tested using tungstated zirconia-alumina (WZA), sulphated tin oxide (STO) and sulphated zirconia-alumina (SZA) (Furuta et al. 2004). Biodiesel production has also been reported from commercial sulfonic ion exchange resin (Reis et al. 2003). Kim et al. (2004) have reported application of Na/NaOH/g-Al_2O_3 as heterogeneous alkaline catalyst to the transesterification of soybean oil with methanol using hexane as co-solvent (Kim et al. 2004). Transesterification of soybean oil using mixed oxides as catalysts has been studied by Monteiro and Cruz (2004). Methanolysis of soybean oil in the presence of NaX faujasite zeolite, ETS-10 zeolite and metal catalysts were studied (Suppes et al. 2001). Methanolysis of rapeseed oil for different methanol: oil ratio has been tested by Leclereq et al. (2001) in the presence of cesium-exchanged NaX faujasites, mixed magnesium-aluminum oxides, magnesium oxide and barium hydroxide.

For transesterification reaction, different types of heterogenous catalysts are being used (Lee et al. 2009), and those include zeolite type solid acid catalyst (De Rezende et al. 2008; Sasidharan and kumar, 2004), Heteropoly acid loaded MCM-14 catalyst (Mabaraka et al. 2003; Demirbas, 2005; Chen et al. 2007; Sakthivel et al. 2008), Sulphated zirconia and tin oxide solid acid catalyst (Yadav and Murkute, 2004; Furuta et al. 2004; Chen et al. 2007; Einloft et al. 2008), tungsten trioxide loaded zirconia type solid acid catalyst (Brei et al. 2003; Lopez et al. 2008; McNeff et al. 2008), alkali metal salt loaded alumina solid base catalyst (Ebiura et al. 2005; Xie and Huang, 2006), zeolite (Philippou and Anderson, 2000; Suppes et al. 2004; Kang and Lee, 2005; Lopez et al. 2005), hydrotalcite (Di Cosimo et al. 1998; Watkin et al. 2004; Serio et al. 2006; Barakos et al. 2008), lithium calcium oxide (Davydov et al. 1995; Gryglewicz, 1999; Philippou et al. 1999; Watkins et al. 2004). Heterogeneous alkali like CaO, $CaTiO_3$, CaO-CeO_2, $Ca_2Fe_2O_5$, KOH/Al_2O_3, KOH/NaY, Al_2O_3/KI, ETS-10 zeolite, alumina/silica supported K_2CO_3 are advantageous in being non-corrosive, environmentally benign, recyclable, fewer disposal problems, easily separation, higher selectivity and longer catalyst life time (Liu et al. 2008; Qian et al. 2008; Di Serio et al. 2007) on the other hand heterogeneous acids like ZnO/I_2, ZrO_2/SO_4^{2-}, TiO_2/SO_4^{2-} Vanadyl phosphate, nicobic acid, sulphated zirconia, Amberlyst-15, and Nafion-NR50, carbon based solid acid catalyst, carbohydrate derived acid catalyst (Di Serio et al. 2007; Dizge and Keskinler, 2008; Lou et al. 2008;

Kawashima et al. 2009) are advantageous as they catalyse esterification and transesterification simultaneously, in addition to being recyclable and eco-friendly.

To promote the preferential adsorption of oily hydrophobic species on the surface of the catalyst a hydrophobic surface is essentially required. Heterogeneous base and acid catalyst(s) also have cetain obvious disadvantages such as Low FFA requirement, anhydrous conditions, more wastewater from purification, high molar ratio of alcohol to oil requirement, high cost, low acid site concentration, low microporisity, diffusion limitation, high cost (Leung et al. 2010; Meher et al. 2006). It is also quintessential to avoid possible deactivation of catalytic sites by strong adsorption of polar by-product such as water and glycerol (Lotero et al. 2005).

2.3 Enzyme catalysed biodiesel production

Another form of transesterification reaction generally referred to as alcoholysis, or through interesterification (ester interchange), is possible through enzymatic reactions involving lipases. Lipases are excellent alternatives to chemical catalysts due to the obvious advantages of the former viz. Requirement of milder mild reaction conditions; specificity; reuse; immobilization possibilities; improvable efficiency by genetic engineering; acceptability for new substrates; more thermo stability and their natural origin which identifies their catalytic reactions as "green" in nature. Other important aspects that get added to the list of advantages include their higher efficiency, high selective property, less energy consumption component (which cause reaction to take place in mild conditions), lesser production of side products or waste (environmental favourability), and better efficacy in immobilized forms (Akoh et al. 2007; Fjerbaek et al. 2009; Robles-Medina et al. 2009; Rottig et al. 2010; Karimpil et al. 2012). Lipases are universally present in all biotic components and are classified extracellular or intracellular, however, both are designed to catalyse hydrolytic reaction. Classification is also made on the basis of origin of lipase eg, microorganisms, animals or plant. Lipases obtained from microorganisms or microbial lipases viz, bacterial or fungal lipases are commonly used by the industry. Lipases are selected for lipid modification and the selection is made on the kind of modification desired. For instance, position specific modification of triacylglycerol, fatty acid specific modification, modification by hydrolysis, and modification by synthesis (direct synthesis and transesterification). Lipases derived from *Aspergillus niger*,

Candida cylindracea, *Candida rugosa*, *Chromobacterium viscosum*, *Geotrichum candidum*, *Fusarium heterosporum*, *Fusarium oxysporum*, *Humicola lanuginose*, *Mucor miehei*, *Oospora lactis*, *Penicillium cyclopium*, *P. roqueforti*, *Pseudomonas aeruginosa*, *P. cepacia*, *P. fluorescens*, *P. putida*, *Rhizopus arrhizus*, *R. boreas*, *R. thermosus*, *R. usamii*, *R. stolonifer*, *R. fusiformis*, *R. circinans*, *R. delemar*, *R. chinensis*, *R. japonicus* NR400, *R. microsporus*, *R. nigricans*, *R. oryzae*, *R. rhizopodiformis*, *R. stolonifer* NRRL 1478, *Rhizomucor miehei*, *Rhodotorula rubra* and *Staphylococcus hyicus* have been reported in literature in addition to animal and plant sources (Sellappan and Akoh 2005; Akoh et al. 2007).

The optimal temperature range for lipase catalyzed transesterification is normally from 30-45°C. This temperature range basically is a compromise between enzyme stability and transesterification rate, the former decreases but the latter increases with the increase in temperature. In addition, molar alcohol:oil ratio and the type of solvent used also influence the range (Rodrigues et al. 2008).

Almost all types of alcohols can be used for enzymatic catalysis, however alcohols like methanol which is short chain readily inactivates the lipase as its solubility in hydrophobic oil is less. 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 and Wu, 2003). Generally higher reaction rates undergo with use of longer chain alcohols but 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: oil plays a vital role with 3:1 alcohol to oil sufficing to high yields with no enzymatic inactivation. Further, increase of alcohol yields marginally higher FAAE at the same time causes lipase inactivation. *C. antarctica* lipase which otherwise inactivated by methanol/oil ratios of 0.5:1 or higher (Shimada et al. 1999; Xu et al. 2003) has however been prevented by stepwise addition of alcohol (Shimada et al. 1999; 2002; Watanabe et al. 2000; 2001).

Biodiesel synthesis by enzyme catalysis is possible in aqueous medium or in water free organic solvents. TAG's are firstly cleaved (into FFA and glycerol) followed by esterification of FFA (Selmi and Thomas, 1998). Another advantage of enzymatic catalysis in process of transesterification is presence of water which on the other hand is in contrast with chemical catalysis wherein water content has to be kept

very low. However the optimal water content remains a compromise between maximum enzyme activity and minimum ester hydrolysis because excess of water shifts the equilibrium towards hydrolysis, thereby lowering the biodiesel yield (Noureddini et al. 2005).

Properties like increased alcohol solubility and high alcohol concentration (alcohol: oil, 6:1) usage (without inactivation of lipases) (Antczak et al. 2009) can be optimized if organic solvents like n-hexane (Nelson et al. 1996; Soumanou and Bornscheuer, 2003), petroleum ether (Lara and Park, 2004; Du et al. 2007), or t-butanol (Li et al. 2006; Wang et al. 2006; Du et al. 2007; Royon et al. 2007), are used instead of aqueous reaction medium. Hydrophobic organic solvents like n-hexane and petroleum ether increase the enzyme activity and the hydrophilic organic solvents like t-butanol yield the highest. Furthermore, the later diminishes the problem of undissolved alcohol droplets (Du et al. 2008). Use of organic solvents is further embellished by features like reduced capacity of the reaction chamber, less environmental hazards and cost (Nielsen et al. 2008).

Rayon et al. (2007) carried out enzymatic production of biodiesel by methanolysis of virgin cottonseed oil using immobilized *Candida 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.

Explanation of utilization of different lipases for biodiesel production is available from many reviews. A brief overview about different oil sources transesterified using different lipases under standardized conditions is shown in Table 2.

Despite various advantages, industrialization of biodiesel production has yet 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 (Rottig et al. 2010).

2.4 Whole cell catalysis for biodiesel production

Use of whole cell biocatalyst instead of purified lipase cuts the cost of isolation, purification and immobilization of pure lipase if used. Different bacteria were used for lipase production, and number of studies on transesterification was carried out by using immobilized lipase of bacteria (Table 2). Devanesan et al. (2007) carried out transesterification of jatropha oil by using immobilized *Pseudomonas fluorescens* MTCC 103, The maximum yield of 72% by using 4 g of beads at 40 ºC, pH 7 for reaction time of 48 h, and 1:4 molar ratio of oil to alcohol was used during this study. Further no reports were seen in literature till date on the utilization of whole bacterial cells either in immobilized or suspension form (Rottig et al. 2010).

Table 2.2. Transesterification of different oils by using lipase as catalyst obtained from different sources

Oil or Fat source	Catalyst used	Acyl acceptor	Yield	Reference
Jatropha oil	Novozym-435	Ethyl acetate	91.3%	Modi et al. 2007
Karanj oil			90%	
Sunflower oil			92.7%	
Soybean oil	*Pseudomonas fluorescens* recombinant LipB68	Methanol	92%	Luo et al. 2006
Crude Jatropha oil	Novozym-435	Propane-2-ol	92.8%	Modi et al. 2006
Karanj oil			91.7%	
Sunflower oil			93.4%	
Cottonseed oil	*Candida Antarctica*	Methanol	97%	Royon et al. 2007
Waste activated bleaching earth	*Candida cylindracea*	Methanol	100%	Kojima et al. 2004
Soybean oil	*Rhizopus oryzae*	Methanol	80-90%	Kaieda et al. 1999
Soybean oil	Immobilized *Pseudomonas cepacia* lipase (lipase PS)	methanol	67 mol%	Noureddini et al. 2005
		Ethanol	65mol%	
Renewable oil	Novozym-435	Methyl acetate	92%	Xu et al. 2003
Soybean oil	Lipozyme IM-77	Methanol	92.2%	Shieh et al. 2003
Palm kernel oil	Immobilized *Pseudomonas cepacia* lipase (lipase PS 30)	Ethanol	72%	Abigor et al. 2000
		t-butanol	62%	
		1-butanol	42%	
		n-propanol	42%	
		Iso-propanol	24%	
		methanol	15%	
Coconut oil		1-butanol	40%	
		Iso-butanol	40%	
		1-propanol	16%	
		Ethanol	35%	
		methanol	Traces	
Cottonseed oil	Novozym-435	Methanol	97%	Royon et al. 2007

Table 2.2. ***Continued***

Soybean oil	Novozym-435	Methanol	97%	Samukawa et al. 2000
Soybean oil	*Candida Antarctica* lipase	Methanol	93.8%	Watanabe et al. 2002
Cottonseed oil	Novozym-435	Methanol	91.5%	Kose et al. 2002
Palm oil	*Rhizopus oryzae* lipase	Methanol	55%	Pizarro and Park, 2003
Rapeseed oil	*Candida rugosa*	2-ethyl-1-hehanol	97%	Linko et al. 1998
Mowrah, Mango, Kernel,Sal	Lipozyme IM-20	C_4-$C_{18:1}$ alcohols	86.8-99.2%	De et al. 1999
Sunflower oil	Lypozyme	Ethanol	83%	Selmi and Thomas, 1998
Fish oil	Candida antarctica lipase	Ethanol	100%	Breivik et al.1997
Recycled restaurant grease	Lipase PS-30 + Lipase SP-435	Ethanol	85.4%	Wu et al.1999
Tallow, Soybean, Rapeseed oil	Lipozyme IM-60	Primary alcohols	94.8-98.5%	Nelson et al. 1996
	Lipase SP-435	Secondary alcohols	61.2-83.8%	
	Lipozyme IM-60	Methanol	19.4%	
	Lipozyme IM-60	Ethanol	65.5%	
Sunflower oil	*Pseudomonas fluorescens* lipase	Methanol	3%	Mittelbach, 1990
		Methanol	79%	
		Ethanol	82%	
Soybean oil	Lipase AK	Methanol	90%	Kaieda et al. 2001
	Lipase PS		80%	
	Candida rugosa		90%	
Sunflower oil	Lipase AK	Methanol	95%	Soumanou and Baornscheuer, 2003
	Lipozyme RM IM		80%	
	Lipozyme TL IM		60%	
Sunflower oil	Novozym-435	Methanol	93.2%	Deng et al. 2005
	Lipozyme RM IM	Ethanol	79.1%	
	Lipozyme TL IM	1-Propanol	89.8%	
	Lipase LA 201	2-Propanol	72.8%	
	Lipase PS-C	1-Butanol	88.4%	
	Lipase AK	Iso-butanol	45.3%	
Mauha oil	Lipase PS	Ethanol	96%	Kumari et al. 2007
			92%	
			99%	
Babasu oil	Porcine pancreatic lipase	Butanol	95%	Paula et al. 2007
Jatropha oil	*Pseudomonas cepacia*	Ethanol	98%	Shah and Gupta, 2007
Tallow	*Candida antarctica*	Methanol	74%	Lee et al. 2002
Rapeseed oil	*Candida* sp. 99-125	Methanol	83%	Deng et al. 2003; Nie et al. 2006; Tan et al. 2006
Salad oil			95%	
Waste oil			92%	

Table 2.2. ***Continued***

Vegetable oil unspecified			96%	
Restaurant grease	*Pseudomonas cepacia* lipase + *Candida antarctica* lipase	Ethanol	96%	Wu et al. 1999
Soybean oil	*Rhizopus oryzae* lipase mixed with *Candida rugosa*	Methanol	99%	Lee et al. 2006
Rapeseed and waste oil	Lipozyme TL IM mixed with Novozym-435	Methanol	95%	Li et al. 2006

Among the established whole-cell biocatalyst systems, filamentous fungi have proven to be the most robust biosystems for industrial applications as compared to prokaryotes it is easy to cultivate eukaryotes like fungi. Furthermore, separation of fungal biomass from reaction mixture and downstream processing for biodiesel industry is easier with fungal system. The use of *Rhizopus oryzae*, *R. chinensis*, recombinant *Saccaromyces cerevisiae* and most recently *Aspergillus niger* as whole-cell biocatalysts, have been studied and reviewed by different research groups (Rottig et al. 2010).

Lipase production in filamentous fungi is induced by different substrate related compounds such as triglycerides as fatty acids (Long et al. 1996). The effect of different oils (refined soybean, refined olive, refined cottonseed, crude rapeseed, crude soybean oils) as carbon source on the growth as well as whole cell catalysed transesterification reaction catalyzed by *Rhizopus oryzae* IFO 4697 was studied by Zeng et al. (2006).

Two types of lipases produced by *R. oryzae*, having different molecular masses of 34 and 31 kDa have been identified of which the former (ROL 34) is cell wall bound and the latter (ROL 31) is cell membrane bound (Hama et al. 2006). Lipases of 43kDa localized in the periplasmic space (releasable to the medium) and 40kDa tightly bound to the membrane (soluble in detergent treatment) were observed to be produced by the fungus *Oospora lactis* (Davranov et al. 1983). In *R. chinensis*, as reported by Nakashima et al. (1990), the cell aggregation followed by immobilization within BSPs is a trigger for the increase in intracellular lipase production. It has been reported by many researches that lipases of filamentous fungi mainly tend to localize in the cell wall (Toskueva, 1988; Hoshino et al. 1991; El Abbadi et al. 1995). Lipase isoforms are produced inside the cells when filamentous fungi is immobilized within biomass support particles as reported by Adamczak and Bednarski (2004). Majority of

the observations on whole cell catalyst transesterification using fungi have been focused on immobilizing whole cells to catalyze the reaction (Rottig et al. 2010).

Different oils contained in the cultivation medium had varied effects on the whole cell catalysis. In the presence of certain oils such as refined soybean oil, refined olive oil, refined cottonseed oil, crude rapeseed oil and crude soybean oil, whole cells expressed better and higher catalytic activity leading to transesterification (Zeng et al. 2006). The maximum yield for methyl ester (86%) could be obtained under optimized conditions. However, the difference between the cells cultivated with refined vegetable oils and those from crude vegetable oils as carbon sources is the expression of higher catalytic activity of the former which is possibly attributed to the negative effect of certain minor components like phospholipids presents in the latter (Du et al. 2004). To produce fatty acid methyl esters (FAME) from transesterification of palm oil, Xiao et al. (2009) isolated *Aspergillus niger* from atmospherically exposed bread and *Jatropha curcas* seed and utilized the same as a whole cell biocatalyst for palm oil methanolysis. 87% FAME yield was observed after 72 h from 30 immobilized BSPs under standard conditions of 8% water content and temperature of 40 °C. Olive oil was found to be the best carbon source as well as lipase inducer amongst different oils (olive, palm and soybean oils). Hama et al. (2007) found that emulsification of the reaction mixture containing soybean oil and water improved the methanolysis reaction rate when *R. oryzae* was immobilized within cuboidal polyurethane foam biomass support particles (BSPs) for transesterification of soybean oil. Immobilization was carried out in 20 l air lift bioreactor, and 30 g/l olive oil was used as lipase inducer. *R. oryzae* lipase, without any organic solvent, effectively catalysed the methanolysis in a water containing system (Hama et al. 2007).

Methanolysis of used cooking oil (UCO) with stepwise addition of methanol was carried out by the immobilized cell of *R. oryzae* (PTCC5174) in biomass support particles (BSPs) (Pazouki et al. 2010). Biodiesel conversion of this pre-treated UCO was brought out using methanol to fatty acid molar ratio of 3:1 at 35°C. Finally, BSPs of the immobilized cells of *R. oryzae* were added for 72 h. The filtered UCO yielded only 46% conversion of free fatty acid to methyl ester.

The two factors that influence the stability of lipase activity during whole cell catalysed methanolysis as reported by Hama et al. (2007) are the physical damage to immobilized cells by the circulation flow of the reaction mixture and the inactivation

of lipase by methanol. The immobilized lipase located on the exterior surface of the particles gets severely damaged due to high degree of shear stress (Arcos et al. 2000). The immobilized fungal cells located near the surface of the BSPs, get exfoliated by fluid shear stress at high flow rate thereby leading to loss of lipase activity, however, it has also been assumed that inefficient mixing of the reaction mixture caused a hydrophilic layer to cover the BSPs leading to remarkable inactivation of the lipase by high methanol concentration in the hydrophilic layer.

Ethyl ester to the extent of 79% and 94% was produced through transesterification of rapeseed oil with ethanol from the two types of whole cell biocatalysis named wild type *R. oryzae* producing tri-acyl glycerol lipase (w-ROL) and recombinant *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase (r-FHL) respectively (Koda et al. 2010). *F. heterosporum* lipase producing *A. oryzae* attained better final methyl ester production and higher lipase stability as compared to *R. oryzae* (Hama et al. 2008). Sun et al. (2010) used different types of vegetable oils like palm, olive, soybean and rapeseed oils as lipase inducer during cultivation *R. oryzae* IFO 4697. The authors reported the influence of the type of oil on lipase production on the performance of whole cell catalysts. In addition, it was observed that the content of unsaturated fatty acid in the oil would directly influence the yield of intracellular lipase that is with higher unsaturated fatty acid results in higher intracellular lipase production.

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 and Lin, 2010). It could further be exploited for the transesterification of soybean oil with methanol. The authors predicted the yield value 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 cell immobilized within BSPs were used with tert-butanol as solvent and 72% yield of methyl ester from soybean oil under the optimal conditions was reported (Li et al. 2007). 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 (Li et al. 2006; 2007).

A heterogeneous lipase encoding gene was transformed by Hama et al. (2008) from *Fusarium heterosporum* to *Aspergillus oryzae* and was immobilized within porous BSPs which enabled the convenient use of *F. heterosporum* lipase producing *A. oryzae* as a whole cell biocatalyst for the transesterification reaction. Water addition of upto 5% to reaction mixture, not only yielded 94% methyl ester but also proved effective in both preventing methanol from inactivating the lipase and facilitating acyl migration in partial glycerides.

Successful conversion of about 99% of triglycerides into fatty acid methyl ester (FAME) and free fatty acid (FFA) could be achieved using *R. oryzae* (ATCC 10260) and following separate hydrolysis and methanolysis reaction for biodiesel production by Jin et al. (2008). The authors also indicated that use of ethanol in place of methanol did not prove significantly successful in improving the yield of biodiesel. However, some studies as conducted by Mittelbach (1990); Nelson et al. (1996); Abigor et al. (2000) and Hsu et al. (2001) observed better yield of transesterification and alcoholysis by using ethanol in place of methanol. The reason is attributed to better tolerance of lipases towards ethanol or effective action of lipases on longer chain fatty acids (Fukuda et al. 2001). In addition, ethanol is less hazardous and can be produced from renewable resources (Shimada et al. 1997; 1998).

For the process of methanolysis of soybean oil to produce fatty acid methyl ester as a whole cell biocatalyst, four different types of whole cell biocatalysts namingly wild type *R. oryzae* producing triacylglycerol lipase (w-ROL) expressing *F.heterosporum* lipase (r-FHL), *Candida antarctica* lipase B (r-CALB), and mono- and diacylglycerol lipase from *A. oryzae* (r-mdlB) were used by Arai et al. (2010). Amongst all the catalysts studied, *R. oryzae* yielded high FAME in ionic liquid biphasic system following a 24 h reaction.

Methyl ester production using tert-butanol and BSP immobilized *R. oryzae* IFO 4697 resulted yield of 84% ME and reaction rate was significantly high when refined and crude rapeseed oils were used as feedstock as compared to acidified rapeseed oil (Li et al. 2007).

Salum et al. (2010) reported transesterification of soybean oil with ethanol, without co-solvent using *Burkholderia cepacia* as whole cell biocatalyst in solid-state fermentation by passing the reaction mixture through fixed bed column. The best yield

conversion of 95% was observed after 46 h at 50 °C and alcohol:oil molar ratio of 3:1 with step-wise alcohol addition and 1% (m/m) water in the reaction medium.

Rhizopus oryzae IFO4697 with a 1,3-positional specificity lipase immobilized within biomass support particles (BSPs) was also used for transesterification of soybean oil with step wise addition of methanol in the presence of 15% water which yielded 90% methyl ester in the reaction mixture as reported by Ban et al. (2001). Also olive oil or oleic acid was observed to enhance the activity of immobilized cells. Jin et al. (2009) used *R. oryzae* (ATCC10260), without immobilization, in aqueous system and reported 75% yield from virgin canola oil, 80% from waste vegetable oil and 55% from brown grease at 72 h transesterification without using excess of methanol. Xiao and Obbard (2010) reported the optimization of transesterification of waste cooking oil comprising fats, oil and grease and analysis of reaction variables and influencing FAME yield using immobilized *Aspergillus niger*. Response surface methodology (RSM) with a five level three factor central composite rotatable design was used by the group in these studies. Under optimized conditions, the validation experiments showed that the predicted value of maximum FAME yield that is 91.3% fell very close to the experimental value of 91.8%.

R. chinensis CCTCC M201021 showed high catalytic ability in the transesterification of soybean oil which yielded 86% methyl ester in solvent free system when compared to the catalytic activity of five commercial lipases by Qin et al. (2008). The gene encoding lipase from *R. oryzae* IFO4697 in *Saccharomyces cerevisiae* MT8–1 was cloned and further used for whole cell catalysed biodiesel production by Matsumoto et al. (2001). The reaction produced 71 wt% of methyl ester after a 165 h reaction at 37 °C when methanol was added stepwise.

Literature revealed that most of the work was carried out by using homogeneous and heterogeneous catalyst for transesterification reaction. However, the review of the literature also showed that very limited studies have been carried out on the whole cell catalysed transesterification; with most of the work was carried out restricted to *Rhizopus sp*. *Aspergillus niger* and *Burkholderia cepacia*. Keeping this in view, the present doctoral work was designed to isolate different culture strains showing maximum oil tolerance and transesterification reaction for biodiesel production.

2.5 Bio-oils as feedstock for biodiesel production

A variety of feedstock with different catalysts for different oils were used as raw material for biodiesel production as in Table 3.

Different reviews from sources spread worldwide reveal possibilities of biodiesel production from vegetable, animals, waste oil and other sources. Vegetable oil source include soybean oil, canola oil, sunflower oil, corn oil, cottonseed oil, mustard oil and palm oil. Expensive raw material including rapeseed (canola) oil and palm oil were among the most commonly used as feedstock for biodiesel production in Europe (Knothe, 2002) however, virgin soybean oil is used as feedstock in US. Animal source comprise of beef tallow or pork lard, restaurant waste oils such as frying oils/used cooking oils, trap grease (from restaurant grease traps), float grease (from wastewater treatment plants), and edible oil technology by products, such as acid oil and soap stock (Hui, 1996; Schuchardt et al. 1996; Gunstone and Hamilton, 2001) (Table 3).

Table 2.3. Biodiesel production from different feedstock

Feedstock	Catalyst for transesterification	Yield	Reference
Pongamia pinnata	H_2SO_4/KOH	97%	Naik et al. 2008; Raheman and Phadatare, 2004
Moringa oleifera	H_2SO_4/$NaOCH_3$	Not Reported	Rashid and Anwar, 2008a
Jatropha curcas	H_2SO_4/KOH	99%	Kumartiwari et al. 2007; Shah et al. 2004; Pramanik, 2003
Madhuca indica	*Pseudomonas cepacia*	96%	Kumari et al. 2007; Puhan et al. 2005
Nicotiana tabacum	H_2SO_4/KOH	91%	Veljkovic et al. 2006
Calophyllum inophyllum	H_2SO_4/KOH	85%	Sahoo et al. 2007
Zanthoxylum bungeanum	H_2SO_4	98%	Zhang and Jiang, 2008
Hevea brasiliensis	H_2SO_4/NaOH	Not Reported	Ramadhas et al. 2005
Heterotrophic microalgal	H_2SO_4	Not Reported	Miao and Wu, 2006; Scragg et al. 2003
Acid oil	H_2SO_4	95%	Haas et al. 2005
Fat from meat and bone meal	H_2SO_4/KOH	45.7%	Nebel and Mittelbach, 2006
Brown grease	Diaryl ammonium/ $NaOCH_3$	98%	Ngo et al. 2008
Waste cooking oil	H_2SO_4/NaOH	90%	Meng et al. 2008
Waste fryer grease	H_2SO_4/KOH	90%	Issariyakul et al. 2007
Tung oil	Amberlyst-15/KOH	90.2%	Park et al. 2008a, b
Tall oil	HCL	Not Reported	Demirbas, 2008
Sorghum bug oil	H_2SO_4	77.4/97.6%	Mariod et al. 2006
Pork Lard	KOH	97.8	Jeong et al. 2009
Rapeseed	KOH	97-96	Rashid and Anwar, 2008
Sunflower	NaOH	97.1	Rashid et al. 2008
Safflower	$NaOCH_3$	98	Rashid and Anwar, 2008
Jojoba	KOH	83.5	Bouaid et al. 2007

Table 2.3. ***Continued***

Rice bran	NaOH	90.2	Sinha et al. 2008
Waste cooking oil	NaOH	89.9	Meng et al. 2008
Jatropha curcus	KOH	99	Kumartiwari et al. 2007
Madhuka indica	KOH	98	Ghadge and Raheman, 2006
Pongamia pinnata	KOH	97-97	Meher et al. 2006
Brassica carinata	KOH	97	Vicente et al. 2005
Used frying oil	NaOH	88.8	Leung and Guo, 2006
Canola oil	NaOH	93.5	Leung and Guo, 2006
Cottonseed oil	KOH	98	Joshi et al. 2008
Raphanus sativus	NaOH	99.1	Domingos et al. 2008
Melia azedaracha	NaOH	63.8	Stavarache et al. 2008
Balanites aegyptiaca	KOH	90	Chapagain et al. 2009
Terminalia catappa	$NaOCH_3$	93	Dos Santos et al. 2008
Asclepias syiaca	$NaOCH_3$	99+	Holser and Harry-O'Kuru, 2006
Cynara cardunculus	NaOH	94	Encinar, 2002
Camelina sativa	KOH	98	Frohlich and Rice, 2005
Carthamus tinctorius	$NaOCH_3$	98	Rashid and Anwar, 2008
Sesamum indicum	NaOH	74	Saydut et al. 2008
Sclerocarya birrea	H_2SO_4	77	Schinas et al. 2009
Curcubita pepo	NaOH	97.5	Maruid et al. 2006
Melon bug	H_2SO_4	79	Schinas et al. 2009
Soybean soapstock	H_2SO_4	99+	Haas, 2005
Municipal sludge	H_2SO_4	14.5	Mondala et al. 2009
Microalgae	H_2SO_4	60%	Miao and Wu, 2006
Rice bran	H_2SO_4	96%	Zullaikah et al. 2005
Karanj oil	KOH, Solid acid catalysed Sulfuric acid Hb,Zeolite, Montmorillonite K-10 and ZnO were also used	92	Sarma et al. 2005
Peanut oil	NaOH	90	Feuge and Gros, 1949
Canola oil	KOH	87%	Dmytryshyn et al. 2004
Sunflower frying oil	KOH	90	Tomasevic et al. 2003
Andiroba (*Carapa guianesis*)	H_2SO_4, Sn(3-hyroxy-2-methyl-4-pyrone)$_2$(H_2O)$_2$, Pb(3-hyroxy-2-methyl-4-pyrone)$_2$(H_2O)$_2$, Zn(3-hyroxy-2-methyl-4-pyrone)$_2$(H_2O)$_2$	3.8, 23.3, 5.2, 11.2	Abreu et al. 2004
Babassu, (Orbignia sp.)	H_2SO_4, Sn(3-hyroxy-2-methyl-4-pyrone)$_2$(H_2O)$_2$, Pb(3-hyroxy-2-methyl-4-pyrone)$_2$(H_2O)$_2$, Zn(3-hyroxy-2-methyl-4-pyrone)$_2$(H_2O)$_2$	12.5, 35.6, 17.0, 26.2	Abreu et al. 2004; Srivastava and Prasad, 2000; Fukuda et al. 2001
Cumaru (*Dipteryx odorata*)	H_2SO_4, Sn(3-hyroxy-2-methyl-4-pyrone)$_2$(H_2O)$_2$, Pb(3-hyroxy-2-methyl-4-pyrone)$_2$(H_2O)$_2$, Zn(3-hyroxy-2-methyl-4-pyrone)$_2$(H_2O)$_2$	0.6, 10.3, 1.4, 7.2	Abreu et al. 2004

Table 2.3. ***Continued***

Camelina (*Camelina sativa*)	KOH	97.7	Frohlich and Rice, 2005
Refined Rapeseed oil	KOH	97.4	Frohlich and Rice, 2005
Soapstock	Lipase	Not reported	Haas, 2005
Coconut oil	Not reported	Not reported	Tan et al. 2004
Cynara cardunculus	NaOH	93.2	Encinar et al. 2002
	KOH	90.4	
L. fendleri	Not reported	Not reported	Goodrum and Geller, 2005

2.6 Used oils as feedstock materials

Low cost feedstock including used frying oils, animal fats, soapstocks and greases prove efficient in producing economical and profitable biodiesel (Canakci and Gerpen, 2001; NBB, 2003; Zhang et al. 2003). Spent frying oil and fats, currently are collected from giant food processing and service facilities where they are rendered and used primarily in soap making or in animal food. The market value of soapstock- a by product of edible oil refining is about one fifth of crude vegetable oil (Haas, 2005) and therefore are attractive due to low costs. Also, animal fats and used frying oils with FFA level lower than 15% is called yellow grease and the one with FFA content more than 15% is called brown grease. Those mentioned above are attractive due to low cost. However, their transesterification is tougher than food grade high quality oil due to presence of FFA between 10-25% and large amount of water which is inconvertible into biodiesel with alkaline catalyst.

Biodiesel production from edible sources faced severe criticism by many quarters as they take 'fuel for food' concept as unethical. Therefore, using waste edible oil as alternative proved a better solution for its abundant (Gui et al. 2008). Advantages like reduction of prices to about half of that for virgin oils and little scope of contamination problems support utilization of waste frying oils dominately over use of virgin oil (Supple et al. 1999; Kulkarni and Dalai, 2006; Canakci, 2007; Veljkovic´et al. 2006). Moreover, utilization of these waste greases reduce the burden on the government for waste water disposal, maintaining public sewerages, treatment of oil waste water disposal, maintaining public sewers, and treating the oil wastewater (Encinar et al. 2005). Properties of pre-used frying oils are different from those of refined and crude oil. High temperature of typical cooking process accelerates the hydrolysis of triglycerides and production of water from food resulted in increasing the FFA content in the oil (Encinar et al. 2005).

Commercialization of biodiesel is majorly concerned with and affected by its cost. Fresh vegetable oils are quite costly for use as raw material for biodiesel production. The feedstock cost contributes more than 70-95% to the overall production cost of biodiesel (Krawczyk, 1996; Connemann and Fischer, 1998; Haas et al. 2004). However easily available and relatively cheap raw material like waste cooking oil, non-edible feedstock and use of by-products in biodiesel may prove significantly advantageous in cutting the production cost of biodiesel (Nisworo, 2005; Zhang et al. 2003). Zhang et al. (2003) have reported that the cost of feedstock oil is one of the most significant factors which affect the economic viability of biodiesel manufacturing. Biodiesel from used frying oil also represents a lower biodiesel break-even price (Zhang et al. 2003). Extensive use of biodiesel is mainly concerned with the economics of biodiesel production as demonstrated by Dorado et al. (2002). Nevertheless, the final manufacturing cost of the process could be reduced upto 6.5% (depending up on the feedstock used) by glycerol which is again a valuable by-product.

Various physical and chemical changes occur in the food constituents when food is heated at different temperatures while cooking, boiling, frying etc. In the modern era, frying is considered one of the most popular food preparation methods as it adds delicacy to the food. Undoubtedly, oils (lipids) are the quintessential for frying during which they are heated in air in the presence of light for very long periods within temperature range of 160-200 °C. In restaurant and household frying, the same oil is used and reused, though; fat exchange takes a long time (Cvengros and Cvengrosova, 2004). Depending upon the composition of oil, various physical and chemical changes are brought in the oils for which, to a certain extent, the frying conditions are also effective. Extended heating (abuse) of vegetable oils leads to their oxidation (degradation) thereby resulting in formation of oxides such as hydroperoxides and epoxides which have adverse consequences pertaining to health exhibited in the form of growth retardation or increased size of liver. Thus are disposed to public sewers causing a number of problems. But proper recycling of WCO can prevent its inappropriate disposal problems. Recycled WCO is mainly used in producing animal feeds and a small proportion of it also helps in manufacturing soaps and biodegradable lubricants, however certain health risks including undesirable level of contaminants, particularly PAHs (polycyclic aromatic hydrocarbons), PCBs (polychlorinated

biphenyls), dioxins and dioxin related substances have been traced in using these recycled cooking oil in animal feeding (Riera et al. 2000). Increase in viscosity and specific heat, change the surface tension and colour, increase in tendency of fat to foam are some commonly observed physical changes (Cvengros and Cvengrosova, 2004). During frying the three major types of reaction that occur are thermolytic, oxidation and hydrolytic (Nawar, 1984; Mittelbach and Enzelsberger, 1999).

If the triglycerides, containing saturated fatty acids, are subjected to very high temperature (180 °C) in the absence of oxygen, thermolytic reaction occurs, thereby producing series of normal alkane, alkenes, lower fatty acids, symmetric ketones, propyl esters, CO and CO_2. On the other hand unsaturated fatty acids either can form dimeric compounds viz. dehydrodimers, saturated dimers, and polycyclic compounds or can undergo Diel- Alder reaction producing dimers and trimmers. Such reaction happens between acyl groups within same molecule, in case of glycerides (Nawar, 1984).

Hydroperoxides are formed as a primary product in free radical mechanism reaction between unsaturated fatty acid and molecular oxygen. These hydroperoxides may further form many compounds viz. isomeric hydroperoxides that contain conjugate diene groups, chemical with remarkable variation in molecular weight, flavour threshold and biological importance. Scission of O-O bond of hydroperoxides leads to formation of alkoxy radical which further may gain or lose H atom(s) to form hydro or keto derivatives respectively. Decomposition of these alkoxy radicals on hand can form different chemicals such as aldehydes, hydrocarbons, semialdehydes and acids and on the other hand, in presence of excess oxygen, alkoxy and peroxy radicals can be transformed into dimeric and oligomeric compounds (Nawar, 1984).

Hydrolytic action on triglycerides by steam produced during food preparation forms FFA, glycerol, mono and diglycerides (Mittelbach and Enzelsberger, 1999). This change in composition of oil can be quantified by measuring the monoglycerides or diglycerides content. The cumulative effect of these thermolytic, oxidative and hydrolytic chemical reactions is the formation of undesirable by-products. Repetitive heating also leads to enhanced polar content of the oil badly affecting the quality of the oil (Guesta et al. 1993). Bastida and Sanchez (2001) have reported normal polar content of fresh unused oil between 0.4 and 6.4 mg/100 g (Bastida and Sanchez, 2001). 20 fryings with sunflower oil increases its polar content 640% and with olive

oil by 480% as per study conducted on sunflower oil, olive oil and a mixture of the two oils (Bastida et al. 2001). In study on sunflower, Bastida et al. (2001) also reported that thermo-oxidative and hydrolytic changes in the frying oil could be minimized if fresh oil is frequently added throughout uptil 20 repeated fryings. European countries have set maximum 25% polar content of edible oils to be normal beyond which the oil needs to be discarded.

The amount and type of undesirable products, especially the type of polar compound, formed during frying adversely affect the biodiesel properties or the transesterification reaction. Different methods have been recommended for efficient and economical production of alternative renewable liquid fuels from waste cooking oil using variety of catalysts.

2.7 Catalysis of transesterification of used oils

A two-step alkaline catalysed transesterification reaction has been proven economic for biodiesel production from used vegetable oil by Dorado et al. (1999) in Brazil, Spain, and Germany. However, Canakcı and Van Gerpen (1999) used acid – catalysed pre-treatment to esterify the FFA before transesterifying the triglycerides with an alkaline catalyst to complete the reaction of reducing the FFA content of restaurant wastes and animal fats. Hsu et al. (2001) used a phyllosilicate sol-gel immobilized lipase to study the optimization of alkyl ester production from grease, resulting in 60% -97% conversion when 10% (w/w) biocatalyst was used with respect to grease. Another notable observation is 90% conversion of waste oil to biodiesel by Watanabe et al. (2001) when transesterification of waste edible oil to biodiesel was studied in a fixed bed bioreactor using three step and one step methanolysis. Lim et al. (2009) tried to extract this waste oil using different solvents viz methanol, ethanol, petroleum ether and hexane and utilized the oil, further in transesterification reaction taking CaO catalysts as heterogeneous base as homogenous catalysts like NaOH and KOH result in yield loss due to saponification due to relatively high FFA content in spent bleaching clay oil. Refaat et al. (2008) too reported successful production of biodiesel from waste/ recycled oil using KOH as catalyst in order to reduce the cost of biodiesel and cost of treating water ways affected by effluents and pollutants present in sewage systems of Egypt where millions L. of oil was used for frying food and discarded each year (Refaat et al. 2008). An essential point regarding biodiesel

production encompasses the slow reaction rate that is low dispersion rate of alcohol in feedstock for which more amount of alcohol is to be used along with vigorous stirring of the mixture (Leung and Guo, 2006; Hanna, 1998; Ma et al. 1999). Though industrially biodiesel production from high FFA feedstocks being carried out using alkali catalysed reaction is the most popular, however, some works in the literature state that the traditional alkali catalysed reaction using waste feedstocks makes no improvement to biodiesel unit price because of the pre-treatment unit addition to reduce FFAs in the feedstock. One of the alternative methods for biodiesel production from low cost feedstocks, other than alkali catalysed process is supercritical transesterification process. Advantages include (1) absence of catalyst; (2) simultaneous esterification and transesterification; and (3) non influence of water or FFA in feedstock on quality of the product. However, the disadvantages that add to increasing cost to use of supercritical process cannot be ignored. These include (a) high alcohol to feed stock ratio as high as 40:1; (2) high pressure and temperature requirement; and occurrence of un-desirable side reactions (Kulkarni and Dalai, 2006; Saka and Kusdiana, 2001).

Acid catalysis is another alternative in the processing of feedstock with high FFA content as it doesn't require any pre-treatment reaction, which further makes it less complex than the traditional alkali catalysed process using these feedstocks. However, high alcohol addition and larger reaction are required in this process in addition to the use of stainless steel reactors because of its corrosive nature, thereby increasing the equipment cost. These are reasons because of which no biodiesel plants at the commercial level have been reported, hitherto, which use acid catalysis process (Zhang et al.2003)

Cao et al. (2008) has tried investigation transesterification of waste cooking oil with high acid value and high water content by using heteropolyacid $H_3PW_{12}O_{40}.6H_2O$ (PW12) as catalyst which proved promising, exhibiting the highest ester yield of 87% and the catalyst also yielded 97% for esterification of long chain palmatic acid. The important point of comparison between food grade vegetable oil and waste frying oil is about the problem of processing of the latter for containing large FFA content that could not be converted into biodiesel using and alkaline catalyst which cause saponification (Canakci, 2007; Kulkarni and Dalai, 2006; Veljkovic et al. 2006).

There are limited reports on use of biocatalysts, especially the whole catalysis process for transesterification of used cooking oils. The cells of *Rhizopus oryzae* (PTCC5174) in BSPs were immobilized and utilized for methanolysis of used cooking oil (UCO) resulting in 88% conversion from heated and filtered UCO, while 46% conversion from only filtered UCO (Pazouki et al. 2010).

2.8 Non-edible oils as feed stock materials

Easily extracted non-edible oils including *Mellia azadirachta* (Neem), *Bussia Latifolia* (Mahua), *Pongamai Pinnata* (Karanja), *Orbignaya maritiana* (Babassu) and *Jatropha curcas* (Ratanjyot), are relatively cheap to use. These are easily extracted in many parts of the world including India. Jatropha oil can be used as fuel in diesel engines directly and by blending with methanol, which is extracted from seeds of *J. curcas*, otherwise toxic to humans and animals consumption (Gubitz et al. 1999). Satisfactory engine performance was obtained from engine tests with Jatropha oil in Thailand (Takeda, 1982) after it was used for the first time during the World War II as a diesel fuel substitute. Eisa (1997) have reported about the studies carried out on the feasibility of the production of fatty acid ethyl esters form jatropha oil for African countries. As per the economic evaluation, biodiesel from jatropha is very profitable if its by-product can be sold as valuable product (Foidl and Eder, 1997). The two stage transesterification process improves the ester yield from jatropha with high FFA content (15%) in which the pretreatment of oil with acid constituted the first step which reduced the FFA level to less than 1%, followed by the second step transesterified the oil using alkali as catalyst resulting in 90% methyl ester yield (Tiwari et al. 2007; Berchmans and Hirata 2008).

Blend of jatropha and palm biodiesel has been studied by Sarin et al. (2007) for their physico-chemical properties and their optimum mixture achievements at low temperature with improved oxidation stability. Interestingly, jatropha seeds themselves contain lipase activity required to catalyse transesterification reaction (Staubmann et al. 1999). Biodiesel production from jatropha oil in solvent free system was worked out by Shah et al. (2004) using three different lipase (*Chromobacterium viscosum*, *Candida rugosa,* and Porcine pancreas), however appreciable yields (71%) were obtained from *Chromobacterium viscosum* in which the lipase was immobilized on celite-545 using free tuned enzyme preparation with process time of 8h at 40 °C.

Moreover, addition of water to free (1%, w/v) and immobilized (0.5%, w/v) enzyme preparation further enhanced the yields to 73 and 92%, respectively. Lipase producing whole cell of *R. oryzae* immobilized onto BSP also proved as promising biocatalysts for producing low cost biodiesel (Tamalampudi et al. 2007).

Canola oil was transesterified by Joshi et al. (2009) by using a 1 : 1 molar mixture of methanol and ethanol (M/E) with potassium hydroxide (KOH) catalyst which yielded 98% of alkyl ester at 25 °C at 2.5 min, when catalyst concentration of 1.1 wt-% and an M/E to canola oil molar ratio of 20 : 1. The yield further improved to 99% when catalyst concentration of 1.15 wt-% at 25 °C at 5 min. The mixture of cottonseed, soybean and castor oil was transesterified using NaOH with alcohol:oil:catalyst as 34:6:1 by Meneghetti et al. (2007). Ramadhas et al. (2005) established a two-step transesterification process to convert the high FFA containing rubber seed oil and it monoester. The first step being reduction of FFA content of the oil to < 2% by acid catalysed esterification process followed by the second step of conversion of the product of the first step to their mono-esters and glycerol by alkali catalysed transesterification (Ramadhas et al. 2005).

In near future, considering many available oils as potential raw materials for the use of other oils like cottonseed oil, jatropha oil (Wood, 2005; Sarin et al. 2007) algae oil (Chisti, 2007), and coconut oil for biodiesel production (Frohlich and Rice, 2005) is seeming crucial not only from economic evaluation point of view but also from environmental protection stand point (Tashtoush et al. 2004, Nelson and Schrock, 2006).

Sharma and Singh (2008) have reported use of Karanja oil in biodiesel production due to furanoflavones, chromenoflavones, flavones and furanodiketones which promote it as non-edible. They produced biodiesel form karanja oil using NaOH and KOH and reported a maximum of 89.5% yield achieved at 8:1 molar ratio for acid esterification and 9:1 molar ratio for alkaline esterification, 0.5 wt.% catalyst (NaOH/KOH) using mechanical stirrer. The two plant species soapnut (*Sapindus* mukorossi) and jatropha (*J. curcas, L.*) oils as the feedstock for biodiesel production have been worked out and compared by Chhetri et al. (2008) which reveals soapnut oil to have an average of 9.1% free FA, 84.43% triglycerides, 4.88% sterol and 1.59% others. Jatropha oil contains approximately 14% free FA, approximately 5% more than soapnut oil. Biodiesel produced from soapnut oil has been reported to contain

approximately 85% of unsaturated FA while jatropha oil biodiesel was found to have approximately 80% unsaturated FA. The dominating FA in both soapnut and jatropha biodiesel was found to be oleic acid. Both oils yielded nearly 97% FAME conversion. Jatropha being grown in marginal and waste lands had shown no possibility of land use competing with food production. Similarly the use of soapnut seeds for biodiesel production entitles waste–to–energy scheme. Prospects of FAME of some of 26 non-tradition plant seed oils including jatropha have been studied by Azam et al. (2005) to use as a potential biodiesel in India. The one among them which are found most suitable for use as biodiesel and meet the major specification of biodiesel for use in diesel engine include *Azadirachta indica*, *Calophyllum inophyllum*, *J. curcas* and *Pongamia pinnata.* Nevertheless over 75 oil bearing plants containing nearly 30% or more oil in their seed, fruit or nut have also been reported. To the list are nearly 300 different species of trees which produce oil bearing seeds (Subramanian et al. 2005). Hence, non-edible oil sources from different plants have significant potential for biodiesel production as an alternative to petrodiesel.

One of the most important characteristic of biodiesel that is its viscosity is determined by the amount and type of FFA in it. Soapnut oil may exhibit a slightly higher viscosity as compared to jatropha oil due to the presence of higher amount of long chain fatty acid. However, viscosity of jatropha oil biodiesel is similar to that of peanut oil, corn oil, palm oil and sunflower oils due to the presence of similar FA in all of these. *J. curcas* is a multipurpose species with many attributes and considerable potential and biodiesel form it has similar characteristics to that of petrodiesel, thereby, proving it to be a strong alternative for diesel replacement (Reddy and Ramesh, 2005). Sarma et al. (2005) have carried out a comprehensive analysis of the fuel properties of biodiesel (*Pongamia glabra)* Koroch seed oil. Anastopoulos et al. (2009) have carried out the transesterification reaction of four different vegetable oils (sunflower, rapeseed, olive oil and used frying oil) with ethanol, using sodium hydroxide as catalyst, resulting in 81.4% wt/wt ethyl esters in one stage transesterification.

Kywe and Oo (2009) carried out the transesterification of Jatropha oil using NaOH and KOH as catalyst which resulted in 92% and 90% of methyl and ethyl esters respectively. A process to obtain biodiesel form non-edible oils of jatropha and karanja was developed by Kalbande et al. (2008). Transesterification of crude

sunflower and jatropha oil by using immobilized lipase on chitosan was studied by Rattanaphra and Srinophakun (2010) under standardized condition 25% of methyl ester was obtained from sunflower oil, however, under similar conditions transesterification of jatropha oil was difficult (Rattanaphra and Srinophakun, 2010).

2.9 Effect of the type of alcohol on alkyl ester yield

Amongst variety of alcohols like ethanol, propanol, iso-propanol and butanol used in the preparation of biodiesel, methanol has been used most commonly across by various research groups (Tables 2 and 4).

Table 2.4. Different alcohols as acyl acceptor for biodiesel production

Oil Used	Catalyst Used	Acyl acceptor	Reference
Soybean	H_2SO_4, NaOBu, $NaOCH_3$	Methanol, butanol	Freedman et al. 1986
Peanut, soybean, safflower, cottonseed, sunflower (crude and refined)	NaOH, $NaOCH_3$	Methanol	Freedman et al. 1984,
Soybean, sunflower, high-oleic safflower	Not reported	Methanol, ethanol	Schwab et al.1987
Soybean, low palmitate soybean, canola, lard, tallow	Sodium alkoxide of alcohol used	Methanol, ethanol, Propanol, iso-propanol, 2-butanol, tert-butanol, Neo-pentanol	Lee et al.1995
Rapeseed	KOH	Ethanol	Peterson et al. 1996
Tallow, grease	Lipase from different sources(*Mucor miehei, Candida Antarctica, Geotrichum candidum, Pseudomonas cepacia, Rhizopus delemar*)	Methanol, ethanol, propanol, butanol, Iso butanol, iso-pentanol	Foglia et al. 1997
Yellow grease, tallow, rapeseed, soybean, olive	Lipase from different sources(*Mucor miehei, Candida Antarctica, Geotrichum candidum, Pseudomonas cepacia, Rhizopus delemar*)	Methanol, ethanol, propanol, butanol, iso-butanol	Nelson et al. 1996
Tallow, grease	*Mucor miehei* lipase, *Candida antarctica* lipae	Ethanol, iso-propanol	Wu et al. 1998
Rapeseed	KOH, H_2SO_4	Methanol, ethanol, propanol, butanol, iso-butanol	Nimcevic et al. 2000
Brown grease, yellow grease	KOH, NaOH, $NaOCH_3$	Methanol	Canakci and Van Gerpen, 2001
Canola and linseed	KOH and Sodium alkoxide	Methanol, ethanol, 2-propanol, butanol	Lang et al. 2001
Canola, soybean	KOH	Methanol, ethanol, butanol	Zhou et al. 2003
Soybean, yellow grease	Sodium iso-propoxide, potassium iso-propoxide	iso-propanol	Wang et al. 2005

Table 2.3. ***Continued***

Sclerocarya birrea oil (SCO), melon bug oil (MBO), sorghum bug oil	H_2SO_4	Methanol, ethanol	Mariod et al. 2006
Castor	H_2SO_4, HCl, $NaOCH_3$, $KOCH_3$	Methanol, ethanol	Meneghetti et al. 2006

The reactivity of different alcohols vary with the type of substrate and catalyst in general methoxide anion formed during transesterification being more reactive than ethoxide anion and thus favours and fastens the process of methanolysis than ethanolysis. Also, the increased length of alkoxide anion in ethoxide brings corresponding decrease in its nucleophilicity, thereby, reducing its reactivity (Sridharan and Mathai, 1974). Transesterification of canola oil with 1:1 mixture of ethanol to methanol (providing overall alcohol:oil of 6:1; 2.7:1; and 1.3:1) at 25°C resulting in 50% or higher methyl ester than ethyl counterpart, is a suitable example to explain the above mentioned fact (Kulkarni et al. 2007; Joshi et al. 2009). The overall result indicated preferential formation of methyl esters at both ambient and elevated temperature. Beside slow formation of ethyl esters, the overall rate of formation of esters is faster than with methanol alone due to better solubility to TAG in a mixture of methanol and ethanol which leads to reduction of mass transfer limitations (Kulkarni et al. 2007). Stavarache et al. (2008) have reported that complete ultrasonically assisted transesterification of *Melia azedarach* (syringa) oil with methanol and ethanol takes 40 min and 20 min respectively. Production of FAME is quite less complicated than base-catalyzed formation of fatty acid ethyl esters (FAEE). On the other hand, during methanolysis, quickly and easily separable emulsions form a lower glycerol-rich and an upper FAME rich phase (Korus et al. 1993; Zhou et al. 2003; Zhou and Boocock, 2006). Ethanolysis involves much more stable emulsions, the separation and purification of which is severly complicated (Zhou et al. 2003; Zhou and Boocock, 2006). Kulkarni et al. (2007) reported about requirement of mechanical agitation during the transesterification reaction to facilitate sufficient mass transfer between the phase, even though ethanol is slightly more miscible with TAG at ambient temperature than methanol (Kulkarni et al. 2007).

Butanolysis of vegetable oil or animal fat encompasses the classical reaction conditions of 6:1 molar ratio of butanol to oil, 0.5wt% catalyst (with respect to TAG), 600+ rpm, 114°C reaction temperature, and 1 h reaction time to produce fatty acid butyl esters and glycerol (Freedman et al. 1984). A variety of feedstock have been used for yielding butyl ester which are used and evaluated as potential biodiesel fuels (Freedman et al. 1986; Schwab et al. 1987; Ali and Hanna, 1994; Foglia et al. 1997; Nimcevic et al. 2000; Lang et al. 2001; Zhou and Boocock, 2006; Rodrigues et al. 2008). Transesterification reactions of butanol are through mono-phasic (Zhou and Boocock, 2006) since it is significantly less polar than methanol and ethanol, thus, completely miscible with vegetable oils and animal fats (Boocock et al. 1996). The rate and the extent of the reaction are quite dependent on mono-phasic nature of butanolysis reaction and also the same fact allows no mass transfer limitations since all the reactants and catalysts are in the same phase. Another report on butanolysis (30°C) found the reaction 50% complete in only 15 s of reaction time, and 60% and 63.5% complete after 90 and 150 s, respectively. Methanolysis (40°C) however was only 55% complete after 10 min (Freedman et al. 1986). Zhou and Boocock (2006a) in a recent study have reported about 15.4 wt.% TAG remaining after 3 min of butanolysis while 84.4 wt.% remained in the case of methanolysis (Zhou and Boocock, 2006a). For 40% conversion to alkyl esters, methanolysis proved about 12–16 times slower than butanolysis if lag time in the case of methanolysis is ignored and it is still much slower if it is not ignored (Freedman et al. 1986; Boocock et al. 1996).

As an alternative to methanol, ethanol is more preferrable because it is less expensive in some parts of the world and the biodiesel prepared from it is absolutely bio-based. In addition, butanol is also biologically obtained (Qureshi et al. 2008), thereby, another eco-friendly alcohol for production of bio-based biodiesel. Issariyakul et al. (2007) reported that during transesterification, ethanol is preferred when compared to methanol as it is renewable, more ecofriendly and is derived biologically. On the other hand rapeseed ethyl ester showed some desirable attributes over its methyl esters being significantly lower in smoke opacity, exhaust temperatures, and pour point (Issariyakul et al. 2006). Some studies have observed better yield of transesterification or esterification using ethanol as compared to methanol (Mittelbach, 1990; Nelson et al. 1996; Abigor et al. 2000; Hsu et al. 2001). Lipase are more tolerant to ethanol as compared to methanol (Fukuda et al. 2001), as

lipase act on long-chain fatty alcohols better than on short-chain ones (Shimada et al. 1997; Shimada et al. 1998). Ethanol resulted in more stable ester in the presence of *Rhyzopus oryzae* lipase as it shifts the FAA/ester equilibrium toward ester (Jin et al. 2008). Ethyl ester (95%) was produced from soybean oil without any solvent using *Burkholderia cepacia* as whole cell biocatalyst in solid-state fermentation by passing the reaction mixture through fixed bed column (Salum et al. 2010). Moreover, Issariyakul et al. (2007), Kulkarni et al. (2007) and Joshi et al. (2009) have reported about the mixtures of ethyl and methyl esters where both ethanol and methanol were used in transesterification reactions. Domingos et al. (2008) reported ethanolysis of crude *Raphanus sativus* oil in which the reaction conditions of 0.60 wt.% NaOH, 11.7:1 molar ratio of ethanol to oil, 38°C reaction temperature, and a 1-h reaction time, quite different from the reaction conditions of the same reaction as already reported by Freedman et al. (1984), resulting in ethyl esters yield reaching upto 99%. The classic vegetable oil or animal fat ethanolysis conditions incorporate 6:1 molar ratio of ethanol to oil, 0.5 wt.% catalyst (with respect to TAG), 600+ rpm, 75°C reaction temperature, and 1 h reaction time to produce fatty acid ethyl esters (FAEE) and glycerol (Freedman et al.1984). A number of feedstocks have yielded ethyl esters for use or evaluation as potential biodiesel fuel (Tables 2 and 4).

2.10 Importance of glycerol as a by-product of transesterification reaction

Transesterification of vegetable oils and animal fats produces glycerol (or glycerin, 1,2,3-propanetriol) in addition to FAAE. The demand to supply ratio for glycerol was relatively balanced before the improved interest of biodiesel production that occurred over the past decade as result to consistent interest in renewable fuels. But the surplus generation of glycerol from emergence of biodiesel industry has spawned countless efforts to find new applications products and markets using this versatile chemical. The industries that have immense use of glycerol includes production of polyuretahanes, polyesters, polyethers, lubricants, wrapping and packing materials, foods, drugs, cosmetics and tobacco product. Behr et al. (2008) have recently published a review, covering the recent development on the chemistry and utility of glycerol. The applications and products which displace present petroderived materials or feedstock have been emphasized to be of particular interest. A notable example of this fact is represented by the development of a synthesis route

to propylene glycerol (1,2-propanediol) from glycerol that displace the classic petrochemical route from propylene (Dasari et al. 2005; Suppes, 2006; Feng et al. 2008). The common antifreeze component ethylene glycol is replaced by this remarkable alternative.

2.11 Biodiesel properties and specification

Successful commercialization and market acceptance of biodiesel is possible with increasing interest and use of the fuel, however, assurance of fuel properties and quality has become the element of paramount interest (Table 5).

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 satisfy the requisites of these standards before inclusion in the respective field.

Therefore, it is imperative to ensure the quality of biodiesel, produced by biocatalysis and using renewable feedstocks, also satisfy the quality parameters as per the international standards, so as to make the product competitive to conventional diesel and chemically catalyzed biodiesel. As on date, this aspect has never been considered for further application of biodiesel at scale-up production or application levels.

Table 2.5. ASTM D6751 and European Committee for Standardization EN 14214 biodiesel fuel standards

Property	Test Method		Limits		Units ASTM/EN
	ASTM	EN14214	ASTM	EN14214	
Flash Point(Closed Cup)	ASTM D93	EN ISO 3679	93 min	120 min	°C
Water and Sediment	ASTMD2709	EN ISO 12937	0.050 max	500 max	% volume/ mg/kg
Kinematic Viscosity, 40°C	ASTM 445	EN ISO 3104, ISO 3105	1.9-6.0	3.5-5.0	mm^2/s
Sulphated ash	ASTM D874	ISO 3987	0.020 max	0.02 max	% mass/ %(mol/mol)

Table 2.5. continued…

Copper strip corrosion	ASTM D130	EN ISO 2160	No.3 max	1	Not mentioned/ Degree of corrosion
Cetane number	ASTM D613	EN ISO 5165	47 min	51min	Not reported
Cloud point	ASTM D2500	Not reported	Report	Not reported	°C
Carbon residue	ASTM D4350	EN ISO 10370	0.050 max	0.30 max	% mass/ %(mol/mol)
Acid Value	ASTM D664	EN 14104	0.50 max	0.50 max	% mass/ mg KOH/g
Free glycerine	ASTM D6584	EN 14105,EN 14106	0.020	0.020 max	% mass/ %(mol/mol)
Total glycerine	ASTM D6584	EN 14105	0.240	0.25 max	% mass/ %(mol/mol)
Oxidation Stability	EN 14112	EN 14112 (110°C)	3.0 min	6.0 min	H
Phosphorous content	ASTM D4951	EN 14107	0.001 max	10.0 max	% mass/ mg/kg
Sodium and Potassium, Calcium and Magnesium	EN 14538	EN 14108, EN 14109, EN14538	5 max	5.0 max	ppm/ mg/kg

2.12 Lacunae

The industrialization of the biodiesel depends on its cost which acts as a main hurdle and can only be overcome by the choice of catalyst, raw material used and the easy recovery of by–product that is glycerol and last but not the least is the product meeting standard specifications. In the literature examined till date, biocatalysis has been noted to gain importance with increasing focus on whole cell catalysis. However, there have been limited reports on (i) the use of non-edible oils like jatropha and karanja as raw materials; (ii) study of different fuel properties of alkyl esters produced through biocatalysis; and (iii) glycerol produced as a by-product of whole cell catalysed transesterification of vegetable oils.

Keeping in view the lacunae of literature, the present doctoral work was designed under the following objectives

1) **Screening and characterization of selected oil tolerant fungal strains for their potential to mediate transesterification**
2) **Optimization of culture and reaction condition to increase the extent of transesterification of used and non-edible vegetable oils**
3) **Studying recyclability and/or regeneration potential of fungal isolate for transesterification**

3.1 Isolation and characterization of fungal strains from contaminated butter

3.1.1 Isolation

Different fungal strains isolated from contaminated butter were incubated in PDA for 72 h at 25 ºC to get active culture. The isolates were obtained by using Warcup method (Aneja, 2001), where in 15-20 ml of medium (PDA-Hi Media), supplemented with Streptopenicillin and rose Bengal, was inoculated with contaminated clarified butter sample with the help of a sterilized loop. The sample was spread evenly and incubated at 25 ºC in inverted position for 15 days.

Purification of isolated fungal strains was carried out by subculturing strains that appeared in plate by preparing slants as well as plates. Sterile loop with spores was inserted in the slant and inoculated by drawing the loop over the hardened surface of the media and incubated at 25 ºC. Same process was repeated until pure colonies of the fungal strains were obtained. Pure culture was maintained in PDA and stored at 4 ºC.

3.1.2 Preparation of inoculum

Inoculum was prepared by using 10^5/ml spores in 500 ml PDB and incubating the culture flask from 120 h and at 28°C and 120 rpm. This freshly grown biomass was further used as inoculum.

3.1.3 Morphological and molecular characterization of isolates

Identification of the fungi isolated from contaminated clarified butter was facilitated by Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

Morphological characterization - Isolated fungal strains were inoculated in Sabouraud agar plate and incubated at 25 ºC in an inverted position for 5-7 days and colony appearance, morphology, hyphae colour, spore colour, underside was observed whereas microscopic characterization using slide culture technique and coverslip culture technique were used. Slide culture technique was used to observe morphological characteristic of molds without disturbing the arrangement of spores and conidiogenous cells. One-two pieces of sterilized filter paper were placed in the sterlized petriplate and moistened with water. Two applicator sticks were placed across the filter papers by using sterilized forceps. Clean sterilized microscope slide

was placed across the applicator. Simultaneously 100 ml Sabouraud Agar was prepared, 15-20 ml of media was poured in another sterilized petri-plates and allowed to solidify. A square agar block of 10mm was cut by using sterilized blade and placed on the slide in a sterilized petri-plate; this agar block was inoculated further with pure stock culture of fungus. A sterilized cover slip was placed over the inoculated agar block. After inoculation, petri-plate was covered with lid and incubated with right side up at 25 ºC.

For preparation of semi-permanent mounts, cover slip from agar plate was gently lifted. A drop of 95% alcohol was put on the fungus side of cover slip and drained off. A drop of lactophenol cotton blue was poured on a clean slide and cover slip was place over it with side containing isolate downwards and later examined microscopically. For preparation of permanent slides, 4-5 drops of histological stain fluid was poured on the mount and covered with another cover slip (22 x 22 mm) which make it permanent slide. The stained slide and cover slip was examined for the hyphae (septate or coenocytic) type of spores, type of fruiting body, arrangement of conidia/spores on the conidiogenous hyphae/sporangiophores, simultaneously the dimensions of various structures (i.e hyphae, spores, reproductive bodies) was determined by using calibrated ocular micrometer and the fungus was identified by consulting the monographs and books on fungal taxonomy.

Molecular Characterization **–** The molecular characterization of the strains were also facilitated by MTCC, IMTECH, Chandigarh, India. The protocol followed was as follows.

The fungal mycelium was ground with liquid nitrogen in a mortar and pestle. The tissue was transferred to SS-34 tube taking care that material did not thawed. Equal volume of 2X Cetyl trimethyl ammonium bromide (CTAB) buffer (equilibrated to 65°C) was added followed by the addition of 0.5 – 1.0% β-mercaptoethanol and heated at 65°C for 30 minutes. Equal volume of chloroform : isoamyl alcohol (24:1) was added and mixed thoroughly so that emulsion was formed. Centrifuged for 15 min at 12,000 rpm, the upper aqueous phase was transferred to a new SS-34 tube and the lower chloroform phase was discarded. 1/5 volume of 5% CTAB was added and mixed. Similar chloroform:isoamyl alcohol (24:1) extraction step was performed. Equal volume of CTAB precipitation buffer was mixed gently and placed on ice for 20-30 min followed by centrifugation for 15 min supernatant was discarded. The

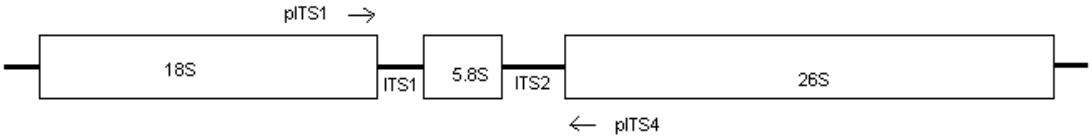
pellet was rehydrated with high salt Tris EDTA (TE) buffer (heat at 65°C for 5-10 min), followed by the addition of two volumes of cold absolute ethanol and mixed gently, centrifuged for 10-15 min at 12,000 rpm the supernatant was discarded again. The pellet was washed with 70% ethanol, centrifuged for 5 minutes at 10,000 rpm, the supernatant was discarded. The pellet was dried at room temperature by keeping in a laminar flow. The pellet was dissolved in 50-100 µl 0.1X TE (depending on the quantity of DNA). Gel was run to see the quality and quantity of DNA and the DNA samples was stored in a –20°C freezer.

All the DNA samples were divided into 2 microfuge tubes and 10µl of RNAase (10 mg/ml) was added in each tube and kept at 37°C for 2 h. Then, it was extracted with phenol: chloroform mixture (equal amounts of phenol: chloroform) and centrifuged at 13000 rpm for 10 min. The supernatant was collected into new tube and chilled ethanol was added. It is precipitated with ethanol and centrifuged at 13000 rpm and rinsed with 70% ethanol. The sample was dried and 500µl of TE was added in each tube and the tubes were kept at 60°C for some time to dissolve the DNA properly.

Sufficient amount of electrophoresis buffer was prepared (Tris Borate EDTA -TBE) to fill the electrophoresis tank and the gel was prepared. 100 ml buffer was taken in a 250 ml Erlenmeyer flask, 0.7 grams of agarose was weighed followed by the addition of the buffer solution (Normally 0.7% agarose gels are used to examined the genomic DNA). Electrophoresis buffer was added to the tank and the gel was kept in the electrophoresis tank. Buffer level was 1 mm above the gel. The DNA sample was mixed with desired gel-loading buffer (4µl loading buffer + 2 µl of DNA) and the samples was loaded in the agarose gel slots (wells). The lid of the electrophoresis tank was closed and leads were attached. Gel was taken out and stained for 30 minutes with ethidium bromide (0.5 µg/ml) in TBE buffer. Destained with distilled water for 15 minutes and examined under transmitted UV light.

The Internal Transcribed Spacer region, comprising of ITS1, 5.8S rRNA gene and ITS2 was amplified with primers pITS1F and pITS4R which were designed from the conserved regions of 18S and 26S rRNA genes respectively.

Amplification of ITS region



The sequence primers used were:

pITS-1 - 5'-TCC GTA GGT GAA CCT GCG G –3'

pITS-4 - 5'-TCC TCC GCT TAT TGA TAT GC –3'

The polymerase chain reactions were set-up in a final volume of 50µl, containing 50 ng genomic DNA, 25 pmols each of primers (pITS1 F and pITS4 R), 200 Mm each of dATP ,dTTP, dGTP and dCTP (Promega Corporation, USA); 2.5mM $MgCl_2$ and 2.0 units of Taq polymerase (Promega). The amplification of reactions were performed in a PTS 100 Mini cycler (MJ Research, USA) with the following cycling parameters: for ITS; initial denaturation for 5 min at 94°c, followed by 30 cycles of 30 sec at 55°C and 1.0 min at 72°C, with a final extension of 10 min at 72°C and cooled to 4°C. The following components were added to the 500 µl reaction tube. Taq polymerase was added after adding all other components.

10 x reaction buffer	- 5.0 µl
$MgCl_2$	- 5.0 µl
dNTP'S (5mM)	- 4.0 µl
primer-1	- 2.0 µl
primer-2	- 2.0 µl
Taq polymerase	- 0.5 µl
Milli 'Q' water	- 31.5 µl

Total	- 50.0 µl

The tubes were spined briefly (for a few seconds in a microfuge) and kept in the thermal cycler and the programme ITS was run.

The PCR program was as follows: Step 1 : 94°C - 5 min; Step 2 : 94°C - 30 sec; Step 3 : 55°C - 30 sec; Step 4 : 72°C - 1 minute; Step 5 : repeat of step 2, 34 more times; Step 6 : 72°C-7 min; Step 7 : 4°C - Till tubes are taken out

After the PCR programme was over, the tubes were taken out from the thermal cycler, spinned briefly. Three to five µl of PCR product was mixed with 3 µl of loading dye and electrophoresed on 1.2% agarose gel to examine the size of amplified product. The PCR product was further used for sequencing using MJ Research Minicycler.

3.2 Screening on the basis of oil tolerance and hydrolytic activity

The seven isolates obtained during the process of isolation were examined for their tolerance to oil as carbon source and the extent of hydrolytic activity. 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) was used for screening. Cottonseed oil was used as carbon source for screening purpose.

Inocula of different strains as prepared above, were subjected to growth in different levels of oil supplementation (10%- 90%) in the basal medium and incubated for 120 h at 28 ºC and 120 rpm, to examine the oil tolerance. Viability of the strains were checked after 120 h of growth by plating the biomass on PDA and observing the re-growth of the strain. Hydrolytic activity of the fungal strains was checked by determining the free fatty acid (FFA) produced after 120 h of incubation.

3.3 Free fatty acid determination

Free fatty acids generated due to the hydrolytic activity of fungal strains were determined by the standard method outlined by AOCS Ca5a-40 (AOCS, 1989). 10 g of oil was dissolved in 1:1 ethanol and diethyl ether and titrated against standardized 0.1 M 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 10.

$$\%FFA\ as\ oleic\ acid = \left[\ Alkali\ volume\ (ml) \times Alkali\ normality \times 28.2\ \right] \div Sample\ weight\ (gm)$$

3.4 Optimization of culture condition for lipase production

3.4.1 Extracellular lipase

Among the various strains screened, *Aspergillus flavus* MTCC 5436 exhibited maximum oil tolerance (80% and maximum hydrolytic activity (77%FFA) under growth conditions mentioned earlier (Sec. 3.2) due to which it was further exploited for the standardization of growth conditions for maximum lipase production. During standardization cottonseed oil used acted as carbon source as well as lipase inducer. Various step-wise modifications were carried out in growth conditions (Table 3.1) so as to standardize parameters to obtain maximum lipase activity. The enzyme activity was determined after day 5 in all the steps outlined.

In the step – I , *A. flavus* was grown in mineral salt medium along with 10% cottonseed oil by varying percentage of different organic (peptone and urea) and inorganic ($NaNO_3$, $(NH_4)_2HPO_4$, KNO_3 and NH_4NO_3) nitrogen sources (0.2 - 2.5%) maintaining pH 7.5 ± 0.2 at 28 ºC. In step – II, pH of the growth medium was modulated by maintaining oil supplementation at 10% and using peptone as nitrogen source (0.5% w/v) with growth conditions set at 28 ºC. In step – III, the temperature of the culture medium was varied from 25-50 ºC in medium containing 10% cottonseed oil, peptone (0.5% w/v) in pH 7.5 ± 0.2. In step – IV, modulation was carried out in the percent supplementation of cottonseed oil (10-50%) to growth medium that contained mineral salt medium and peptone (0.5% w/v) in growth condition set at 35 ºC.

Table 3.1. Experimental layout for lipase production

	Constant Parameter	Variable Parameter	Range
Step 1	Minimal salt medium + 10% oil as main carbon source + pH 7.5 ± 0.2 + growth temperature 28°C	Nitrogen source: Peptone; bi-ammonium hydrogen orthophosphate (BAHP); urea; ammonium nitrate; sodium nitrate; or potassium nitrate	0.5 to 2.5%
Step 2	Minimal salt medium + 10% oil as carbon source + 0.5% peptone + growth temperature 28°C	pH	4.0 to 9.5
Step 3	Minimal salt medium + 10% oil as carbon source + 0.5% peptone + pH 7.5 ± 0.2	Growth temperature	25 to 50°C
Step 4	Minimal salt medium + 0.5% peptone + pH 7.5 + growth temperature 35°C	Percentage of oil as main carbon source in the growth medium	10- 50%

For all the steps outlined (Table 3.1), the total volume of the test medium including inoculum was maintained at 200 ml and the orbital shaking was set at 120 rpm. The lipase activity was determined by the separation of biomass from the growth medium (Sigurgisladottir et al. 1993) 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 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 0.01 M pNP (p-nitrophenol laurate) in ethanol. The mixture was incubated at 60°C for 30 min, followed by addition of 0.25 ml of 0.1 M

Na_2CO_3 on cooling to room temperature. 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 (molar extinction coefficient 1.336×10^7 cm^2/mol at 420 nm) with pNP-laurate as substrate under standard assay conditions in 30 min.

3.4.2 Intracellular lipase

Intracellular/cell bound lipase generation in *A. flavus* was examined by varying concentration of cottonseed oil (10- 90%), in growth medium inoculated with test fungus and incubated for 120 h, 120 rpm at 35 °C. Cottonseed oil was used as carbon source as well as lipase inducer. Peptone and bi-ammonium hydrogen orthophosphate (0.5%w/v) were used as nitrogen source. Hexane (20 ml) was added after 120 h of incubation with continuous stirring for additional 12 h to separate out oil and fatty acid from the biomass. Biomass was separated by filtration and air dried for 12 h followed by overnight drying in oven at 40 °C and weighed. The products obtained after 120 h were further used for the determination of FFA produced using method outlined in section *3.3*. For the determination of lipase activity, biomass was crushed to powder with liquid nitrogen in a mortar and pestle.

The intracellular/cell bound lipase activity of the biomass was determined by modifying titrimetric method (Pinsirodom and Parkin, 2001). 5 ml of cottonseed oil was taken in 50 ml round bottom flask as substrate and pre-incubated for 15 min at 35 °C. To this, 1 g biomass was added to initiate lipolysis on the oil substrate with continuous stirring. Sample was removed after 1 h. Samples were transferred to a separate flask with cocktail containing 10 ml of 95% (v/v) ethanol and 2 to 3 drops of 1% (w/v) phenolphthalein indicator and swirled immediately to stop the reaction. The content of the flask was titrated with 0.05 N NaOH until end point was reached. In another 100 ml Erlenmeyer flasks containing titration cocktail, 5 ml of oil was added and titrated with 0.05 N NaOH and used as blank.

Free fatty acids liberated were estimated on the basis of equivalents of NaOH used to reach the titration end point, with the following equation:

$$\mu\ mol\ Fatty\ acid/ml = \frac{(ml\ NaOH\ for\ sample - ml\ NaOH\ for\ blank) \times N \times 1000)}{5\ ml}$$

where *N* is the normality of the NaOH titrant used (0.05 in this case).

3.5. Optimization of culture conditions for transesterification /esterification reaction

3.5.1 Transesterification studies with wet biomass

The optimization of parameters for transesterification was carried out using *A. flavus*. For the transesterification reaction, edible (used cottonseed oil) as well as non-edible oils (jatropha and karanj oils) were used as carbon source as well as lipase inducer. Before inoculation, medium was sterilized at 120 ^{o}C, 115 psi for 15 min.

Inoculum was prepared as in section 3.1.2. Standardization of transesterification was carried out in steps (Table 3.2). Primarily, time interval of ethanol addition was optimized by stepwise (0, 2, 4, 8 and 12 h) addition of ethanol, *A. flavus* was inoculated in 70:30 oil (cottonseed, jatropha and karanj oil): Mineral medium, and incubated for 72 h at 28^{o}C and 120 rpm. Mycological peptone and di-ammonium hydrogen ortho-phosphate (0.5% w/v) were used as nitrogen source. Following incubation, ethanol (5 ml/12 h), was added in 1:3 (oil:ethanol) molar ratio. In addition to the volume based on molar ratio, an additional amount of approximately 3- 4 ml of ethanol was added to avoid the reverse reaction and ensure maximum transesterification reaction. After an additional 24 h of incubation, biomass was separated from reaction mixture using Whatman filter paper. The ethyl ester layer was separated from the aqueous layer using separating funnel and further used for analysis.

Further reactions were carried out using either cottonseed, jatropha or karanj oil and varying the levels of oil supplementation (50:50, 60:40, 70:30, 80:20 and 90:10) in the medium with stepwise addition of ethanol at an interval of 12 h and at conditions mentioned above. Ethyl ester layer was separated for further analysis. Biomass was washed with *n*-hexane followed by water to remove adhering oil and further air-dried overnight in oven, at 40 °C to remove excess water, before weighing.

Attempt was made to enhance the extent of transesterification of 80 and 90% of oils (cottonseed, jatropha and karanj oil) by varying the concentration of peptone (0.5, 1.0, 2.0, 3.0, 5.0 and 7.0%) as nitrogen source keeping other parameters constant in all the experimental conditions.

Reusability potential of biomass was observed for different cycles (5 cycles) wherein for each cycle same biomass was repeatedly used by replacing media and oil after every reaction, and transesterification reaction carried out after each cycle.

After reaction was over biomass was separated by filteration, upper layer of ethyl ester was removed using separating funnel, glycerol was separate using rota-evaporator from lower layer.

Studies on whole cell catalyzed transesterification were also carried out for determining methyl ester generation at standardized conditions of ethyl ester.

Table 3.2. Reactions conditions altering different parameters for transesterification reaction in the presence of used cottonseed, jatropha and karanj oil as carbon source

Composition of Growth media	Variables
70:30 Mineral medium: oil; 0.5% peptone; 0.5% BAHP	Step wise addition of ethanol at variable intervals of 0, 2, 4, 8, 12 h
	Methanol addition at an interval of 12 h
0.5% peptone; 0.5% BAHP, Ethanol addition at an interval of 12 h	Oil percentage in the growth medium (50, 60, 70, 80 and 90%)
	Oil percentage in the growth medium (80 and 90%), Peptone in the growth medium (0.5, 1.0, 2.0, 3.0, 5.0 and 7.0%)

Observations on whole cell catalyzed transesterification was also studied with other isolates (*Fusarium subglutians, Aspergillus sydowii* (MTCC 10397), *A. aculeatus, Curvularia pallescens* (MTCC 10390), *Penicillium citrinium* (MTCC 10398) and *Periconia sp*) strains, following the optimized conditions determined for *A. flavus*.

3.5.2. Transesterification reaction with dried biomass

Similar to the observations carried out with wet biomass, study was also carried out to examine the potential of dry biomass of *A. flavus* to catalyze the transesterification reaction. The biomass was separated as outlined in sec. 3.5.1, air dried and crushed to powder in liquid nitrogen. Dried biomass, thus, obtained was used for further study. The conditions optimized to achieve maximum transesterification with dried biomass as catalyst, is outlined in the Table 3.3.

Transesterification reaction was standardized by varying the parameters like amount of biomass (5 - 40%) with respect to oil, temperature range (25- 65 °C), time interval of alcohol addition (1, 2, 4, 8 and 12 h). Molar ratio of 10 g oil (1:3,1:4, 1:5 and 1:6) to ethanol ranging across 1.6 g, 2.1 g, 2.7 g and 3.3 g was optimized to get

maximum transesterified product and the reactions was carried out for total reaction time of 36 h . Under conditions standardized with cottonseed oil, similar experiment was performed with Jatropha and Karanj oils.

Table 3.3. Reaction condition altering various parameters for transesterification by using dried biomass

Steps	Reaction Parameters	Variables
Step 1	10 g oil, molar ratio of oil to ethanol - 1:5, 35°C, alcohol addition at an interval of 8 h	Biomass percentage with respect to oil (5, 10, 20, 30 and 40%)
Step 2	10 g oil, 2 g biomass, molar ratio of oil to ethanol - 1:5, reaction temperature - 35°C	Time interval of alcohol addition (0, 2, 4, 6, 8 and 12)
Step 3	10 g oil, 2 g biomass, molar ratio of oil to ethanol - 1:5, alcohol addition at an interval of 8 h	Reaction temperature (25, 35, 45, 55, 65°C)
Step 4	10 g oil, 2 g biomass, alcohol addition at an interval of 8 h, reaction temperature - 35°C	Molar ratio of oil to ethanol (1:3, 1:4, 1:5 and 1:6)
Step 5	10 g oil, 2 g biomass, Molar ratio of oil to ethanol 1:5, reaction temperature - 35°C, alcohol addition at an interval of 8 h	Water content in the reaction mixture (0-50%)

3.5.3. Effect of frying on transesterification reaction

Effect of frying was examined on the extent of transesterification, for which frying oil was generated by using cottonseed oil and frying the same for 7 h. An average of 7-9 fryings were carried out each hour at 160-200 °C and samples were collected at hourly interval. No fresh oil was supplemented during frying (Prakash et al. 2010). Transesterification was carried out under standardized conditions for 36 h, (Table 3.3) after proper filtration oil to remove debris formed during frying.

3.6. Transesterification reaction by using immobilized biomass

3.6.1 Whole cell immobilization on Luffa (Luffa cylindrica) and polyurethane foam

Dried fruits of *Luffa cylindrica,* collected from the field were peeled and seeds were removed after cutting both ends. Other adhering substances and resins were removed by immersing the luffa in boiled water for 1 h followed by air-drying for 12 h. Luffa, thus obtained was further cut into pieces of 1cm x 1cm with thickness of 2-3

mm. In addition, polyurethane foam was cut into pieces of 1cm^3. Weight of the cut pieces of dried luffa and polyurethane foam was recorded.

Pieces (20) of luffa and polyurethane foam were taken in separate 500 ml Erlenmeyer flasks containing 70 ml mineral salt medium and 30 ml cottonseed oil (as 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. The medium was sterilized at 115 psi for 20 min. On cooling, the media was inoculated with freshly grown biomass of *A. flavus* and incubated for 120 h at 28 ºC and 120 rpm. On colonization of fungus over luffa and polyurethane foam, the biomass filled matrices were removed from the culture flasks and used as such for transesterification reaction. After the reaction, the matrices containing immobilized biomass were washed with hexane to remove oil or/and fatty acids followed by washing with sterile distilled water. The matrices were air-dried overnight and the weights were recorded. The matrices were stored at -4 ºC to avoid sporulation. Axenic conditions were maintained to avoid contamination across washing till storage.

3.6.2 Transesterification reaction with immobilized whole cells

Transesterification was carried out by using 20 pieces of immobilized luffa/ polyurethane foam in separate 500 ml Erlenmeyer flasks containing oil (cottonseed) and mineral medium in the ratio of 100:0, 90:10, 80:20, 70:30, 60:40,50:50 (V/V) and incubated individually for 72 h at 28 ºC and 120 rpm. Following incubation, ethanol was added in the ratio of 1:3 (oil:ethanol) at different time intervals (0, 2, 4, 8 and 12 h) to optimize the time for maximum ethyl ester production. On completion of ethanol addition, followed by incubation for additional duration of 12 h, 20 ml of hexane was added to facilitate release of oils and fatty acids. At the end of the reaction with total reaction time of 36 h, the matrices were separated and discarded. After removal of the matrices, the upper layer of the reaction medium containing alkyl ester was separated using separating funnel and collected for further analysis. The FFA content in alkyl ester was further estimated using method followed in Sec. 3.3.

For examining the reusability of immobilized matrices, the matrices were used over five cycles replacing the used medium with fresh medium and oil, after each cycle, in the ratio of 20:80 that was observed to be the optimum ratio for transesterification reaction.

3.7 Identification and quantification of the transesterified products

3.7.1 Identification of alkyl ester and glycerol

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

The ester was further quantified using proton nuclear magnetic resonance spectroscopy (^{1}H NMR) (400 MHz; Bruker-Advance II-400 with 5mm BBO probes). $CDCl_3$ (deuterated chloroform) was used as solvent and tetra methyl silane as internal standard. ^{1}H NMR spectra were recorded with pulse duration of 2.72 sec with a relaxation delay of 1 sec and 16 scans. Glycerol, obtained as by-product was also analyzed similarly using ^{1}H NMR.

3.7.2 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 triacylglycerols. The strong singlet at 3.6 ppm indicates methyl ester ($–CO_2\ CH_3$) formation. The signals at 2.3 ppm result from the protons on the CH_2 groups adjacent to the methyl or glyceryl ester moieties ($–CH_2\ CO_2\ CH_3$ for methyl esters).

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

Wherein

C - conversion of triacylglycerol of feedstock (vegetable oil) to the corresponding methyl ester.

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

$A_{\alpha\text{-}CH2}$ - 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.

3.7.3 Quantification of ethyl ester

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 oil and the $-OCH_2$ from ethyl ester in biodiesel where partial conversion was obtained. However, the reaction where peak due to glyceryl methylenic

hydrogens in oil at 4.25-4.35 ppm completely disappeared, the process of transesterification was considered to be nearly completes. In case of incomplete transesterification, ethyl ester quantification was carried using the equation proposed by Ghesti et al. (2007).

$$\% C_{EE} = 100 \left(\frac{4(I_{TAG+EE} - I_{TAG})}{4(I_{TAG+EE} - I_{TAG}) + 6(2\, I_{TAG})} \right)$$

Where

(i) (I_{TAG}) integration of glyceryl methylenic hydrogens at 4.25-4.35 ppm;

(ii) (I_{TAG+EE}) integration of glyceryl methylenic hydrogens and -OCH_2 of ethoxy hydrogens superimposed at 4.10- 4.20 ppm; and

The numbers 4 and 6 in above equation are related to four glyceryl methylenic hydrogens present in TAG molecules and to six hydrogens formed in three ethyl ester products.

Quantification of ethyl ester was also further validated using gas chromatography for those samples where maximum transesterification was observed. Percentage of ethyl 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 ethyl 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.

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

Where

$\sum A$- was the total peak area of ethyl esters;

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

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

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

m - was the mass in milligrams of the sample

3.8 Whole cell catalyzed esterification using active culture and dry biomass of *A. flavus*

Esterification with wet biomass was carried out in 500 ml Erlenmeyer flask, containing 200 ml of oleic acid: mineral medium (50:50) with oleic acid acting as carbon source, peptone and bi-ammonium hydrogen ortho phosphate (0.5%) as nitrogen sources, and pH of the medium set at 7.0 ± 0.2. Fresh biomass of *A. flavus* was inoculated and incubated for 72 h at 28ºC and 120 rpm. The influence of chain length of alcohols on the esterification process was studied by taking various alcohols (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol and decanol) which were added stepwise at an interval of 12 h. After the completion of reaction, biomass was separated out by simple filtration. Upper layer of ester was separated by using separation funnel and used further for analysis. Identification of the product was carried out using thin layer chromatography as in section 3.7.1 and quantification was carried out by using ^{1}H NMR.

Similarly, to examine the esterification process with powdered biomass, *A. flavus* was used to carry out esterification of fatty acids with varying chain length of alcohol (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol and decanol) for biodiesel production. Esterification was carried out in round bottom flask on hot plate magnetic stirrer at 35 °C, using 2 g of biomass and 10 g oleic acid. Alcohols are added stepwise at an interval of 6 h, with total reaction time of 36 h (Aulakh et al. 2011).

3.9 Quantification of alkyl ester produced by esterification of fatty acid

Alkyl ester produced by esterification was quantified by using the formula of Satyarthi et al. (2009). The principle underlying method outlined by author is detailed as follows, whereas remaining un-esterified FFA amount was determined based on the fact that R-CH_2 peaks of fatty acids appear at δ values higher than those of the alkyl esters. The difference in chemical shift (between the acid and ester) is due to the greater deshielding effect of the carboxylic group compared to the ester group. Due to this shift, one of the peaks of the triplet of FFA (at 2.38 ppm) shifts out of the R-CH_2 region of the ester, and the other two peaks (2.34 and 2.30 ppm, respectively) are merged with those due to the ester at 2.35 and 2.31 ppm, respectively. In other words, a sample containing FFA and ester shows a quartet like spectral pattern in the R-CH_2 region of the ^{1}H NMR spectrum with the intensity of the peaks depending on the content of FFA in esters. The unmerged peak of the FFA triplet can be used to determine the FFA content in esters. The area of the unmerged peak of the FFA triplet (appearing around 2.38 ppm, out of the ester triplet) can be determined by integration of the spectral region 2.37-2.41 ppm. The triplet appears with an intensity ratio of 1:2:1. The total areas corresponding to the R-CH_2 groups of the FFA will thus be four times the area of the single unmerged FFA peak around 2.38 ppm. The total area corresponding to R-CH_2 of both FFA and ester can be determined by integrating the spectral region 2.20-2.41 ppm. The concentration of FFA (wt %) in alkyl esters is thus

$$\% \text{ of FFA} = \frac{4\times \text{ Area of unmerged peak of } \alpha\text{-}CH_2 \text{ of FFA}}{\text{Total area of } \alpha\text{-}CH_2 \text{ of both FFA and ester}} \times 100$$

3.10 Physico-chemical properties of ethyl ester

Samples from reactions resulting in maximum transesterification with all the three oils tested and collected over a series of experiments, were further analyzed for physico-chemical properties as per Indian (BIS) Standards.

3.10.1 Calorific value

Calorific value of biodiesel produced was determined by using Indian Standard 1350 Part II-1970, making use of calorimetric bomb immersed in a static or isothermal water jacket.

One gram of sample taken in capsule was kept in tarred crucible and placed in the hanger of bomb. Bomb was filled with oxygen upto pressure of 25 kg/cm^2 and operated further using other standard operations. 2000 ml of water was poured in the bucket where in the temperature of water was kept 2°C below as that of the calorimeter jacket. Initial temperature after waiting for (at least) 10 min was noted. The sample was fired and the final temperature was noted when the temperature remained constant after firing. Calculation was carried as per Indian Standard: 1350(Part II)-1970.

3.10.2 Flash point

Flash point was determined using Pensky Martens closed tester according to Indian Standard 1448 [P:21]:1992. The sample was heated at a slow, constant rate with continual stirring. The cup with inserted thermometer was covered with lid and placed in the stove. Test flame was lightened and adjusted to 4mm in diameter. Heat was supplied allowing 5 to 6°C rise in temperature per minute. Stirring was maintained 90-120 rpm in downward direction. The flash point was noted on the thermometer at the time, when the test flame application caused a distinct flash in the interior of the cup. Calculation was carried out as per Indian Standard 1448 [P:21]:1992.

3.10.3 Pour Point

Indian Standard IS: 1448 [P:10]- 1970 was used for the determination of pour point, the sample was first heated and then cooled at a specified rate. It was examined at intervals of 3°C. The lowest temperature at which the oil was observed to flow was noted and recorded as the pour point of the material. The test was continued until point was reached at which the oil in the test jar showed no movement when the test jar was held in a horizontal position for exactly 5 sec. The reading of the test thermometer was recorded and calculated as per Indian Standard: 1448 [P:10] -1970.

3.10.4 Kinematic viscosity

Kinematic viscosity was determined using Indian Standard IS: 1448 [P:25] -1976 was used for the determination of kinematic viscosity. The time was measured in seconds for a fixed volume of liquid to flow under gravity through capillary of a calibrated viscometer under a reproducible driving head and at a closely controlled temperature. Calculation was carried out as per Indian Standard: 1448 [P:25] -1976.

3.10.5 Density

Capillary stoppered pyknometer method was used as per Indian Standard 1448[P:32]: 1992, for the determination of density. In this method the weight of equal volumes of the sample and of water were compared. Pyknometer was filled with the sample in such a way that no air bubble was produced. Pyknometer was brought to 30 ^{o}C test by immersing it up to its neck in the constant temperature bath for 20 min. Dry capillary stopper was inserted firmly. Pyknometer was removed from the bath when it reached the required temperature. All traces of sample and water was removed from the exterior surface of the pyknometer using lint free cloth and weighed to precision and calculation was carried out as per Indian Standard 1448[P:32]: 1992.

3.10.6 Acid number

For the determination of total acidity, 10g of sample was taken in 250ml conical flask. Alkali blue 6 B indicator and a drop of standardized HCl was added in a mixture of 60ml toluene and 40ml of rectified spirit in another flask and the mixture was neutralized with standard alcoholic potassium hydroxide solution. The sample was dissolved in it and titrated immediately with standardized alcoholic potassium hydroxide till the purple blue colour was changed into a wine red colour which persisted for 15 seconds. Volume of KOH used was noted and calculation was carried as per Indian Standard:1448[P:2]-1967.

3.10.7 Water content

Water in oil was determined with coulometrically generated Karl Fischer reagent. In this titration method, the sample is mixed with a base/alcohol solution, iodide solution and sulphur dioxide. Iodine is generated electrolytically and reacts with water. One mole of iodine reacted with one mole of water stoichiometrically wherein 1 mg of water is equivalent to 10.72 coulombs and the amount of water was determined directly from the quantity of electricity required for the electrolysis.

3.10.8 Sediment

Following the method outlined in Indian standard 1866 – 1983, the sample was thoroughly agitated in the original container of the equipment until the sediments were homogeneously suspended in the oil. 10 g of sample was taken in a stoppered conical flask and 100ml of n-heptane was added to it. Sample and solvent were thoroughly mixed and allowed to stand in glass flask in the dark for 18-24 h. The solution was filtered through a tared Grade 4 sintered glass crucible with the assistance of vacuum

rinsing the flask with fresh n-heptane to ensure complete transfer of the precipitate to the crucible. The crucible was washed and precipitated with the heptane until free from oil. Heptane was evaporated and crucible was dried in an oven at a 100-110°C for one hour, cooled in a desiccator and weighed. The increase in weight of the crucible as a percentage of the weight of oil was calculated.

3.10.9 Sulphur content

General Bomb method was used as per Indian standard D 129-95 for the determination of sulphur content. The sample was oxidised by combusting in a bomb containing oxygen under pressure. Interior of the bomb, the oil cup and inner surface of the bomb were washed with fine jet of water and collected in 600 ml beaker.10ml of saturated bromine water was added to the washing in the beaker. Sample cup was placed in another 50 ml beaker. 5 ml of saturated bromine water, 2 ml of HCl and enough water just to cover the cup was added. The contents of the 50 ml beaker was heated to just below its boiling point for 3 to 4 min and added to earlier beaker containing the bomb washings. Sample cup and the 50 ml beaker were washed thoroughly with water. Precipitates in the cup were removed by means of a rubber policeman. Washing from the cup and the 50 ml beaker, was added to the bomb washing in the 600 ml beaker and evaporated on hot plate. Heat was adjusted to slow boiling of the solution. 10 ml of $BaCl_2$ was added drop wise with continuous stirring. Boiling was continued by covering the solution with a fluted watch glass till 75 ml of the solution remained in the beaker. Beaker was removed from the hot plate and allowed to cool to room temperature for 1 h. Supernatant was filtered through ash less filter paper; precipitates were washed with water until free from chlorine. Paper and precipitates were dried at low heat until the moisture was evaporated. The paper was charred without igniting and finally ignited at a bright red heat until white residue was obtained, cooled to room temperature and weighed. Calculation was carried out as per the standard protocol.

3.10.10 Ash content

Ash content was determined following the procedure outlined in Indian standard 4/94. Crucible was heated at 700 to 800 °C for 10 min in muffle furnace, cooled to room temperature in a suitable container and weighed to the nearest 0.1 mg. The sample of sufficient mass, to the nearest 0.1% to give up to 20 mg of ash was weighed into the crucible. The crucible and sample were heated until the contents can

be ignited with time. The temperature was maintained such that sample continues to burn at a uniform and moderate rate, leaving only ash and carbon when the burning ceased. The residue was heated in the muffle furnace at 775 ± 25°C till all carbonaceous material disappeared. The crucible was cooled to room temperature in a suitable container and weighed. Calculation was carried out as per Indian Standard 4/94.

4.1 Isolation and identification of fungal strains

The fungus strains isolated from the contaminated clarified butter were identified as *Aspergillus flavus* (MTCC 5436), *Fusarium subglutians, Aspergillus sydowii* (MTCC 10397), *A. aculeatus, Curvularia pallescens* (MTCC 10390), *Penicillium citrinium* (MTCC 10398) and *Periconia sp* (MTCC 10391) by Microorganism Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. *Fusarium subglutians* and *A. aculeatus* were not deposited at MTCC.

4.1.1 Morphological characterizations of isolated fungal strains

4.1.1.1 *Aspergillus flavus* (MTCC 5436)

Colonies were yellowish-green with a cream reverse showing rapid growth, consisting of a dense belt of conidiophores or mature vesicles bearing phialides over their entire surface. Texture was woolly to cottony and brown in colour. Effuse, lime green colonies bore rough conidiophores and smooth to very finely roughed conidia. Hyphae were septate and showed dichotomous, 45^{o}C angle branching. Conidial heads were radiate to loosely columnar with age. Conidiophores were coarsely roughened, uncolored, up to 800 µm long x 15–20 µm wide, vesicles spherical (20–45 µm), metulae (8–10 x 5–7 µm) covering nearly the entire vesicle in biseriate species. Conidia were pale green and conspicuously echinulate, smooth to very finely roughened, (sub) spherical, 3.5 - 6 µm in diameter.

4.1.1.2 *Aspergillus sydowii* (MTCC 10397)

Colonies were bluish green, moderately growing and reverse in shade of red. The conidial heads exhibited globose patter with conidiophores colorless and smooth; and conidia having 4.0-5.0 dia. Conidia were echinulate, green in mass, spherical to subspherical and 2.5-4.0 µm in dia.

4.1.1.3 *Aspergillus aculeatus*

Colony diameter at 7 days and 25^{o}C on PDA was 5-6 cm. Conidiophores were produced abundantly, conidial areas were light brown; sclerotia were present, small, globose to subglobose, creamish to light yellow. Reverse of the colony was light yellow to light brown and furrowed. Conidial heads were radiate, splitting into poorly

defined columns, phialides flask-shaped and covered the entire surface of the vesicle, conidia subglobose to ellipsoidal, echinulate.

4.1.1.4 *Curvularia pallescens* (MTCC 10390)

After 5 days of growth on over PDA, colonies of *C. pallescenss* produce well developed mycelium with some anastomosis and formed conidia on sympodially proliferating conidiogenous cells. Colonies were effuse, grey and became black when older. Mycelium was mostly immersed, formed straight or flexuous and smooth conidiophores which appeared rough as a result of condensation of exudates. The conidiogenous cells were broader at the apex and seemed inflated first with sympodial formation of conidia. Conidia were slightly curved, three septate with the medium septum off-center. In general, central cells were broader and darker than the end cells. Some mature conidia were slightly collapsed after being liberated from the conidiophore.

4.1.1.5 *Perconia sp*. (MTCC 10391)

Colonies were effused, with grey/brown, conidiophores which were dominantly macronematous but some extent micronematous. Conidiogenous cells were polyblastic. Conidia were catenate, chain often branched, simple, usually spherical to subspherical, echinulate and o-septate.

4.1.1.6 *Penicillium citrinium* (MTCC 10398)

Colonies grew restrictedly, attaining a diameter of 1-1.5cm within 7 days, consisting of dense felt of conidiophores, sometimes appearing leathery, blue green. Reverse normally yellow to orange. Conidiophores, smooth walled with 3-5 divergent metulae in a whorl. Conidia were produced in columns, globose to subglobose, smooth walled or finely rough, hyaline to greenish.

4.1.1.8 *Fusarium subglutians*

Colonies formed sparse white aerial mycelium on PDA after incubation in the dark with more dense and tinged with purple at the colony centre. The undersurfaces of the Petri dishes revealed cream-white with a peach or purple mid-point, which, after 14 d, led to a prominent variation of purple and blue pigmentation. Macroconidia were fairly abundant and produce mainly in cream to orange sporodochia. Three septate macroconidia are most abundant with production of few one- and two-septate macroconidia (mesoconidia). Conidia were sickle-shaped to almost straight with the ventral side curved to straight and the dorsal side always curved, forming a dorsal-

ventral curvature widest at the middle or near the apical cell. Microconidia were abundant on mono- and polyphialides in false heads. Conidiophores were situated laterally or terminally on hyphae, very short to quite long and seldom branch tended to narrow towards the apical opening.

4.1.2 Molecular characterization of *Aspergillus flavus*

Based on the NCBI –BLAST carried out by IMTECH, Chandigarh, search analysis of the internal transcribed spacet ITS/5.8S subunit Ribosome RNA gene and partial β-tublin gene, the fungal isolate MTCC 5436 was confirmed as *Aspergillus flavus* (Annexure I).

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

Isolated fungal strains were further screened on the basis of hydrolytic activity and oil tolerance. Result of the present study (Table 4.1) shows that three strains, *A. flavus, A. aculeatus* and *F. subglutians* showed growth in medium supplemented with 80% of cottonseed oil in the medium, whereas, *A. sydowii* (MTCC 10397) and *P. citrinium* (MTCC 10398) showed growth at 50% and 30% supplementation. *C. pallescens* (MTCC 10390) and *Perconia sp*. (MTCC 10391) were viable in medium supplemented with 20% and 10% oil in the growth media with no viability beyond the mentioned levels of oil in medium.

Study on hydrolytic activity by different fungal stains revealed that *A. flavus* exhibited maximum hydrolytic activity (77% FFA) with 40% oil supplementation in the growth media. On the other hand, *A. aculeatus* showed maximum hydrolysis of oil upto 76.4% FFA with 50% oil (Table 4.1) followed by *A. sydowii, P. citrinium* and *C. pallescens* wherein 63.6% (10% oil), 28.6% (20% oil) and 25% (10% oil) of hydrolysis was seen. *Periconia sp.* showed tolerance only upto 10% oil resulting in 31% FFA generation. In contrast, *F. subglutians* showed maximum oil tolerance that is upto 80% oil without any lipolytic activity.

A notable observation in the study was the nature of *F. subglutians* to exhibit growth even at greater than 80% oil supplementation in the growth medium. However, the organism did not show any potential to facilitate hydrolytic activity at any level of oil supplementation.

Table 4.1. Growth (+/-) strains at different levels of oil supplementation and FFA(%) produced after growth for 120 h

oil supplementation (%)	Growth / FFA(%)						
	Aspergillus flavus	*Aspergillus aculeatus*	*Aspergillus sydowii*	*Penicillium citrinium*	*Curvularia pallescens*	*Periconia sp*	*Fusarium subglutians*
10	+ ve / 58.0	+ ve / 52.6	+ ve / 63.6	+ ve / 23.3	+ ve / 25.0	+ ve / 31.0	+ ve / Nil
20	+ ve / 67.0	+ ve / 59.3	+ ve / 54.0	+ ve / 28.6	+ ve / 21.2	- ve	+ ve / Nil
30	+ ve / 73.0	+ ve / 55.7	+ ve / 61.3	+ ve / 24.4	- ve	- ve	+ ve / Nil
40	+ ve / 77.0	+ ve / 73.0	+ ve / 30.5	- ve	- ve	- ve	+ ve / Nil
50	+ ve / 75.6	+ ve / 73.3	+ ve / 28.7	- ve	- ve	- ve	+ ve / Nil
60	+ ve / 76.0	+ ve / 75.0	- ve	- ve	- ve	- ve	+ ve / Nil
70	+ ve / 75.0	+ ve / 76.4	- ve	- ve	- ve	- ve	+ ve / Nil
80	+ ve / 74.6	+ ve / 68.3	- ve	- ve	- ve	- ve	+ ve / Nil

4.3 Optimization of culture conditions for lipase production

A. flavus resulted in maximum hydrolytic activity and oil tolerance. This strain was further used for the standardization of lipase activity.

4.3.1 Extracellular lipase

4.3.1.1 Effect of various percentages of nitrogen sources

The strain, *A. flavus* was grown in increasing concentrations (0.2-2.5%) of various organic (peptone and urea) and inorganic nitrogen ($NaNO_3$, $(NH_4)_2HPO_4$, KNO_3 and NH_4NO_3) sources, with 10% oil (cottonseed) as carbon source to evaluate the effect of nitrogen concentration on the enzyme activity. Other growth components were maintained as mentioned in Step 1 (Table 3.1). In the present study, supplementation was most effective in the case of addition of peptone, with the highest lipolytic activity (16 U/ml) followed by $(NH_4)_2HPO_4$ that resulted in 15 U/ml among the inorganic nitrogen sources. With increase in the percentage of peptone from 0.3 to 0.4% there was a steep increase in lipolytic activity (Fig.4.1) and with increase in percentage of inorganic nitrogen sources, lipolytic activity was increased to a certain extent after which it decreased sharply, however, in the case of organic nitrogen source, it remained constant.

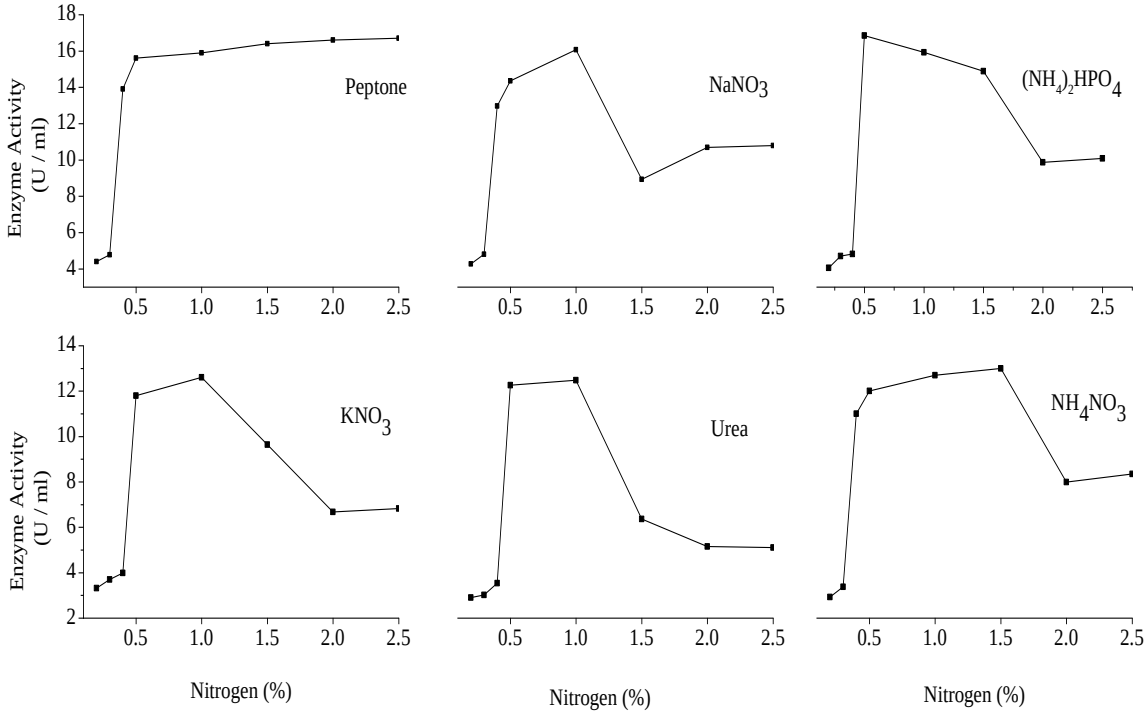


Fig. 4.1. Effect of nitrogen sources and concentration on enzyme activity

4.3.1.2 Effect of pH

To observe the effect of pH on the lipase activity by *A. flavus,* the strain was grown in experimental conditions as outlined in Step 2 (Table 3.1). The lipase activity, thus obtained, at various pH levels is shown in Fig 4.2. Maximum lipase activity of 17.2 U/ml was obtained at pH 7.5. Further increase in pH from 7.5 to 9.0 resulted in reduced lipase activity i.e. from 17.2 U/ml to 7.5 U/ml.

4.3.1.3 Effect of incubation temperature

To observe the effect of incubation temperature on activity of lipase, the reaction medium was incubated in temperature ranging from 25 to 50 °C following the parameters as in step 3 (Table 3.1). The activity of lipase was observed to increase in media incubated from 25 to 35 °C with activity reaching up to 16.5 U/ml at 35 °C (Fig. 4.3). Further increase in temperature was observed to decrease the activity of the enzyme with very low activity observed after 40 °C.

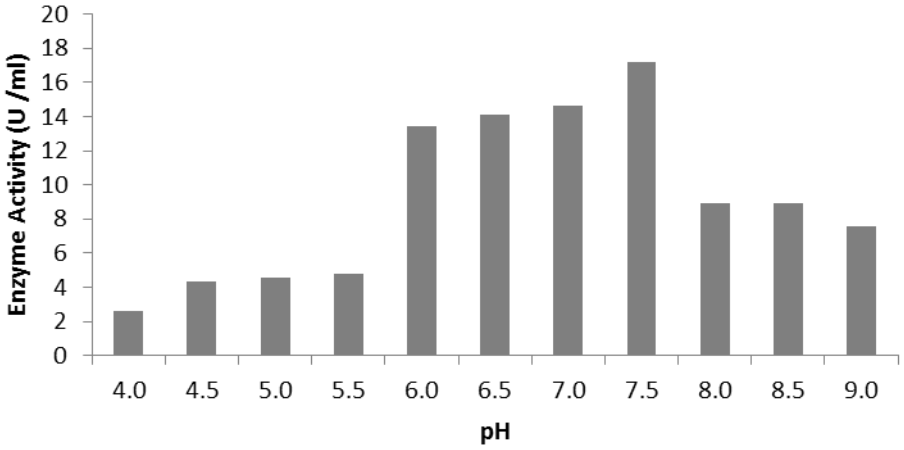


Fig 4.2. Effect of pH on enzyme activity

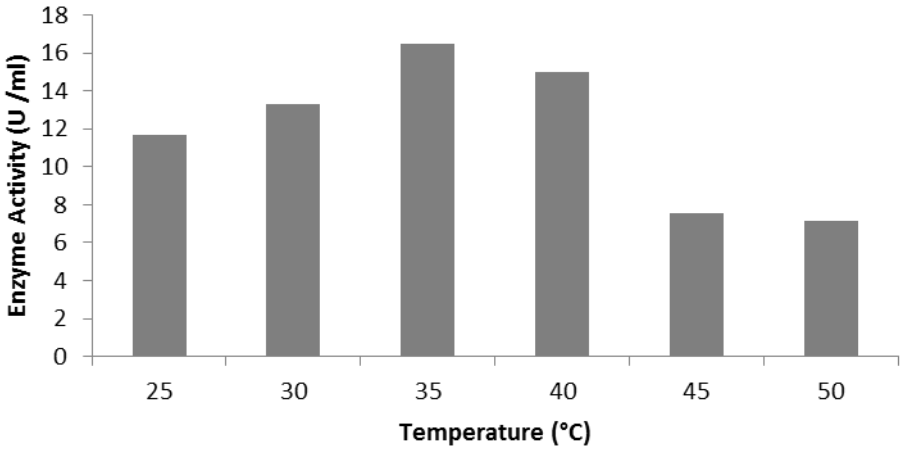


Fig. 4.3. Effect of temperature on enzyme activity

4.3.1.4 Effect of different oil concentration on lipase activity

The concentration of carbon source also significantly influences the lipase production and hence lipolytic activity. In the present study, different levels of supplementation (10%–50%) of cottonseed oil were taken as the carbon source, other parameters remaining unaltered. Maximum of lipase activity 21.8 U/ml (Fig 4.4) was obtained by supplementation of 50% oil in the growth medium. The extent of oil supplementation as the main carbon source i.e. 50% of the growth medium is significantly higher than the observations reported till date.

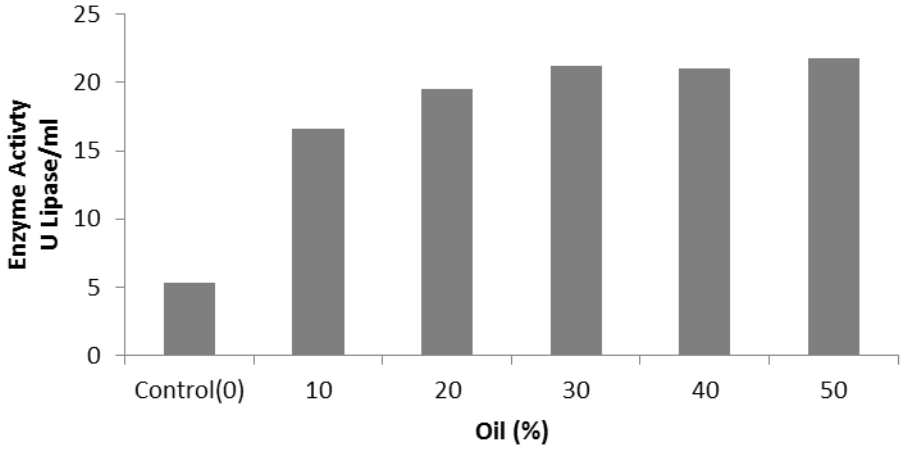


Fig 4.4. Effect of oil concentration on enzyme activity

4.3.2 Intracellular/cell bound lipase

Intracellular/cell bound lipase production was carried out under standardized condition. After 120 h of growth period, maximum 83% (with 70% oil in the growth medium) of FFA was produced. It was observed that with increase in oil percentage, FFA increased till 70% oil supplementation in the growth medium. Further increase in oil percentage resulted in decrease of the FFA yield wherein only 68.3% and 44.0% FFA was produced during growth in 80 and 90% oil, respectively (Table 4.2). It was also observed that with increase in oil percentage, biomass production in the growth medium also increased till 70% oil in the growth medium.

In term of biomass yield, a maximum of 8.8 g biomass was produced (dry weight) in 200 ml growth medium that contained 70% oil, in contrast to control devoid of oil wherein, only 0.7 g of biomass produced.

Furthermore, intracellular /cell bound lipase activity was determined in dried biomass of *A. flavus* which was prepared by drying and powdering in liquid nitrogen. Maximum of 1811 µmol/ml FFA was produced by 1 g biomass obtained from 70% oil in the growth medium, whereas in case of control (devoid of oil in growth medium) resulted in only 142 µmol/ml FFA (Fig 4.5).

4.4 Identification and quantification of the products of transesterification reaction

Under the standardized condition of extracellular lipase production, the potential of *A. flavus* to tranesterify different oils (used cottonseed, crude karanj and crude jatropha oil) was determined. The formation, of ethyl ester was initially identified through thin layer chromatography (TLC) with chemically synthesized ethyl ester as standard (Fig 4.6 a & b).

Table: 4.2. FFA production due to hydrolysis of oil during growth of *A. flavus* in medium supplemented with oil

Oil percentage	FFA (%) produced	Biomass produced (g)
Control (No oil)	Nil	0.7
10	65.5	3.3
20	70.7	4.6
30	70.5	5.0
40	75.0	5.0
50	77.0	5.1
60	80.3	6.3
70	83.0	8.8
80	68.3	4.7
90	44.0	4.6

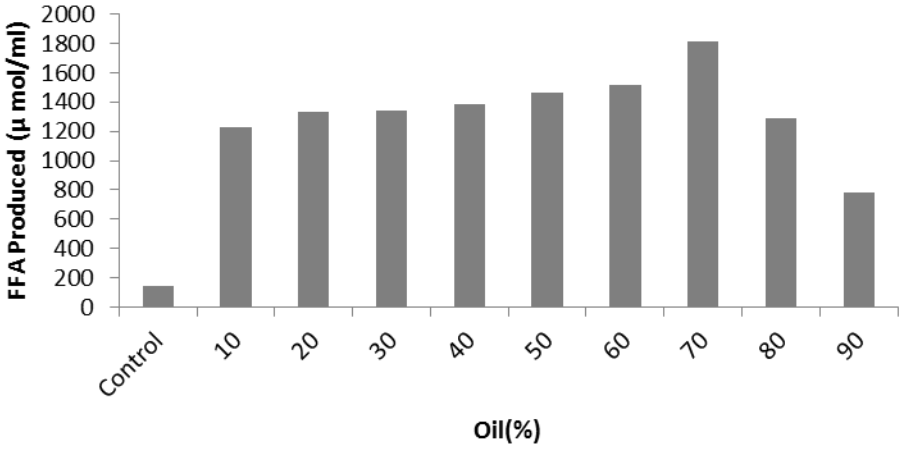


Fig. 4.5. Intracellular/cell bound lipase activity of dried biomass of *A. flavus* at different oil supplementation

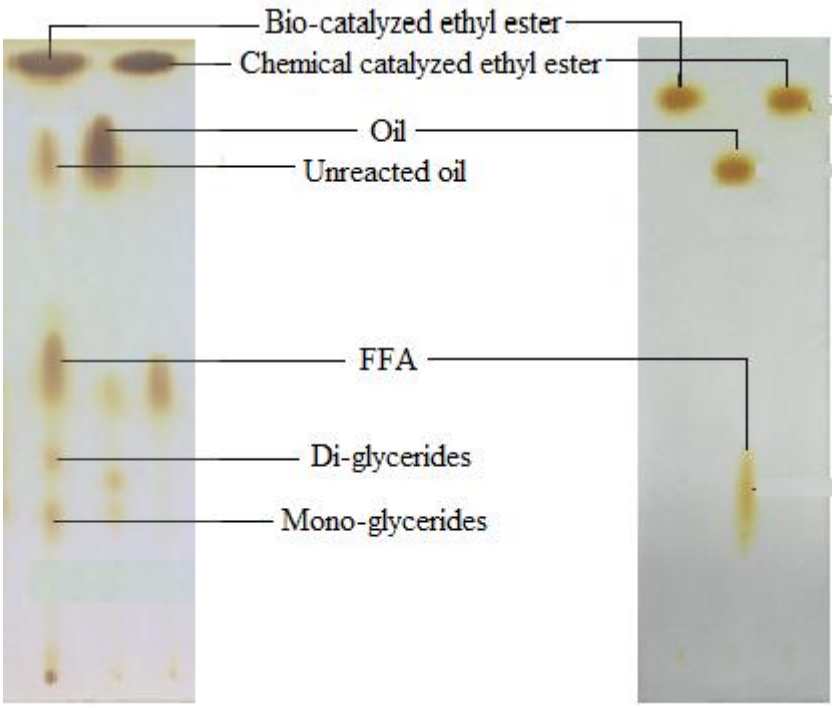


Fig.4.6. Thin layer chromatography of (a) incomplete conversion and (b) of complete conversion of oil to ethyl ester

Furthermore, ethyl ester obtained in the reaction was quantified using ^{1}H NMR and following equation derived by Ghesti et al. (2007). Figure 4.7 to 4.9 (a) depict the ^{1}H NMR of oil, where the peaks due to glyceryl methylenic hydrogens appear at integration values of 4.00-4.40 ppm. Figures 4.7 to 4.9 (b) are the ^{1}H NMR of ethyl ester obtained after transesterification where the peak due to glyceryl methylenic hydrogen at 4.25-4.35 ppm disappears, and only the peak due to $-OCH_2$ of ethoxy hydrogens of ester appears at integration value of 4.10 ppm. In the reaction where incomplete conversion was obtained, calculation was carried out using the integration values of peaks due to glyceryl methylenic hydrogens at 4.25-4.35 ppm and intergration value of glyceryl methylenic hydrogens and $-OCH_2$ of ethoxy hydrogens superimposed at 4.10- 4.20 ppm.

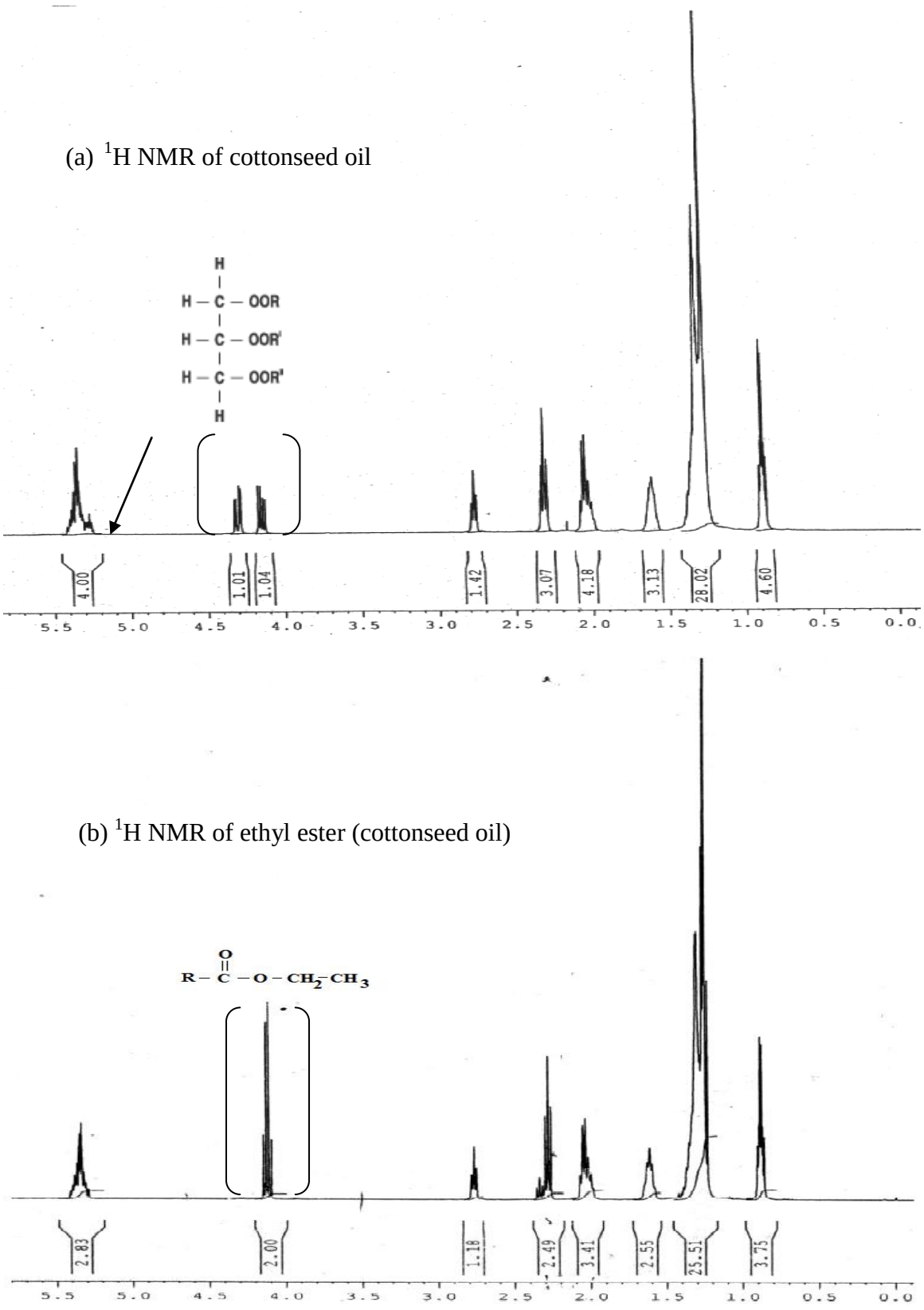
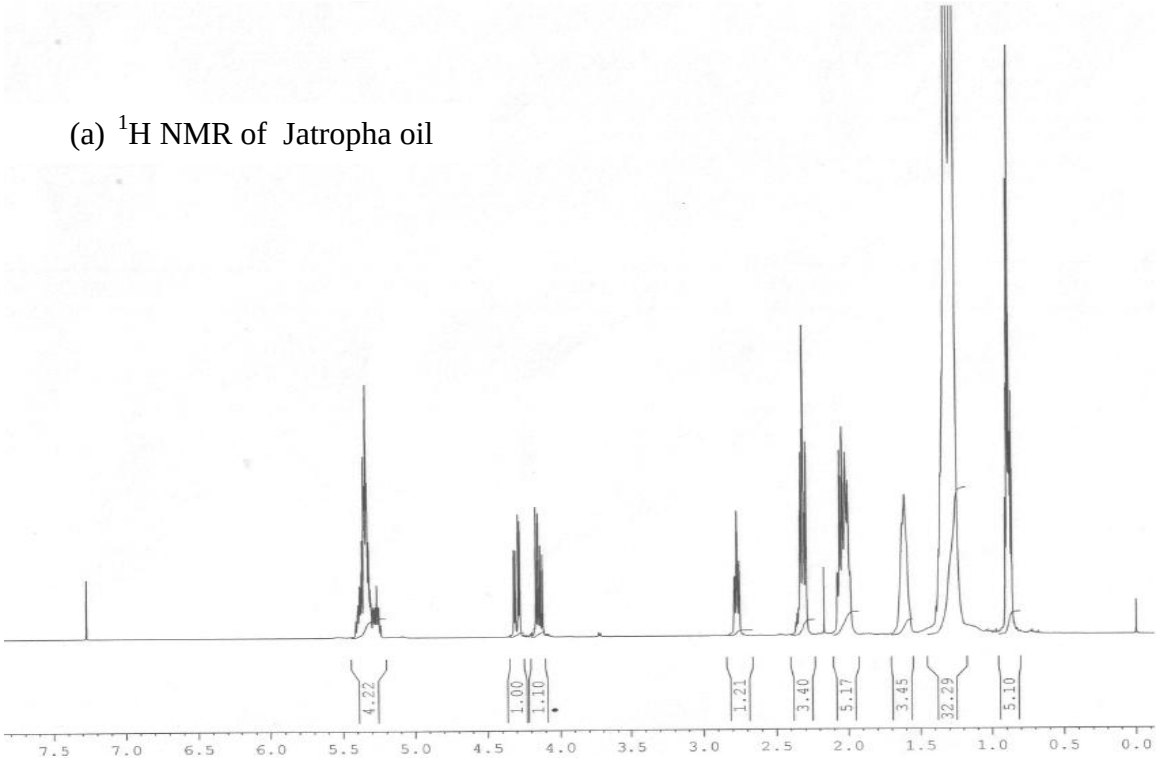


Fig. 4.7. ^{1}H NMR of (a) cottonseed oil and (b) ethyl ester (cottonseed oil)

(a) ^{1}H NMR of Jatropha oil



(b) ^{1}H NMR of ethyl ester (Jatropha oil)

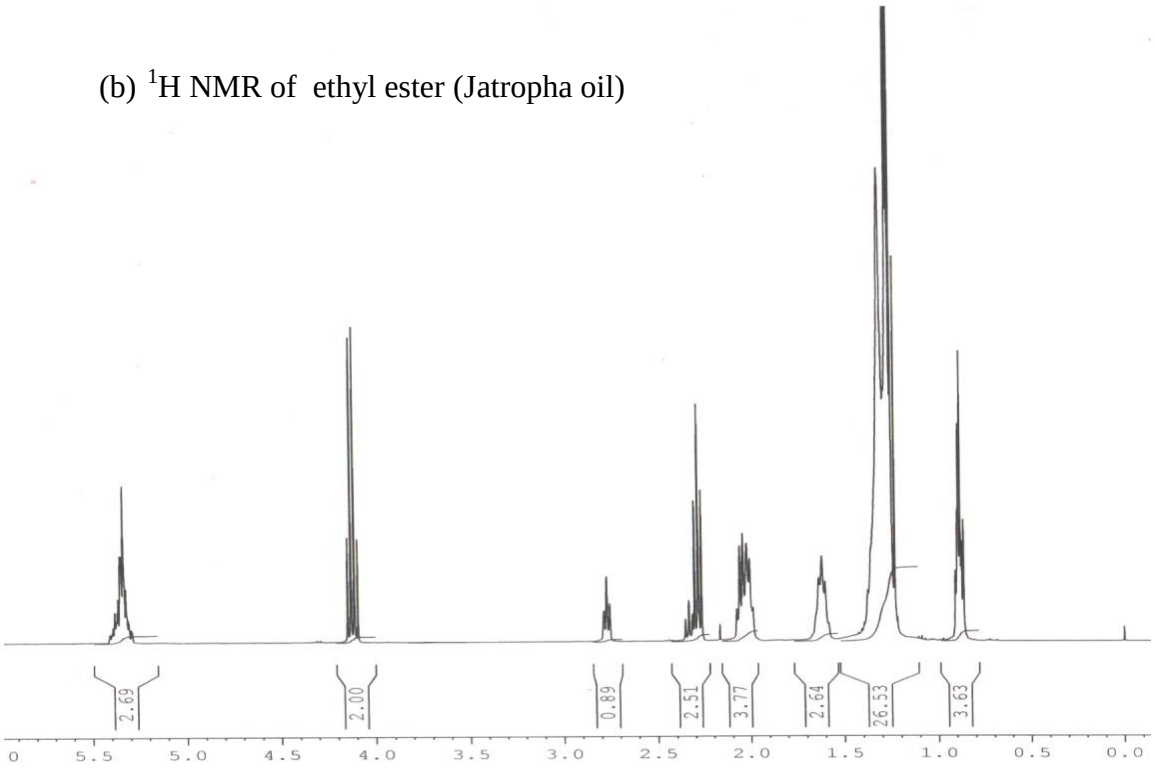
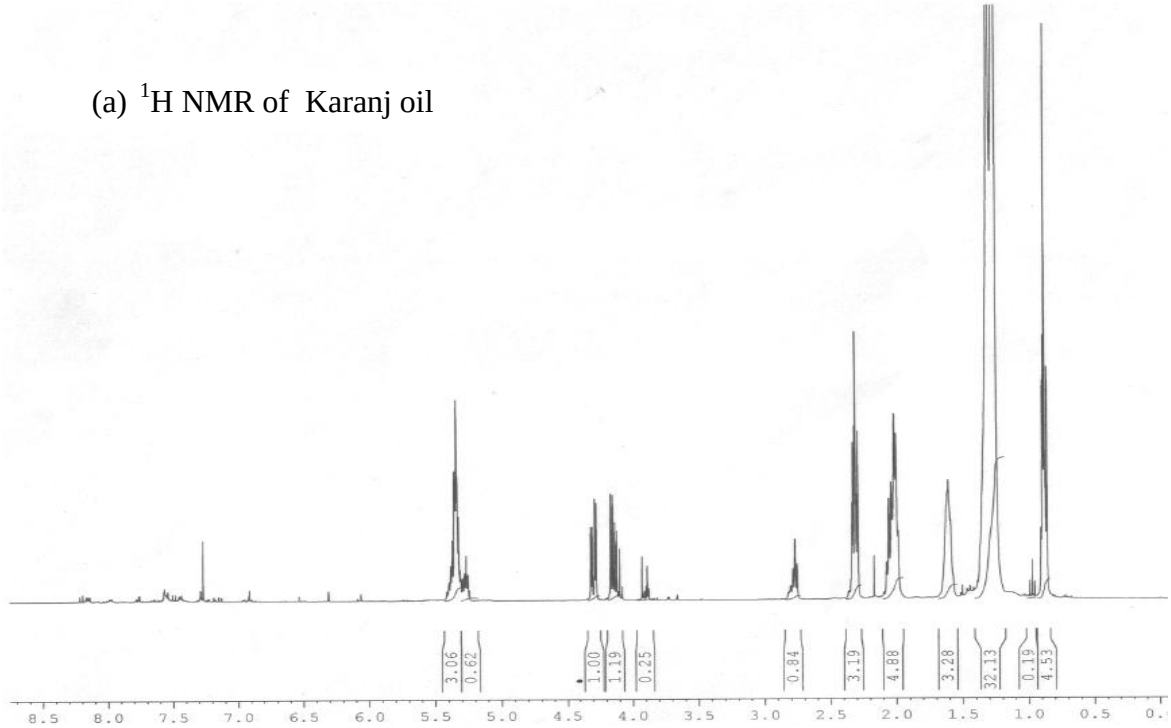


Fig. 4.8. ^{1}H NMR of (a) jatropha oil and (b) ethyl ester (jatropha oil)

(a) ^{1}H NMR of Karanj oil



(b) ^{1}H NMR of ethyl ester (Karanj oil)

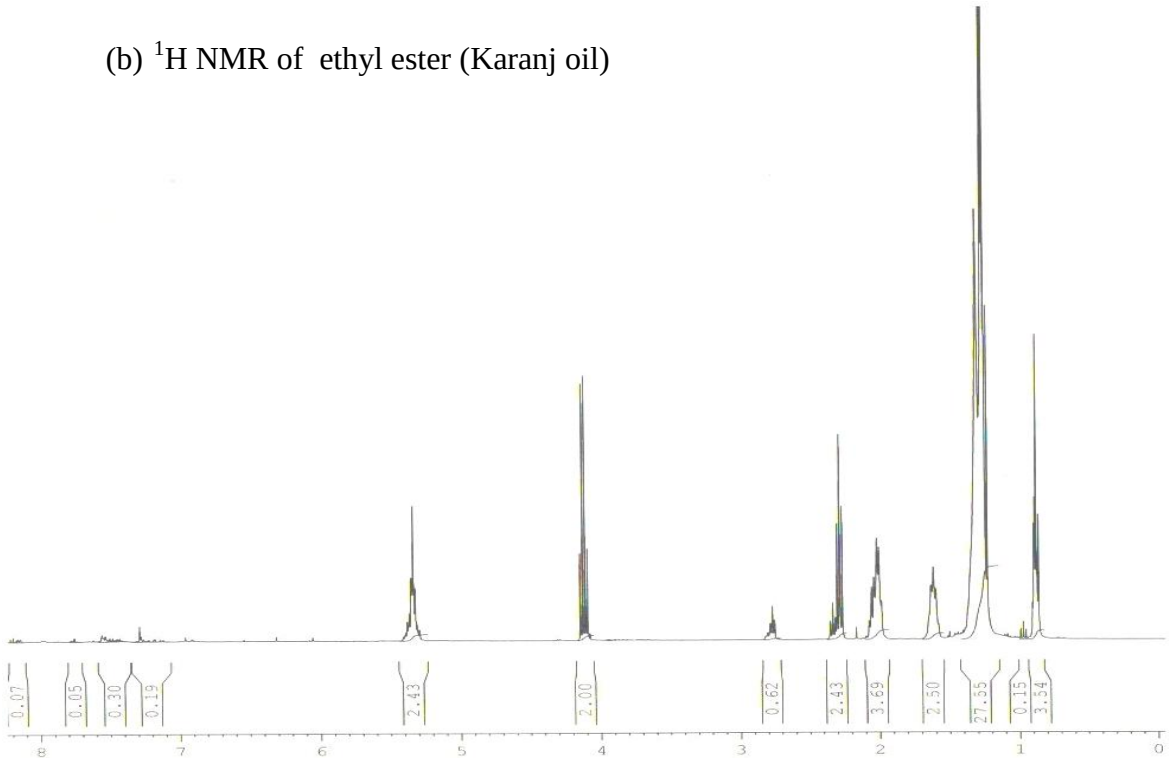


Fig. 4.9. ^{1}H NMR of (a) karanj oil and (b) ethyl ester (karanj oil)

4.5 Optimization of conditions for transesterification reaction with wet biomass

4.5.1 Generation of ethyl ester

Following the conditions optimized for maximum lipolytic activity, further studies were carried out to determine the factors associated with optimum transesterification with supplementation of three different oils namely used cottonseed, jatropha and karanj with wet biomass as a catalyst.

4.5.1.1 Effect of time interval of alcohol addition

Initially, transesterification was optimized by varying the time interval of ethanol addition for all the oils. It was observed that stepwise addition of ethanol at an interval of 12 h resulted in near-complete conversion of oil to ester (Fig. 4.10), however one-time addition of ethanol resulted in 42.6%, 51.3% and 78% yield of ethyl ester (EE) from karanj, jatropha and used cottonseed oil, respectively. The extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl esters of cottonseed, karanj and jatropha oils, (Ref. Annexure II; Fig: 4.5.1.1- I, II and III) showed that with increase in the time interval of alcohol addition, peaks due to glyceryl methylenic hydrogens at 4.25-4.35 ppm disappeared, as the yield of ethyl ester increased.

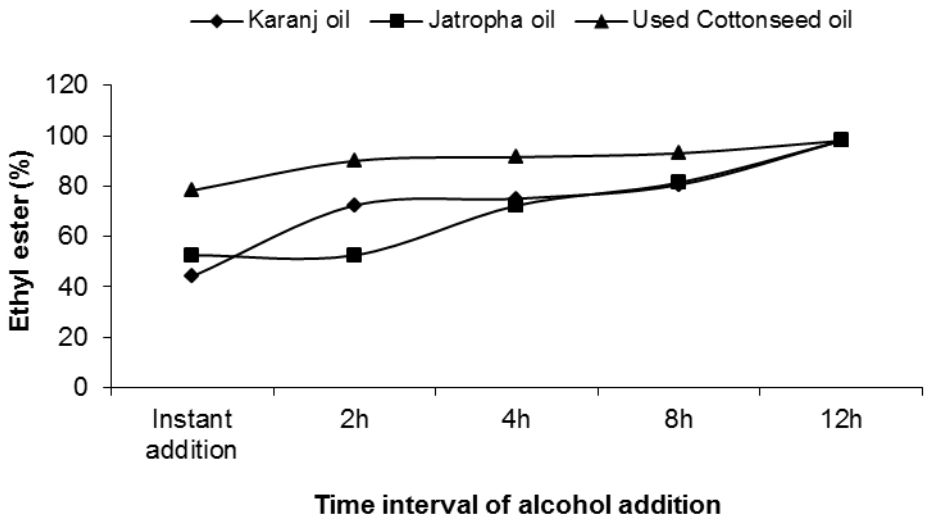


Fig. 4.10. Effect of time interval of alcohol addition on the extent of transesterification

4.5.1.2 Effect of oil supplementation (%) on the extent of transesterification

A near-complete transesterification from oil to ethyl ester (≥98%) was achieved with 50, 60 and 70% oil supplementation after 48 h of reaction from different feedstocks (used cottonseed, jatropha and karanj) (Fig 4.11). Increase in

the concentration of oil upto 90% in the reaction media, however, was noted to decrease the yield of ethyl ester. About 2.9 g, 2.5 g and 2.7 g of biomass was produced during transesterification of 70 g oil in the reaction medium from used cottonseed, jatropha and karanj oil respectively.

The extended region of ^{1}H NMR from 4.00-4.40 ppm of ethyl ester of cottonseed, jatropha and karanj oils, respectively are depicted in Annexure II (Fig: 4.5.1.2: I, II and III). As the concentration of oil increased, the decrease in the ethyl ester yield was indicated by the appearance of peaks due to glyceryl methylenic hydrogens at 4.25-4.35 ppm.

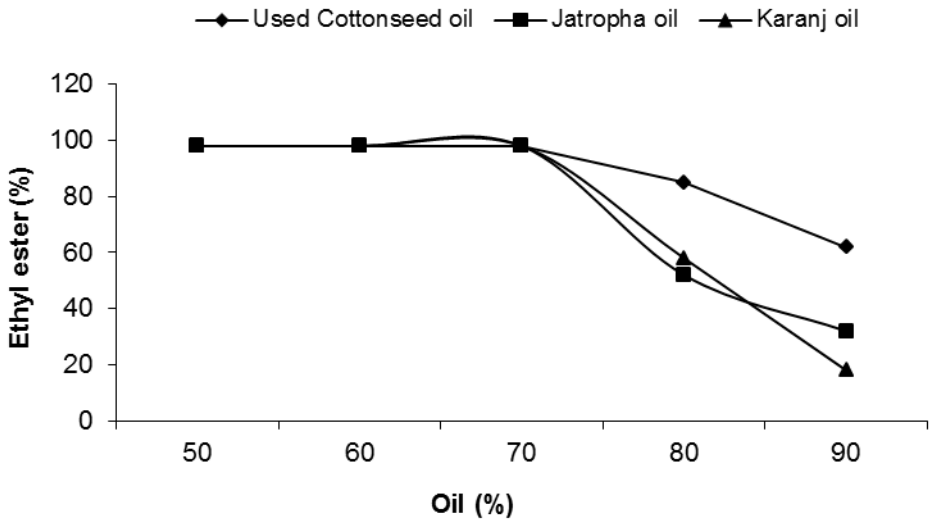


Fig. 4.11. Extent of transesterification (% ethyl ester) with different percentages of various oils

Selected samples that were confirmed to be completely converted to ethyl esters by ^{1}H NMR were further validated using gas chromatograph and the yield percent of ethyl ester was calculated as explain in methodology. The yield percent (%) of product as indicated by gas chromatography was 95.2 ± 3.52, 94.7 ± 3.13 and 95.9 ± 3.60 for used cottonseed, jatropha and karanj oil respectively, that was resulted in ≥98% yield of ethyl ester using ^{1}H NMR. The variations in extent of transesterification as estimated with ^{1}H NMR and GC are attributed to the different analytical approach followed although the difference is not very significant.

4.5.1.3 Transesterification of different oils (at higher supplementation) by varying the percentage of peptone as nitrogen source

Attempts were made to enhance the yield of ethyl ester at higher oil

supplementation (80% and 90%) by modulating the concentration of peptone as nitrogen source. An increasing concentration of peptone enhanced the yield of ethyl ester to a limited extent with maximum of 89% of ethyl ester with 2% of peptone at 80% used cottonseed oil in the growth medium, whereas in the case of 90% oil, 3% peptone in growth medium resulted in maximum of 84% ethyl ester, beyond which ethy ester yield decreased (Fig. 4.12 a & b). The enhancement in yield was observably dependent upon the type of oil in addition to concentration of peptone [Ref. Annexure II; Fig: 4.5.1.3 (a and b)].

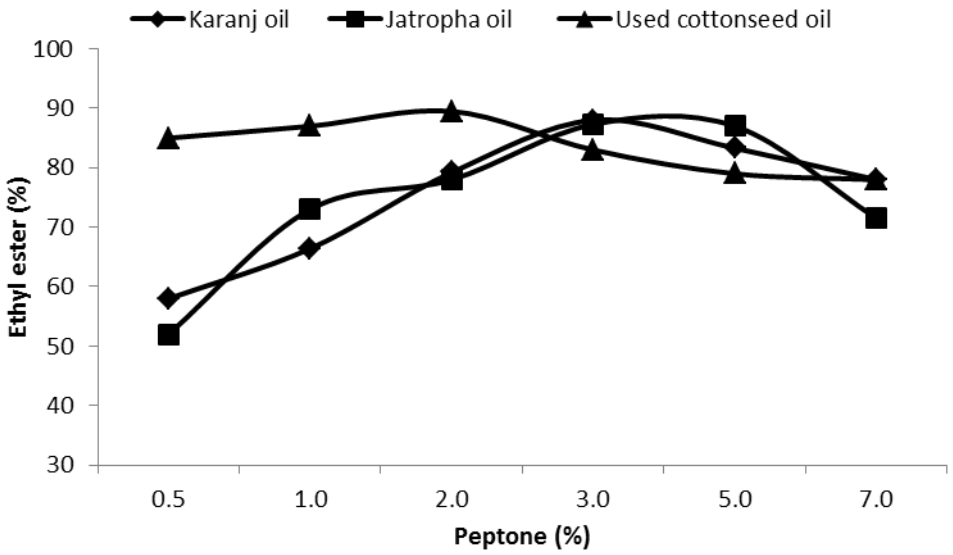


Fig. 4.12 (a). Extent of transesterification (ethyl ester %) at variable supplementation of peptone as nitrogen source at 80% oil supplementation

4.5.1.4 Reusability potential of biomass

Reusability potential of the biomass resulted in decrease in yield of ethyl ester after each cycle. Nearly 50% decrease in the yield of the product was obtained from 1st batch to 5th batch (Fig. 4.13).

Annexure II (Fig 4.5.1.4 (a-e)) presents the variation in ^{1}H NMR of ethyl ester over 5 cycles of biomass reuse. Appearance of peak due to glyceryl methylenic hydrogen indicated the presence of oil at 4.25-4.35 ppm, confirming the decreasing trend in extent of transesterification with repeat use of biomass.

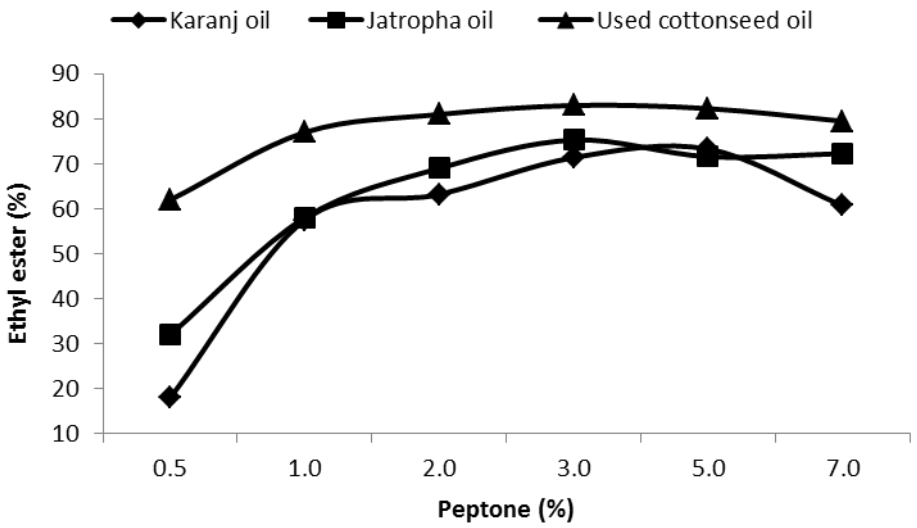


Fig. 4.12 (b). Extent of transesterification (ethyl ester %) at variable supplementation of peptone as nitrogen source at 90% oil supplementation

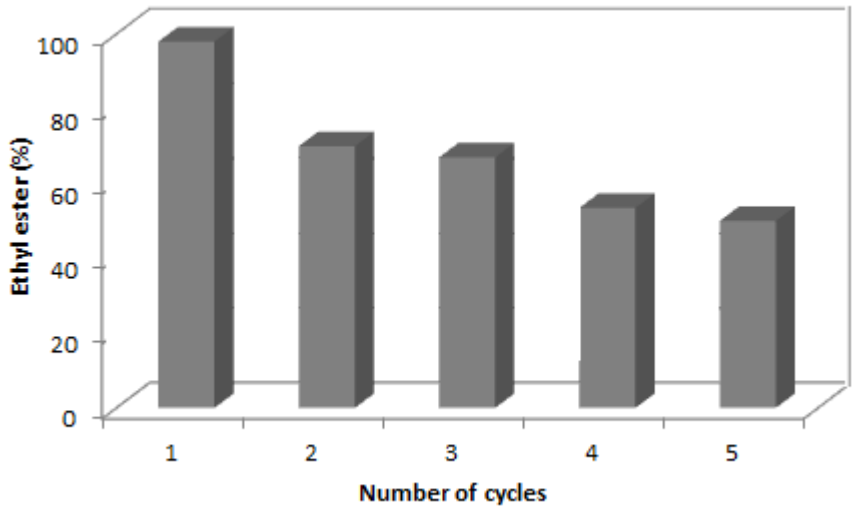


Fig. 4.13. Change in yield of ethyl ester (%) over reuse of biomass

4.5.1.5 Effect of FFA content of oil on the extent of transesterification

The effect of the amount of free fatty acids (FFA) in the oil was also estimated to study its influence on the yield of alkyl ester. The FFA content was 9.8% (acid number of 19.48) in jatropha oil, followed by karanj (4.5%; acid number of 8.95) and used cooking oil (0.28%; acid number of 0.56). The FFA content of the final product (% ethyl ester), represented as acid number, was reduced to 0.52, 0.48 and 0.20 for jatropha, karanj and used cottenseed oil which was within the range of ASTM D 6751-02 standard (Gerpen et al. 2004). Further, it was observed that the extent of

transesterification was not significantly affected by the FFA content (9.8% FFA in jatropha, 4.5 % FFA in karanj oil and 0.28% FFA in used cottonseed oil) in oil as near complete transesterification was obtained with 70% oil supplementation with all the oils.

4.5.1.6 Repeatability of the transesterification reaction using wet biomass

It was observed that across the different experiments carried out for optimizing the conditions for transesterification reaction with wet biomass, the extent of transesterification was near complete under studied reaction conditions [Fig. 4.7 -4.9 (b); Annexure II: Fig. 4.5.1.1 (I- III) e; Fig. 4.5.1.2 (I – III) a; Fig. 4.5.1.4 (a)] in all the cases, indicating the reproducibility of the test conditions.

4.5.2 Transesterification of different oils with methanol

In contrast, the extent of transesterification of methanol to methyl ester with different oils was only 70%, 66% and 62% from used-cottonseed, jatropha and karanj oils (Fig 4.14) respectively.

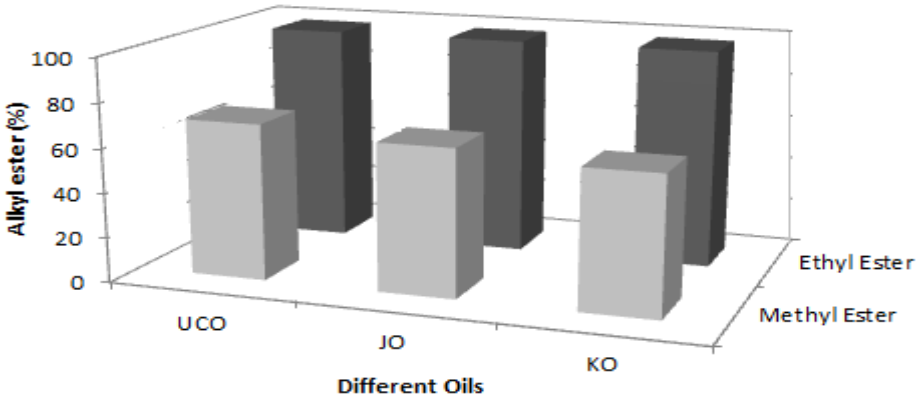


Fig 4.14. Comparative yield of methyl and ethyl esters at 70% oil supplementation (UCO-used cottonseed oil, JO- jatropha oil, KO- karanj oil)

The ^{1}H NMR of methyl ester of used cottonseed, jatropha and karanj oils indicated strong singlet in the region of 3.60 ppm due to the proton of methyl ester (Annexure II; 4.5.2 (a-c)). Protons of the methylenic group at 2.30 ppm adjacent to the ester moiety in triglycerols and the protons in the alcohol moiety of the product methyl ester at 3.60 ppm were considered for monitoring the yield.

4.5.3 Potential of different fungal strains as whole cell catalysts for transesterification

Different fungal strains isolated and screened were also examined for their potential to carry out whole cell catalyzed transesterification. Among the strains tested, *A. aculeatus* resulted in maximum of 77% of ethyl ester from 60% oil in the growth medium, followed by decrease in the yield to 50% ethyl ester in the 80% oil (Fig. 4.15). Whereas, rest of the fungal strains *A. sydowii* and *P. citrinium*, resulted in maximum of 61% and 25% ethyl ester with 20% and 30% oil supplementation respectively, followed by *C. pallescens* and *Perconia sp*. yielding 22% ethyl ester from 10% oil in the growth medium. It was also observed that *F. subglutians* that showed oil tolerance up to 80% oil in the screening experiment, did not catalyze transesterification.

The extended region ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester generated from cottonseed oil, catalyzed by different fungal strains as detailed in Annexure III [Fig. 4.5.3 (I-V)], indicate the appearance of the peaks due to glyceryl methylenic hydrogens at 4.25-4.35 ppm with increase in oil concentration vis-à-vis decrease in yield of ethyl ester.

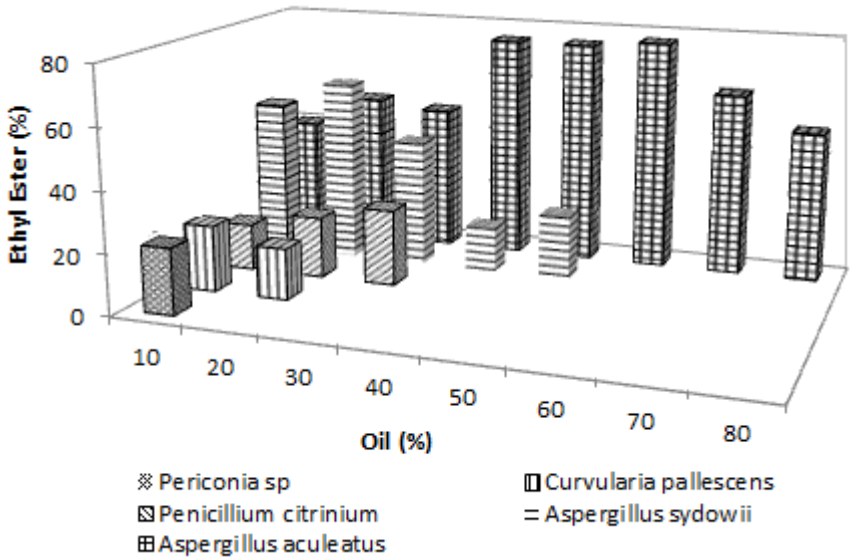


Fig. 4.15. Transesterification potential of different fungal strains

4.5.4 Separation of glycerol

The yield of glycerol, which was separated and concentrated was 6.5(±0.2) g after tranesterification with 70% of oil supplementation in case of near complete transesterification. ^{1}H NMR analysis of glycerol confirmed the similarity of the present by-product with that of commercial glycerol (Fig 4.16 a & b).

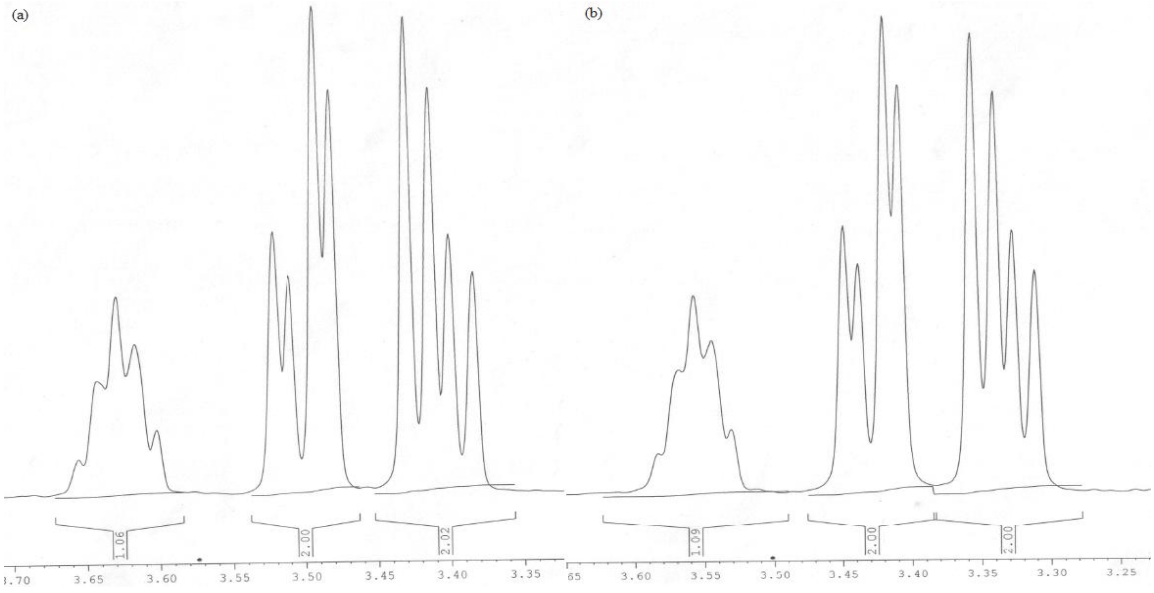


Fig. 4.16. H^{1} NMR spectra of (a) pure glycerol and (b) that obtained by biocatalyzed transesterificaiton

4.6 Optimization of transesterification reaction with dried biomass

A. flavus grown in 30:70 (oil:mineral media) was separated, dried and powdered in liquid nitrogen so as to increase the surface area and maintain homogeneity. The powdered biomass was subjected to transesterification reaction using cottonseed oil and ethanol. Influence of various process parameters on the catalytic potential of dried biomass was further examined.

4.6.1 Amount of biomass

Initially the reaction was standardized by varying the percentage of biomass, wherein the biomass was taken in weight percent (in g) with respect to constant weight of oil (10 g), under temperature set at 35°C, and ethanol addition (1:5 oil to ethanol) at an interval of 8 h.

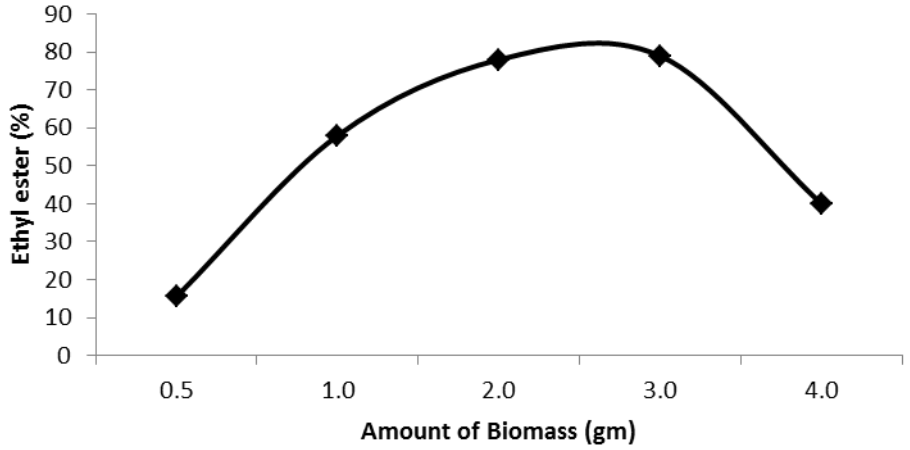


Fig. 4.17. Extent of transesterification (ethyl ester %) using different biomass percentage

It was observed that with increase in the percentage of biomass, the yield of the ethyl ester increased from 5% (15.5% ethyl ester) biomass to 30% (79% ethyl ester) (Fig. 4.17). Subsequent increase in the percentage of biomass (40%) resulted in decrease in yield of the ethyl ester (39%) [Ref. Annexure IV; Fig. 4.6.1 (a-e)].

4.6.2 Effect of time interval of alcohol addition

Further, the time interval of alcohol additon was optimized by adding ethanol (1:5 oil to ethanol) stepwise at an interval of 0, 2, 4, 6, 8, and 12 h, reaction was carried out using 2 g biomass, 10 g oil, reaction temperature 35°C, with total reaction time of 36 h. Ethanol required for transesterification (3.5 ml ethanol) was divided into three parts (1.0; 1.0 and 1.5 ml) and added at different time intervals. It was observed that with increase in time interval of alcohol addition, the extent of transesterification increased as noted with wet biomass. Fig. 4.18 shows that instant addition of alcohol resulted only 36.2% conversion of oil to ethyl ester followed by increase to 83% at 8 h. However, further increase in the time interval to 12 h between the sucessive alcohol additions did not enhance the ethyl ester yield (Fig 4.18). The results varied from wet biomass where in the yield of ethyl ester was 90% at 2 h of alcohol additon and reached up to ≥ 98% at 12 h interval [Ref. Annexure IV; Fig. 4.6.2 (a-f)].

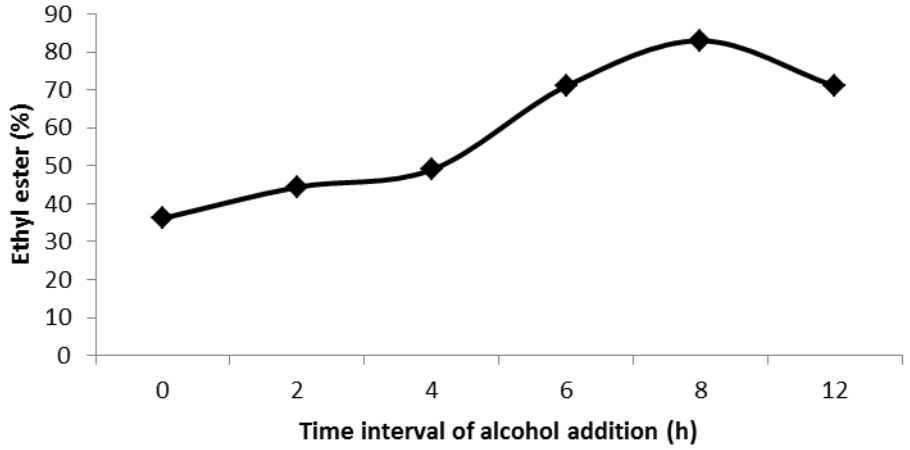


Fig. 4.18. Extent of transesterification (ethyl ester %) at different time interval of alcohol addition

4.6.3 Effect of temperature

Influence of different temperature on the extent of transesterification was also examined under similar reaction conditions. It was observed that yield of ethyl ester enhanced with increase in reaction temperature from 25 °C (41% ethyl ester) to 35 °C (80.5% ethyl ester). Further increase in the temperature from 45 °C (62% ethyl ester) to 65 °C (18% ethyl ester) resulted in sharp decrease in the yield of ethyl ester (Fig. 4.19) [Ref. Annexure IV; Fig 4.6.3 (a-e)].

4.6.4 Effect of molar ratio of oil to alcohol

The influence of molar ratio of oil to alcohol was examined with reference to the excess of alcohol added to carry out reaction in forward direction as transesterification is reversible in nature. The reaction conditions were similar to those followed earlier (2 g biomass, 10 g oil, reaction temperature 35 °C, ethanol added stepwise at an interval of 8 h, with total reaction time of 36 h).

Increase in the molar concentration of alcohol with respect to oil, enhanced the extent of transesterification and yield of ethyl ester 16% (1:3 oil: alcohol) to 81.5% (1:5 oil: alcohol). Further, increase in alcohol (1:6 oil: alcohol) concentration resulted in decrease in the yield of ethyl ester (72% ethyl ester) (Fig. 4.20) [Ref. Annexure IV; Fig. 4.6.4 (a-d)].

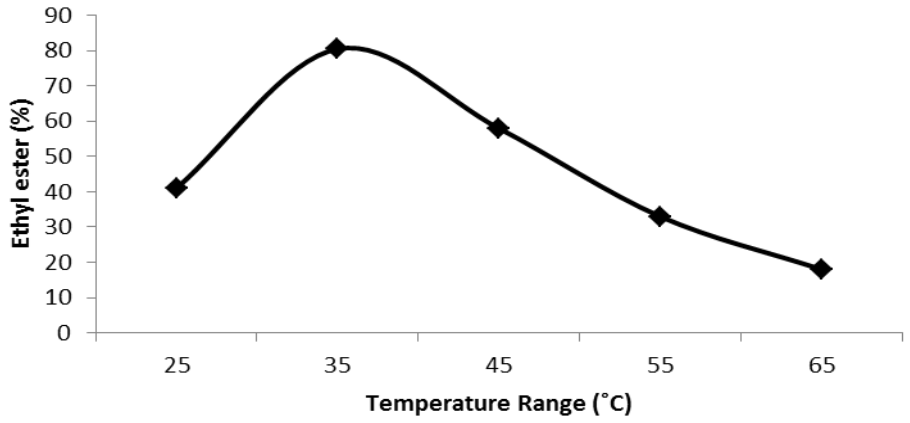


Fig. 4.19. Extent of transesterification (ethyl ester %) at different reaction temperatures

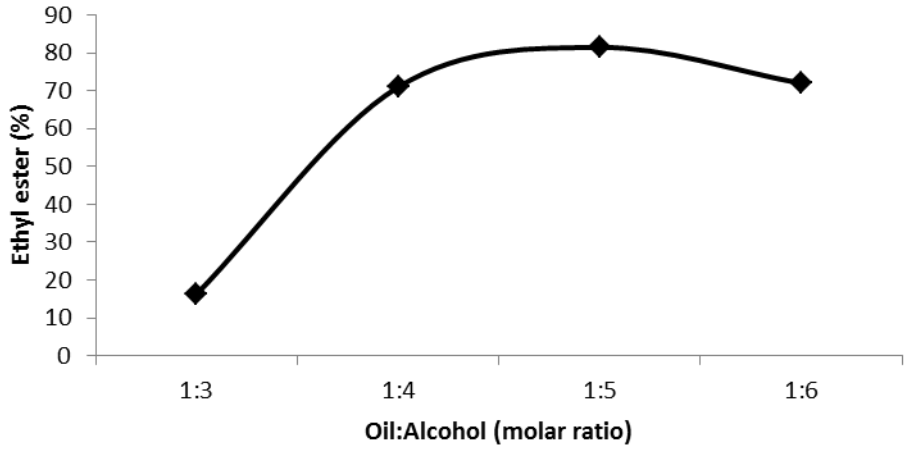


Fig. 4.20. Extent of transesterification (ethyl ester %) at different oil : alcohol molar ratio

4.6.5 Effect of water content on the extent of transesterification reaction

Transesterification using dried biomass was further carried out by varying the percentage of water content in the reaction mixture. Reaction was carried out at conditions mentioned earlier.

With increase in water content from 0 (81.5% ethyl ester) to 2% (83% ethyl ester) with respect to oil, no significant difference was observed in the yield of ethyl ester. However, further increase in water content from 4% to 50% resulted in decrease in the yield from 76% to 24.6% (Fig 4.21), whereas in the case of wet biomass, complete conversion of oil to ethyl ester was obtained from 50% water

in the growth medium [Ref. Annexure IV; Fig. 4.6.5 (a-k)].

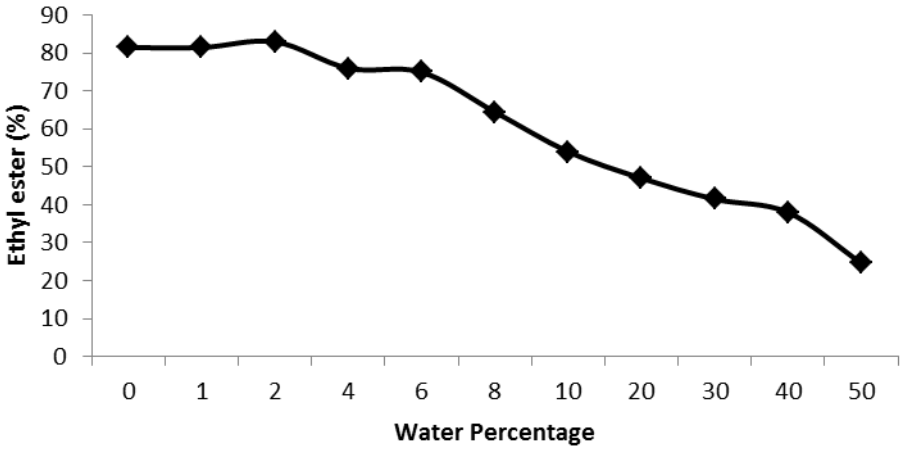


Fig. 4.21. Extent of transesterification (ethyl ester %) in the presence of different water percentage

4.6.6 Effect of frying on transesterification of oil

Transesterification was further carried out with cottonseed oil used for frying and collected at different time interval. Reaction was carried out using 2 g biomass under standardized conditions mentioned earlier.

It was observed that with increase in time of frying the extent of transesterification decreases from 84% (1 h) to 30% (7 h). However from 1st frying to 3rd frying, decrease was only 10% followed by sharp decrease in the yield from 4th (50.7% ethyl ester) frying to 7th (30% ethyl ester) frying (Fig 4.22) [Ref. Annexure IV; Fig. 4.6.6 (a-g)]. Previous studies with wet biomass carried out by our group (Prakash et al. 2010) showed ≥98% of ethyl ester yield upto 3 h frying followed by decreasing trend from 61% to 51%, from 4th h frying to 7th h frying respectively

4.6.7 Transesterification of different oils under standardized conditions

Cottonseed oil yielded maximum of 84% of ethyl ester followed by jatropha (75% ethyl ester) and karanj oil (78.2% ethyl ester) when transesterified using dried biomass under standardized conditions identified with cottonseed oil (Fig 4.23).

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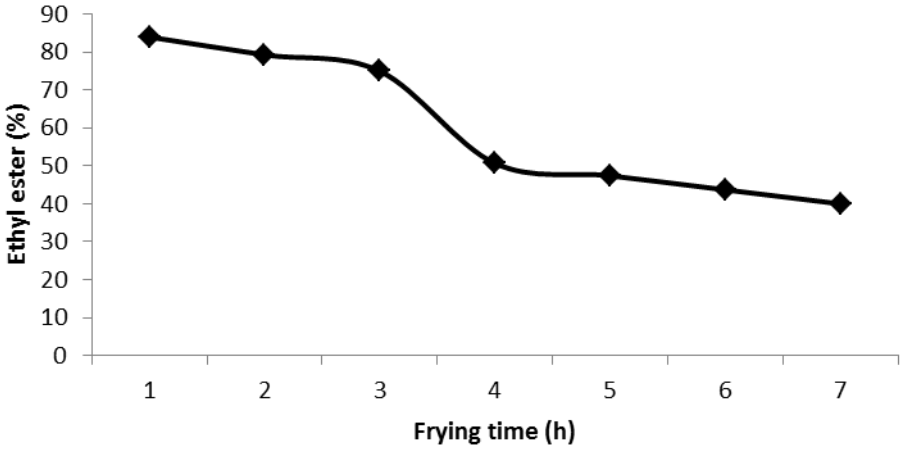

Fig. 4.22. Effect of frying on the extent of tranesterification (ethyl ester %)

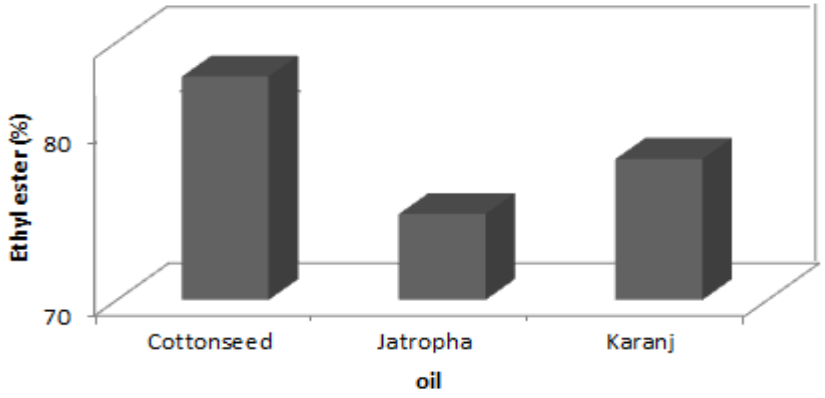

Fig. 4.23. Extent of transesterification (ethyl ester %) of different oils using dried biomass

Annexure IV (Fig. 4.6.7 (a-c)) presents ^{1}H NMR of extended region from 4.00 ppm to 4.40 ppm of ethyl ester obtained from reaction catalyzed by dried biomass using different oils.

4.6.8 Repeatability of the transesterification reaction using dried biomass

The experiments carried out with 2.0 g dried biomass also indicated reproducibility in percent yield of ethyl ester [81.5% (±2.6)] under optimized conditions at various steps of the study [Annexure IV; Fig. 4.6.1(c), 4.6.2(e), 4.6.3(b), 4.6.4(c) and 4.6.5(a)].

4.7 Transesterification reaction by using biomass immobilized over luffa sponge and polyurethane foam

4.7.1 Whole cell immobilization on luffa sponge (*Luffa cylindrica*) and polyurethane foam (PUF)

Immobilization of *A. flavus* after 120 h of submerged fermentation in optimized culture conditions, resulted in 2.8 (±0.3) g and 2.4 (±0.3) g of biomass within polyurethane foam and luffa sponge used as immobilization matrices respectively. The electron micrographs of the matrices with and without immobilization indicated dense mycelial formation on the fibrous network of both the matrices. The fibrous network of the luffa sponge (Fig. 4.24 a) was noted to be completely covered by immobilized fungal cells whereas in the case of polyurethane foam, a uniform network of fungal mycelia was observed over and within the matrix (Fig 4.24 b).

4.7.2 Transesterification of oil (cottonseed) using immobilized matrices

Biomass immobilized in PUF/Luffa (20 pieces each) were taken for carrying out transesterification reaction of cottonseed oil at 28 °C, 120 rpm and ethanol addition at an interval of 8 h, the total reaction time was 36 h. Different levels of oil supplementation (50–100%) were used to examine the extent of transestrification using the immobilized biomass. The percent yield of ethyl ester increased from 24% to ≥98% in the case of PUF and 22% to 74% in the case of luffa sponge when the percentage oil supplementation in the growth medium was 100% oil and 80% oil respectively. Further decrease in oil percentage from 70% (82.8% ethyl ester) to 50% (30% ethyl ester) in the case of PUF and 70% (70% ethyl ester) to 50% (38% ethyl ester) in case of luffa sponge, decreased the yield of the product (Fig. 4.25). In addition, immobilization using PUF resulted in better transesterification over that of luffa sponge [Ref. Annexure V; Fig. 4.7.2 (I-II)]. The FFA content in the ≥ 98% ethyl ester obtained from PUF immobilized biomass was observed to be 0.67 ± 0.04.

With reference to time interval of ethanol addition, in case of PUF, yield of the product increased from 37% EE to 98% EE when ethanol was added at an interval of 0 h and 8 h respectively. Further increase in time interval did not affect the yield. In case of luffa sponge, the yield of the product increased from 36% to 77% when ethanol was added at an interval of 0 h and 8 h respectively. Further increase in time interval (12 h) decreased the yield to 75% ethyl ester (Fig 4.26)

[Ref. Annexure V; Fig. 4.7.2 (III – IV)].

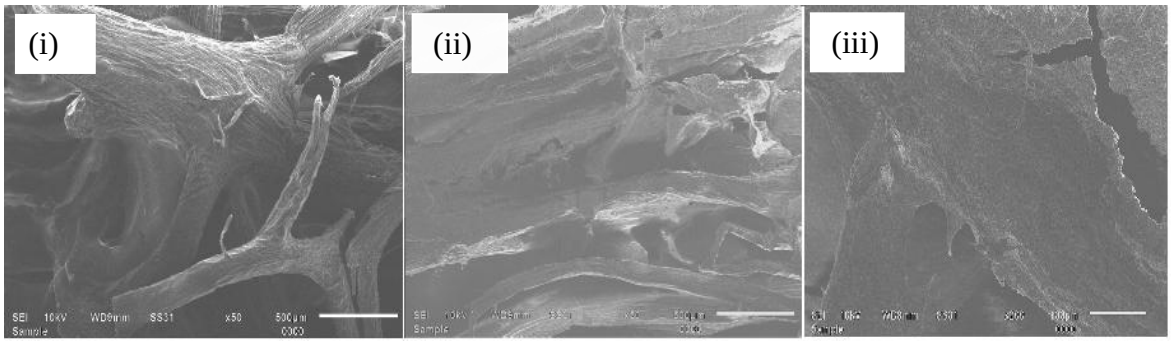

Fig 4.24 (a). SEM images of (i) *Luffa cylindrica* [Magnification: 50X]; *Luffa cylindrica* with immobilized biomass [(ii) Magnification: 50X; (iii) 200X]

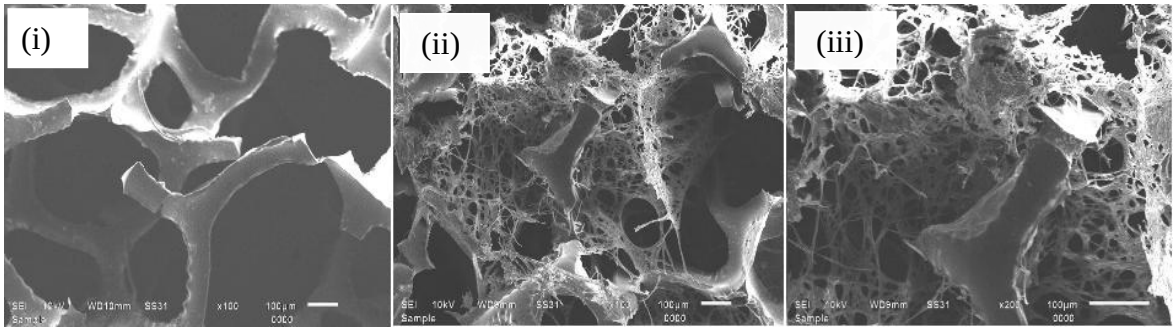

Fig 4.24 (b). SEM images of (i) polyurethane foam [Magnification: 100X], polyurethane foam with immobilized biomass [(ii) Magnification: 100X (iii) 200X]

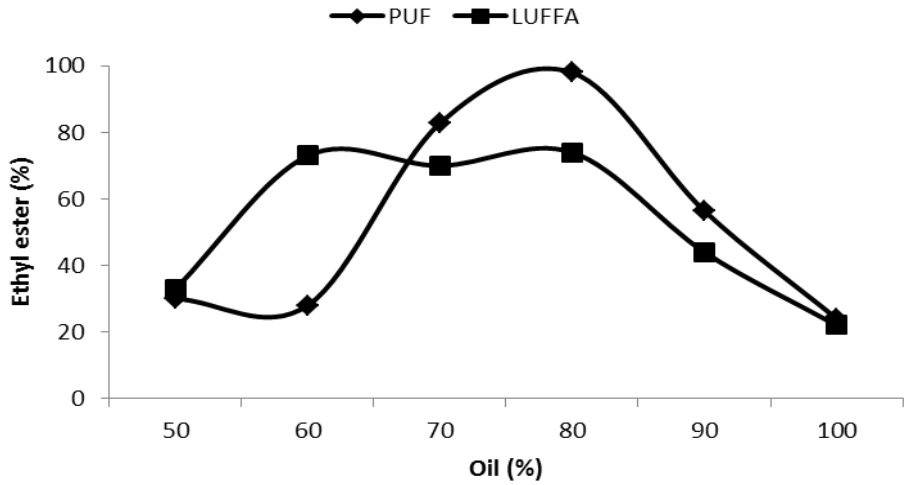

Fig 4.25. Extent of transesterification (ethyl ester %) with varying oil supplementation in reaction medium using immobilized matrices (PUF and Luffa)

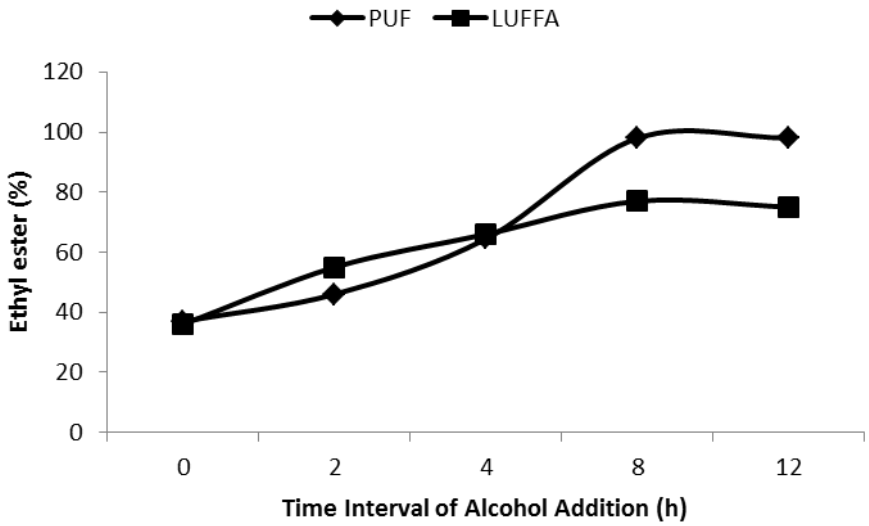

Fig. 4.26. Extent of transesterification (ethyl ester %) of oil with varying time interval of alcohol addition using immobilized matrices (PUF and Luffa)

Re-usability potential of the immobilized biomass on PUF / Luffa was examined by carrying out transesterification reaction at optimum conditions stated earlier over 5 cycles. The potential of the immobilized biomass to catalyse transesterification reaction decreased after each cycle (Fig 4.27). Across five cycles, catalysis with immobilized biomass on PUF resulted in 17.5% decrease from 1st cycle (98% ethyl ester) to 2nd cycle (80.5% ethyl ester). From 2nd cycle (80.5% ethyl ester) onwards, further decrease of 60% in yield of ethyl ester was observed by 5th cycle (17% ethyl ester).

On the other hand, in the case of Luffa, 15% decrease in the yield was observed from 1st cycle (81.5% ethyl ester) to 2nd cycle (67% ethyl ester), followed by only 38% decrease in the extent of transesterification from 2nd cycle (67% ethyl ester) to 5th cycle (28.5% ethyl ester).

Annexure V (Fig. 4.7.2 (V –VI) shows the extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester, produced by immobilized matrices in various standardized conditions using cottonseed oil as raw material, indicating the decrease in extent of transesterification with both the matrices.

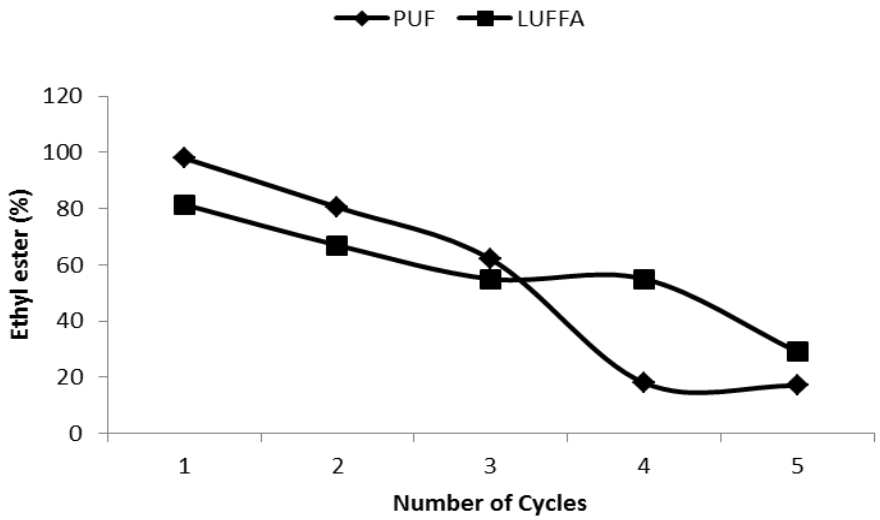

Fig. 4.27. Reusability potential of immobilized matrices (PUF and Luffa) for transesterification (ethyl ester %)

Further, immobilized matrices were used for examining the extent of transesterification of jatropha and karanj oils along with cottonseed oil tested earlier. Polyurethane foam resulted in 98, 95 and 85% ethyl ester with cottonseed, jatropha and karanj oils respectively where as luffa sponge resulted in 78, 75 and 62% ethyl ester respectively (Fig 4.28). Whole cell immobilized in polyurethane foam resulted in better yield of ethyl ester as compared to that in luffa sponge.

During optimization of transesterification using immobilized matrices, it was observed that PUF resulted in near complete transesterification under optimized conditions. However, luffa sponge resulted in an average yield of 77.6% (± 3.6) ethyl ester, which was comparatively lesser than that obtained with immobilized PUF [Ref. Annexure; Fig. 4.7.2 (VII- VIII)].

4.8 Catalysis of esterification reaction with active culture condition and dried biomass of *A. flavus*

In addition to whole cell catalysed transesterification, esterification reaction was carried out with oleic acid as substrate and using either active culture or dried biomass as catalysts. Further, different alcohols were used as acyl acceptor, to examine the influence of chain length of alcohol on the extent of transesterification.

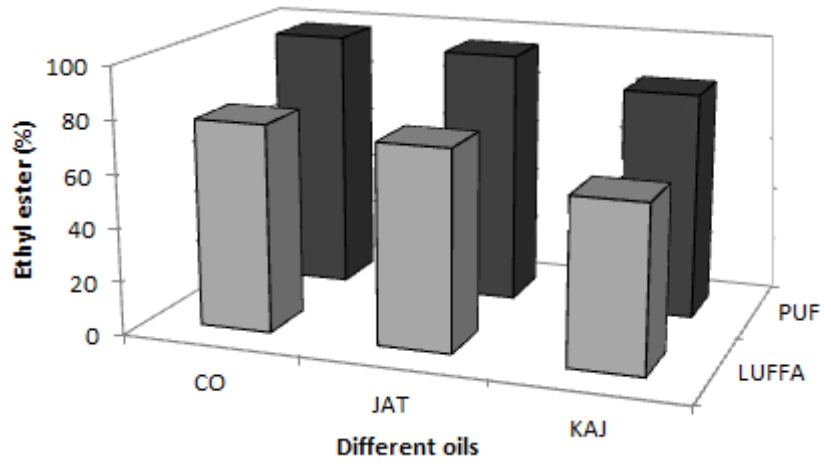

Fig. 4.28. Transesterification (ethyl ester %) of different oils (CO: cottonseed, JAT: jatropha and KAJ: karanj) by using immobilized matrices (PUF and Luffa)

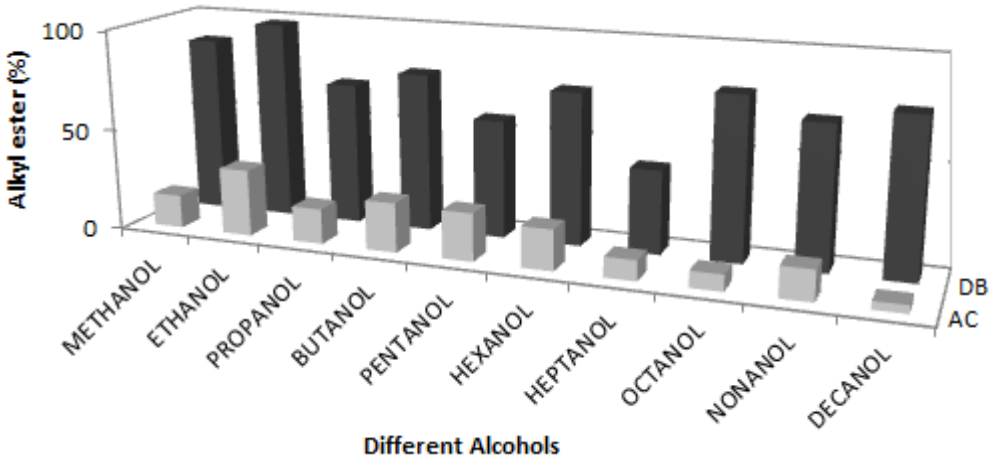

Fig. 4.29. Transesterification (alkyl ester %) of cottonseed with different alcohol using active culture (AC) and dried biomass (DB) of *A. flavus*

It was observed that dried biomass resulted in complete conversion of fatty acid to ethyl oleate. Extent of esterification increases from methanol (87.5% methyl oleate) to ethanol (98% ethyl oleate). Further increase in chain length of alcohol resulted in decrease in the yield from ethyl oleate (98% EO) to decyl oleate (77% DO), however, there were certain variation in decreasing order between wet and dried biomass (Fig. 4.29). It was observed that alcohols with even carbons resulted in better yield as compared to alcohols with odd carbons.

On the other hand, wet biomass resulted in lesser yield as compared to the dried biomass. The yield of ethyl oleate (32.8%) was maximum when compared to the rest of the alcohols, with trend of odd and even carbon chains remaining nearly same.

Annexure VI presents the extended region of ^{1}H NMR from 2.00 ppm to 2.40 ppm of ^{1}H NMR of alkyl esters, produced by dried biomass (I) as well as active culture (II) using different alcohols and oleic acid as raw material.

4.9 Physico-chemical properties of ethyl ester produced

Samples of ethyl ester which obtained from maximum transesterification were generated by carrying out same reaction repeatedly using wet biomass and 70:30, oil: mineral medium under standardized condition of section 4.5, were further examined for different physico-chemical properties as per standards outlined for biodiesel by Indian standard 15607:2005. Table 4.3 present the characteristics of the ethyl ester with reference to standard specified for biodiesel in ASTM D 6751 as well as Indian standard 15607:2005.

Table 4.3. Different physico-chemical properties of ethyl ester produced from karanj, jatropha and cottonseed oil

Parameters	**Karanj ethyl ester**	**Jatropha ethyl ester**	**Cottonseed ethyl ester**	**ASTM D 6751 Standard for biodiesel fuel**	**Indian Standard 15607:2005 for biodiesel**
Calorific Value (kcal kg^{-1})	9385	9575	9440	7870	NA
Flash Point (°C)	105	110	180	130	120
Pour Point (°C)	-1.0	-2.0	-1.0	-15.0 to 10.0	NA
Kinematic Viscosity @ 30°C mm^2/s	6.37	5.53	4.7	4.0-6.0[a]	2.5-6.0
Ash	NIL	NIL	0.01	NA	NA
Density @31°C (g/ml)	0.8786	0.8679	0.8709	0.8780[b]	0.8600 - 0.9000
Acid Number (mg KOH/g oil)	0.52	0.48	0.20	0.80	0.50
Sulphur (%)	0.2	0.14	0.08	0.05	0.005
Water (%)	<0.05	<0.05	0.1	0.05	0.05
Sediment (%)	0.01	0.01	0.09	0.05	0.05
Ester Content (%)[c]	≥98	≥98	≥98	NA	96.5
Ester Content (%)[d]	95.2	94.7	95.9		

[a]@ 40 °C; [b]@15°C; [c] ^{1}H NMR; [d]GC; NA: Not available

5.0 Discussion

Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion bio-industry (Joseph et al. 2008). The availability of lipases has increased significantly since the 1980s. This is mainly due to the huge advancements made in the field of cloning and expression of enzymes from microorganisms as well as increase in demand for these biocatalysts with novel and specific properties such as regio/stereo specificity, stability; and tolerance to wide range of pH and temperature (Bornscheuer et al. 2002; Menoncin et al. 2010). Many microorganisms are known as potential producers of extracellular lipases (including bacteria, yeast, and fungi) (Abada, 2008). Solid-state fermentation (SSF) as well as submerged fermentation (SmF) techniques were employed for the cultivation of microorganisms (Dutra et al. 2008).

Lipase production by filamentous fungi varies according to the strain, the composition of the growth medium, cultivation conditions, pH, temperature and the kind of carbon and nitrogen sources (Cihangir and Sarikaya, 2004). The industrial demand for new sources of lipases with different catalytic characteristics stimulates the isolation and selection of new strains. Lipase-producing microorganisms have been isolated in different habitats such as industrial wastes, vegetable oil processing factories, dairy plants, and soil contaminated with oil and oilseeds among others (Sharma et al. 2001).

Due to the utmost importance of lipase in bio-catalysis and bio-transformations, seven different fungal strains viz., *Aspergillus flavus* (MTCC 5436), *Fusarium subglutians, Aspergillus sydowii* (MTCC 10397), *A. aculeatus, Curvularia pallescens* (MTCC 10390), *Penicillium citrinium* (MTCC 10398) and *Periconia sp* (MTCC 10391) were isolated from contaminated clarified butter sample. Nwuche and Ogbonna (2011), isolated twelve lipase producing fungal strains belonging to *Aspergillus, Penicillium, Trichoderma* and *Mucor* genera from palm oil mill effluent composts. Similarly, 59 lipase-producing fungal strains were isolated from Brazilian savanna soil using enrichment culture techniques (Colen et al. 2006). Winaynuwattikun et al. (2011) isolated 70 different fungal strains from oil-contaminated soil and water sample, which were subsequently screened for potential lipase production using palm oil as enzyme inducer. Further, the best lipase producer,

F. solani was grown in liquid medium by using palm oil as lipase inducer (Winaynuwattikun et al. 2011). The studies outlined in the literature and others indicate that the fungi exhibiting significant activity of lipase can be found in diverse variety of environments. However, most of these organisms have limited tolerance to the presence of oil or other lipophilic substances in the ambient environment.

These isolates mentioned above were further screened on the basis of their oil tolerance and hydrolytic activity. Among all the fungal strains *A. flavus* (MTCC 5436) exhibited maximum oil tolerance upto 80% oil and simultaneously generated 77% FFA during hydrolysis of (40% oil) cottonseed oil. Other strains including *A. aculeatus* showed maximum hydrolysis of oil upto 76.4% FFA with 50% oil followed by *A. sydowii*, *P. citrinium* and *C. pallescens* wherein 63.6% (10% oil), 28.6% (20% oil) and 25% (10% oil) hydrolysis was seen. *Periconia sp.* showed tolerance only upto 10% oil resulting in 31% FFA generation. In contrast, *F. subglutians* showed maximum oil tolerance of nearly 80% oil without any hyrolytic activity. Most of the studies carried out on determining lipolytic activity, have used diverse variety of oils (1-5%) as lipase inducer with limited observation where in oil has also been used as carbon source (Romero et al. 2007; Treichel et al. 2010; Menoncin et al. 2010; Griebeler et al. 2011). Fadıloglu and Erkmen (1999) used different carbon sources as lipase inducers and obtained maximum lipase and biomass production at 3% olive oil supplementation in the growth media. Jonsson and Syngg (1974) optimized the lipase production of four different microorganisms (*Saccharomycopsis lipolytica, Micrococcus caseolyticus, Bacillus licheniformis*, and a *Staphylococcus* sp.) by supplementing growth medium with 2% olive oil. Similar observations have also been reported with coconut oil (Lu and Liska, 1969); olive, corn and soybean oils (Cihangir and Sarikaya, 2004); olive, corn, soy and sunflower oils (Lima et al., 2004), as lipase inducers with variety of microbial systems.

Various biochemical and physical parameters are known to be involved in increasing the enzyme activity, and it is already known that the culture environment has significant influence on enzyme production (Elibol and Ozer, 2001). Yield of the enzyme can be enhanced several folds by providing the suitable cultural conditions. Keeping this in view the oil-tolerant *A. flavus* was examined for its extracellular as well as intracellular lipase production, during growth in oil supplemented medium. Extracellular lipase activity was monitored by modulating pH of the growth medium,

temperature for growth, source of nitrogen and carbon (supplemented as cottonseed oil). During standardization of extracellular lipase production, different nitrogen sources were used for optimization. Peptone (0.5% w/v) as organic nitrogen source and bi-ammonium hydrogen orthophosphate (0.5% w/v) as inorganic source were proved to be appropriate sources of nitrogen resulting in maximum lipase activity of 16 U/ml and 15 U/ml respectively. Observations outlined by Iwai et al. (1884) indicate that high concentrations of nitrogen sources are typically used for the production of fungal lipases. Salleh et al. (1983) obtained maximum extracellular lipase activity *Rhizopus oryzae* with growth medium containing peptone as nitrogen source. High production of lipase also occurred in peptone-supplemented medium in the case of *Pseudomonas* sp., *P. fragi*, *A. wentii*, *Mucor hiemalis*, *M. racemosus* and *R. nigricans* (Chander et al. 1980; Akhtar et al. 1980; Chander et al. 1981; Chopra et al. 1981). Organic nitrogen sources were also found to increase lipase synthesis *by R. oryzae* grown in the presence of oil (Fadiloglu and Erkmen, 1999). Sarkar et al. (1998) and Ginalska et al. (2007) reported decrease in yield of lipase by microorganisms at higher concentrations of inorganic nitrogen. Similar trend was also noted in the present study with reduced enzyme activity corresponding to increase in inorganic nitrogen sources.

The extracellular lipase production was optimized at pH 7.5 and growth temperature of 35 °C. Maximum lipase activity of 17.2 U/ml was obtained at pH 7.5 with *A. flavus*. Further increase in pH from 7.5 to 9.0 resulted in reduced lipase activity i.e. from 17.2 U/ml to 7.5 U/ml. The results are in accordance with studies on *Geotrichum*- like R59, in which the optimum pH for lipase activity was observed to be near by neutral (Ginalska et al. 2007) with significant reduction in activity at pH above 8.0. With reference to optimum temperature for lipase activity, similar results were presented by Corzo and Revah (1999) for lipase of *Yarrowia lipolytica* 681. The activity of lipase, as reported by the authors, was observed to increase in media incubated from 25 to 35 °C with activity reaching up to 16.5 U/ml at 35 °C. Further increase in temperature was observed to decrease the activity after 40 °C. Most of the fungal lipases exhibit maximum activity in the range of 22–35 °C as observed in case of *A. wentii* (Chander et al. 1980), *M. heimalis* (Akhtar et al. 1980), *R. nigricans* (Chander et al. 1981), *M. racemosus* (Chopra et al. 1981), *R. oligosporus* (Nahas et al.

1988) and *P. aeruginosa* (Gilbart et al. 1991). Abbas et al. (2002) found that *Mucor sp.* showed maximum lipase activity at 35 °C which was very similar to our findings.

The extracellular lipase activity of 21.8 U/ml was obtained with 50% (v/v) oil acting as the main carbon source and peptone (0.5% w/v) as nitrogen source under standardized conditions of pH 7.5 at 35 °C. Majority of the reports, till date, have used oil as an inducer (1-3%) only for microbial synthesis of lipases, with limited reports available on use of oil as the main carbon source. Triglycerides such as olive, groundnut and cotton seed oils; and fatty acids such as oleic acid, linoleic acid and linolenic acid stimulated lipase production by *P. mephitica* (Jonsson and Snygg 1974). Mladenoska and Dimitrovski (2001) tested several lipids (sunflower oil, olive oil and sunflower oil sediment) for their effect on lipase production by *Geotrichum candidum-M2* and obtained highest lipolytic activity of 0.28 U/ml by using sunflower oil sediments followed by sunflower oil (0.2 U/ml) and olive oil (0.1 U/ml). Lima et al. (2003) evaluated the effect of carbon source (1% olive oil supplementation) on the production of lipase by *Penicillium aurantiogriseum.* The lipolytic activities in the culture broth were approximately 5.0 U/ml when sunflower, corn and soybean oils were used, as compared to an activity of 12.5 U/ml in the presence of olive oil. The observations are of significance due to limited reports on supplementation of oil as carbon source to the extent of 50% of oil resulting in significant extracellular lipase activity of 21.8 U/ml.

Under the similar standardized conditions applied in determining the activity of extracellular lipase i.e., pH 7.5, temperature 35°C, the activity of intracellular/cell bound lipase was examined by varying the concentration of cottonseed oil (10-90%) in the growth medium. With increase in oil percentage, intracellular lipase activity enhanced upto 70% oil concentration in the growth medium resulting in 1811 µmol/ml FFA production in 1 h. During the growth of 120 h, FFA production increased along with biomass from 10% oil (3.3 g biomass, 65.5% FFA) to 70% (8.8 g biomass, 83% FFA), in contrast to control (devoid of oil) only 0.7 g biomass was produced. The results clearly indicate that oil, in the current context, played the role of lipase inducer as well as carbon source. Further increase in oil percentage did not correspondingly increase the biomass and also the lipase activity was relatively lesser. This is presumably being due to decrease in water content in the reaction mixture. Fadiloglu and Erkmen (1999) also reported that in the presence of oil biomass

production increases. Yan and Yan (2008) and Burkert et al. (2004) tested a combination of different experimental designs to optimize the production conditions of cell-bound lipase from *Geotrichum sp.* According to the authors, the most suitable carbon source was a mixture of olive oil and citric acid and the most suitable nitrogen source was a mixture of corn steep liquor and NH_4NO_3 in the experimental conditions followed for the study. Yan and Yan (2008) reported 23.15 U/ml of cell bound lipase (CBL) activity by growing the *Geotrichum* sp in 1% olive oil. Long et al. (2007) reported the hydrolysis of soybean, corn, cottonseed, rapeseed, palm and coconut oil, after 1 h reaction, containing 10 g oil in water saturated n-hexane, 0.5 g mycelium resulting in 0.28, 0.32, 0.20, 0.27, 0.40 and 0.42 mmol of FFA respectively.

Transesterification is one of the reactions catalysed by lipase and of utmost importance for biodiesel industry so as to convert oil to alkyl esters whose properties are very similar to the diesel fuel (Akoh et al. 2007). In the present study, *A. flavus* (MTCC5436) was further exploited for the transesterification of different edible (used cottonseed oil) and non-edible oils (jatropha and karanj oils). Three approaches with varying forms of biomass, viz., active wet culture, dried biomass and immobilized biomass (in polyurethane foam and luffa sponge) were followed for catalyzing the transesterification reaction. In addition, the effect of chain length of alcohol on esterification of oleic acid was also checked by using active culture as well as dried biomass.

5.1 Studies with wet biomass

With reference to the studies with active wet biomass, initially, time interval of alcohol addition was optimized by adding alcohol at different time intervals ranging from 0 to 12 h. It was observed that with increase in time interval between alcohol additions, the extent of transesterification increased in terms of conversion of oil to alkyl ester. The instant addition resulted in very limited yield of only 42.6, 51.3 and 78% of ethyl ester from karanj, jatropha and used cottonseed oil, respectively. Whereas, a distributed addition of 15 ml at regular intervals of 12 h was observed to facilitate a nearly complete conversion of oil to alkyl ester with maximum yield of ≥98%. Further, transesterification was carried out by using different levels of oil supplementation in the growth media. Supplementation of growth medium with 50, 60 or 70% of oil (jatropha, karanj or used cottonseed) followed by whole cell catalysis

resulted in nearly complete conversion of all the oil (≥98%) to alkyl esters in 48 h of reaction time. However, further increase in oil percentage (80-90%) resulted in decrease in the extent of the conversion. The growth of the biomass was associated with the hydrolysis of oil to free fatty acids and glycerol, followed by esterification of fatty acids to ethyl ester in the presence of ethanol. Further, the step wise addition of ethanol facilitated better result as compared to one time addition which may be attributed to the fact that when polar substrate gets accumulated in the microenvironment of the enzyme it may cause the denaturation of the proteins (Jin et al. 2008; Villeneuve et al. 2000; Trubiano et al. 2007). Rottig et al. (2010) reported that most of the transesterification work was carried out with edible oils, with no report available till date on the utilization of non-edible oils (jatropha and karanj oil) as a raw material for the transesterification with whole cell as a catalyst. Furthermore, whole cell catalysis reported the utilization of methanol with incomplete conversion (Rottig et al. 2010).Jin et al. (2008) reported a yield of 85.2% ethyl ester through transesterification of canola oil using *R. oryzae*. Further, Koda et al. (2010) reported the production of 79% and 94% of ethyl ester, respectively, by using *R. oryzae* and a recombinant *A. oryzae* expressing *F. heterosporium* lipase in 96 h of reaction time. Attempt was made to enhance the alkyl yield at 80% and 90% oil supplementation by varying the percentage of peptone as nitrogen source. Modulating the nitrogen sources in the media so as to enhance the yield of ethyl ester was observed to have only limited influence on the yield of ethyl ester. .

Based on the observations reported by other research groups and those obtained in the present study, it is indicative that the yield of alkyl esters under whole cell catalyzed transesterification is dependent on the type and concentration of oil; type and concentration of nitrogen source; type of acyl acceptor; and total reaction time.

Observations on whole cell catalyzed transesterification, as on date, have been mainly focused towards generation of methyl esters with limited reports on using ethanol as an acyl acceptor and to target complete conversion of oil to ethyl ester (Rottig et al. 2010). Under similar conditions examined for ethyl ester generation, transesterification was also carried out using methanol wherein 70, 66 and 62% methyl ester was obtained from 70% supplementation using cottonseed, jatropha and karanj oil respectively, in growth medium. Rottig et al. (2010) indicated that most of

the reports on transesterification carried out with edible oils (soybean, rapeseed, olive oil) using whole cell catalysis are attributed to *Rhizopus* sp. and methanol. Recent observations of Xiao and Obbard (2010) showed 81.2% methyl ester conversion with virgin soybean/palm oil as carbon source with 8% water content using *Aspergillus* sp. as whole cell biocatalyst. With reference to comparison of ester yield with methanol or ethanol as acyl acceptor, yield of ethyl ester was ≥98% with either of the oil used in the study (cottonseed oil, jatropha or karanj oil) whereas of methyl ester yield was maximum to an extent of 70% with used-cottonseed oil and lesser yields with other oils. Issariyakul et al. (2006) reported that during transesterification, ethanol is preferred to methanol for it is renewable, eco-friendly, and favourable solvent property. In addition, rapeseed ethyl ester showed some desirable attributes over its methyl esters for having significantly lower smoke opacity, lower exhaust temperatures, and lower pour point (Issariyakul et al. 2006). The yield of transesterification or esterification products using ethanol was also observably better as compared to methanol (Mittelbach, 1990; Nelson et al. 1996; Abigor et al. 2000; Hsu et al. 2001; Jin et al. 2008). With reference to the catalytic activity, lipases are more tolerant to ethanol as compared to methanol (Fukuda et al. 2001), as lipases act on long-chain fatty alcohols better than on short-chain ones (Shimada et al. 1997; Shimada et al. 1998). Ethanol also results in more stable esters in the presence of whole cell lipase as it shifts the FFA/ester equilibrium towards ester (Jin et al. 2008). Moreover, Issariyakul et al. (2007), Kulkarni et al. (2007) and Joshi et al. (2009) also reported generation of mixtures of ethyl and methyl esters using ethanol and methanol at varying transesterification reactions. As an alternative to methanol, ethanol is also preferred in some parts of the world, where biodiesel (alkyl esters) are prepared from absolutely bio-based resources (Abigor et al. 2000; Hsu et al. 2001; Mittelbach 1990; Nelson et al. 1996).

In recent past, other alcohols such as butanol were used as acyl acceptor due to their eco-friendly nature (Qureshi et al. 2008). The classic vegetable oil or animal fat ethanolysis conditions incorporate 6:1 molar ratio of ethanol to oil, 0.5 wt.% catalyst (with respect to TAG), 600+ rpm, 75°C reaction temperature, and 1 h reaction time to produce fatty acid ethyl esters (FAEE) and glycerol (Freedman et al.1984). Ethyl ester to the extent of 79% and 94% was produced through transesterification of rapeseed oil with ethanol from two types of whole cell biocatalysts named wild type *R. oryzae*

producing tri-acyl glycerol lipase (w-ROL) and recombinant *A. oryzae* expressing *F. heterosporum* lipase (r-FHL) respectively (Koda et al. 2010). There should be several factors which affect the transesterification efficiency including the denaturation of lipase by alcohol, alcohol accessibility to active site of lipase, and nucleophilicity of alcohol etc. Therefore, we could not readily conclude the reason for the effect of alcohol species (Koda et al. 2010).

With the observations obtained on transesterification reaction using *A.flavus*, the study was extended to the other strains isolated from the clarified butter. Under optimized conditions determined for the lipase activity in *A.flavus*, strains namely *A. aculeatus*, *A. sydowii*, *P. citrinium*, *C. pallescens* and *Perconia* sp. were used as whole cell catalysts for generation of ethyl esters (EE), out of which tranesterification reaction with *A. aculeatus* resulted in maximum of 77% of EE 60% oil supplementation in the growth medium, whereas, with *A. sydowii* and *P. citrinium*, the yield of ethyl esters were 61% and 25% EE from 20% and 30% oil respectively, followed by *C. pallescens* and *Perconia sp*. resulting in only 22% EE from 10% oil in the growth medium. Till date, most of the work has been carried out using *Rhizopus* sp (Rottig et al. 2010) and *A. niger* (Xiao et al. 2009), thus the present study, expands the horizon of exploring other strains of oil-tolerant fungi for such potential reactions.

An attempt was made to reuse the biomass of *A. flavus* over 5 cycles, to determine its potential to catalyze transesterification reaction. Nearly 30% decrease in the yield of the product was obtained from 1st cycle to 2nd cycle and from 2nd cycle to 5th cycle, decrease was only 20%. Majority of studies on the reuse of biomass have been associated with immobilized systems (Ban et al. 2002; Aria et al. 2010)

The significant features of this study when compared to the observations reported by earlier researchers are the simultaneous (a) growth of biomass through utilization of oil as lipase inducer as well as carbon source leading to hydrolysis for oil to fatty acids (FFAs); and (b) transesterification process through esterification of FFAs to ethyl esters, which hitherto has been less reported at oil supplementation of upto 70%. In addition, ease in generation and seperation of glycerol is an additional advantage associated with this mode of ethyl ester synthesis, which has not been reported till date from whole cell catalyzed transesterification reactions (Gerpen et al. 2004).

5.2 Studies with dry biomass

With the promising observations recorded with the active wet biomass on the significant yield of alkyl esters, attempt was made to examine the potential of dry biomass of *A. flavus* to catalyze transesterification reaction under similar conditions as mentioned earlier, but in a non-aqueous environment.

Initially, the amount of biomass required for the catalysis, was optimized by varying the percentage of biomass as catalyst in the reaction with respect to oil. It was observed that with increase in the biomass percentage, yield of EE increased wherein maximum yield of 79% EE was obtained with 30% biomass. Further increase in biomass concentration decreased the product yield, presumably due to increased viscosity of the reaction mixture in which homogeneous stirring couldn't take place. The observations on the excess biocatalyst not contributing to increase in percentage conversion is also further supported by observations of Torres and Otero (2001) showing that the excess of enzyme does not necessarily increase the percentage conversion and sometimes leads to decrease in the yield of product.

In case of dried biomass, observations on time interval of alcohol addition showed that instant addition of alcohol resulted in only 15.5% yield of EE, whereas maximum yield (83% EE) was observed when alcohol was added at an interval of 8 h, with 20% biomass. A yield of 36.3, 44.4 and 48.6% EE was obtained when alcohol was added at an interval of 0 h, 2 h and 4 h, respectively, thereby indicating inactivation of the reaction due to excess alcohol possibly due to denaturation of lipase by interfering with hydrogen bonding between water and amino acid residues (Adelercreutz, 2000; Paez et al. 2003; Trubiano et al. 2007; Villeneuve et al. 2000). Comparatively, low yields (71% EE) were obtained when the time interval of alcohol addition was increased from 8 h to 12 h, due to reversible nature of reaction.

The influence of alcohol on the extent of transesterification by dry biomass was further examined by varying the oil/alcohol molar ratio, maximum yield of ethyl ester (81.5%) was obtained at an oil/alcohol molar ratio of 1:5 with 20% biomass. Further increase in alcohol concentration to a ratio of 1:6 decreased the yield of the product to 72%. The decrease in activity is also presumed to be due to inactivation of enzyme by excess alcohol as explained earlier. Further studies were carried out by varying the water content in the reaction mixture. Presence of 2% moisture in dry biomass lead to 83% EE production, followed by decreasing trend in yield with

increase in moisture, that is 24.6% EE at 50% water content. This is presumed to be due to the reduced availability of interfacial region for transesterification reaction. Xiao and Obbard (2010) reported that the water content in the feedstock had a variable influence on the transesterification reaction, where the highest fatty acid methyl ester (FAME) content i.e. 81.1%, was obtained with 10% water content. One of the most important variables affecting the yield of ester is the molar ratio of alcohol to vegetable oil. The stoichiometry of this reaction requires 3 mole of alcohol per mole of triglyceride to yield 3 mole of fatty ester and 1 mole of glycerol. Bradshaw and Meuly (1942) stated that a 4.8: 1.0 molar ratio of methanol to vegetable oil led to 98% conversion, and step wise addition of alcohol can further reduce 4.8:1.0 to 3.3:1.0. In the ethanolysis of peanut oil, a 6:1 molar ratio liberated significantly more glycerol than did a 3:1 molar ratio (Feuge and Gros, 1949).

Further, transesterification was optimized for reaction temperature of 35 °C with EE yield reaching 80%. Whereas, at lower and upper range of temperature, the ester yield was less due to the fact that at lower temperature enzyme was presumably inactive, whereas increasing the temperature above optimum value caused the enzyme to lose its activity as observed with Lipozyme RM-IM by Vierira et al. (2006) and Trubiano et al. (2007). Xiao and Obbard (2010) conducted experiments for determining optimal reaction temperature over a range from 25 to 50 °C. At an optimum temperature of 30 °C, 86.4% FAME yield was achieved after 72 h. A sharp decrease in yield above 40 °C was likely due to thermal inactivation of the enzyme, as has been observed by Rodrigues et al. (2008).

Dried biomass of *A. flavus* was further used for the transesterification of frying oil generated at different time intervals. It was observed that with increase in frying time (from 1 h to 7h), the yield of EE (from 84% to 30%) decreased. Maximum yield of 84% EE was obtained using oil collected of 1 h of frying. This observation was further supported by our earlier observations (Prakash et al. 2010), wherein active culture of same organism *A. flavus* was used for transesterification. The observations on FFA generation due to frying indicated increase in FFA content from 0.2% to 8.3% with increase in frying time from 10 min to 7 h, during biocatalyzed transesterification, which is reversible in nature, the tri-glycerides and partial glycerides are first hydrolyzed to partial glycerides and FFA respectively, after which ethyl esters are synthesized from FFA and ethanol (Jin et al. 2009) and the FFA which

was generated during frying, easily got transesterified. The yield of ethyl esters was consistently >98% with oil collected till 3 h frying, beyond which it reduced to 51% with 7 h frying oil. The reduced EE yield is presumably due to factors other than FFA influencing the transesterification reaction with used frying oil.

Furthermore, under similar optimized conditions viz., 2 g biomass, 10g oil and reaction temperature of 35°C identified for cottonseed, the FFA content in jatropha and karanj oil were found to be 9.8% and 4.5% and the corresponding EE yield was 75 and 78.2% respectively in 36 h of reaction time. Jin et al. (2009) examined the use of a whole-cell biocatalyst to transesterify triglycerides, including high-FFA containing waste greases, in a water-containing system. The whole-cell biocatalyst derived from *R. oryzae* (ATCC10260) was grown and incubated at room temperature. Results showed that whole-cell biocatalyst was able to produce alkyl esters with a yield of about 75% for virgin canola oil, 80% for waste vegetable oil (0.6%-3.7% FFA) and 55% for brown grease (80% FFA) with a 72 h transesterification reaction using methanol. The reduced yield of methyl esters as observed by Jin et al. (2009) was suggested by the authors to be due to the use of methanol as reactant. Comparatively, the present study demonstrated 83, 75 and 78.2% of ethyl ester yield with ethanol as acyl acceptor using cottonseed oil, jatropha and karanj oil, in 36 h of reaction time, using dried biomass.

5.3 Studies with immobilized biomass

Further, the transesterification studies were extended towards use of immobilized biomass grown on two different biomass supporting matrices viz., polyurethane foam and luffa sponge. In both the matrices, the mycelial growth of *A. flavus* was observed to be uniformly distributed both in polyurethane foam and the fibrous network of the luffa sponge. Whole-cell biocatalysts are generally immobilized in porous polyurethane based biomass support particles (BSPs), a technique developed by Atkinson et al. (1979) which allows the biocatalysts to be reused in subsequent reactions.

Dry matrix of *Luffa cylindrica* (luffa sponge) is an excellent carrier for immobilizing microorganisms; as well as plants and animal cells (Roble et al. 2002) in addition to being a renewable and biodegradable natural material. Factors such as high surface area per volume, strong and durable structure, low specific gravity and cost-

effectiveness are characteristics of Luffa making it a suitable alternative for use as a packing medium (Mazali and Alves, 2005). The use of luffa sponge has been explored to a limited extent only with studies restricted to biosorption of heavy metals from wastewater (Ahmadi et al. 2006; Iqbal and Edyvean, 2004), development of biofilms for metabolizing organic compounds (Tavares et al. 2008) and for bioconversion of raw cassava starch to ethanol (Roble et al. 2002). Use of luffa sponge was explored for immobilizing fungal biomass involved in transesterification reaction, keeping in view that such naturally available, cost-effective matrices have hitherto not been reported, attempt was made to use *A. flavus* immobilized in luffa sponge for catalyzing reactions in oil rich conditions in general and tranesterification reactions, in particular, in comparison to polyurethane foam which has been widely used for such reactions (Rottig et al. 2010).

Using immobilized matrices, transesterification was carried out by varying the percentage of oil in the reaction mixture, to examine the influence of moisture content on transesterification reaction in an immobilized system. Present study resulted in maximum of 98% and 70% EE in the presence of 80% oil and 20% water in the reaction mixture using polyurethane foam and luffa sponge respectively. Xiao and Obbard (2010) reported that 10% water content, a reaction temperature of 30 °C and use of support particles with biomass resulted in 86.5% FAME. Low water content in the reaction media deactivates the enzyme whereas excess water led to the formation and growth of water droplet clusters within the active site of lipase, thereby inducing a change in the protein structure and activity of the enzyme (Xiao et al. 2009, Xiao and Obbarda, 2010).

Saluma et al. (2010) reported that optimum water concentration was an important factor that influences the yield of alkyl esters. Qin et al. (2008) further indicated need of optimum water content as a crucial parameter for maintaining enough conformational flexibility of the enzyme. Wu et al. (2004) reported that excessive water content in the system not only makes enzyme conglobate, but also increases diffusion resistance, and decreases the catalytic ability of enzyme. Tamalampudi et al. (2008) found that the presence of 5% (v/v) water in the crude jatropha oil had significant effects on the rate of methanolysis. Xiao et al. (2009) and Chen and Lin (2010) examined the effect of water content on *A. niger* whole cell catalyzed methanolysis and observed the highest yield of 81.2% and 72.6% of methyl

ester could be obtained in the presence of 8% and 10.9% of water in the reaction media. The optimum water content is therefore, a compromise between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction.

Rottig et al. (2010) reviewed the immobilization of *R. oryzae* within and over polyurethane foam as biomass support particle (BSPs) by number of groups for biodiesel production. Xiao et al. (2009) reported the immobilization of *A. niger* on polyurethane foam as BSPs and its utilization for biodiesel production. Reticulated polyurethane foam was used as BSPs to immobilize *A. oryzae* whole-cell biocatalyst which co-expresses *F. heterosporum* lipase (FHL) and mono- and di-acylglycerol lipase B (mdlB) in the same cell to improve biodiesel production. Ban et al. (2001) and Oda et al. (2005) reported generation of biodiesel using whole-cell biocatalysts immobilized on similar BSPs.

A comparative study was carried out to examine the influence of immobilization on the transesterification potential of the fungal biomass with reference to its activity as wet and dry biomass. Studies similar to those carried out with wet and dry biomass were carried out with the immobilized biomass particles also. With reference to the time interval of alcohol addition, instant addition resulted in only 37 and 36% EE using polyurethane foam and luffa sponge respectively. Maximum yield of 98 and 75% EE was observed when alcohol was added at an interval of 8 h from polyurethane foam and luffa sponge respectively. It was observed that the yield remained constant in the case of PUF when alcohol was added at an interval of 12 h, however the same decreased, in case of Luffa sponge. The transesterification of cottonseed, jatropha and karanj oil resulted in 98, 95.6 and 85% of EE with biomass immobilized in PUF. Whereas in case of luffa sponge, the yield was 78, 75 and 62% using cottonseed, jatropha and karanj oil as a raw material respectively. It was observed that cottonseed resulted in better yield as compared to jatropha and karanj oils.

Salum et al. (2010) reported transesterification of soybean oil with ethanol, without co-solvent using *Burkholderia cepacia* as whole cell biocatalyst in solid-state fermentation using sugarcane bagasse and sunflower seed meal, by passing the reaction mixture through fixed bed column. The best yield conversion of 95% was observed after 46 h at 50 °C and alcohol:oil molar ratio of 3:1 with step-wise alcohol addition and 1% (m/m) water in the reaction medium.

Based on literature reviewed, to the best of the knowledge, there have been very limited studies on transesterification of oils with immobilized biomass using ethanol as acyl acceptor. All the studies examined till date, dominantly involved use of methanol for such reactions. Chen and Lin (2010) demonstrated the transesterification of soybean oil using *R. oryzae* bed immobilized on non-woven fabric as matrix The authors predicted the yield value of 72.6% under the optimum conditions that is 11% (w/w) water content, 0.64% molar ratio of methanol to oil, 2.25 (w/w) cell weight and reaction time of 23 h. Further, *R. oryzae* IFO4697 whole cell immobilized within BSPs in tert-butanol as solvent resulted in 72% yield of butyl ester from soybean oil under similar optimal conditions reported earlier (Li et al. 2007). 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 alkyl ester (biodiesel) production (Li et al. 2006; 2007). *R. oryzae* IFO4697, with a 1,3-positional specificity lipase, immobilized within biomass support particles (BSPs) was also used for transesterification of soybean oil with step wise addition of methanol in the presence of 15% water which yielded 90% methyl ester in the reaction mixture as reported by Ban et al. (2001).

A heterogeneous lipase encoding gene was transformed by Hama et al. (2008) from *F. heterosporum* to *A. oryzae* and the organism was immobilized within porous BSPs enabling the convenient use of *F. heterosporum* lipase producing *A. oryzae* as a whole cell biocatalyst for the transesterification reaction. Addition of water upto 5% to reaction mixture not only yielded 94% methyl ester but also proved effective in both preventing methanol from inactivating the lipase and facilitating acyl migration in partial glycerides. *F. heterosporum* lipase producing *A. oryzae* attained better final methyl ester production and higher lipase stability as compared to *R. oryzae* (Hama et al. 2008).

Methanolysis of used cooking oil (UCO) with stepwise addition of methanol was carried out by the immobilized cell of *R. oryzae* (PTCC5174) in biomass support particles (BSPs) (Pazouki et al. 2010). Biodiesel conversion of this pre-treated UCO was brought out using methanol to fatty acid molar ratio of 3:1 at 35 °C. Finally, BSPs of the immobilized cells of *R. oryzae* were added for 72 h. The filtered UCO yielded only 46% conversion of free fatty acid to methyl ester. The lower yield of methyl esters might be due to presence of some unknown substances present in fried oils that

influence the transesterification process as noted in our previous study on generation of ethyl esters using UCO (Prakash et al. 2010)

To produce fatty acid methyl esters from transesterification of palm oil, Xiao et al. (2009) isolated *A. niger* from atmospherically exposed bread and *Jatropha curcas* seed and utilized the same as a whole cell biocatalyst for palm oil methanolysis. 87% FAME yield was observed after 72 h from 30 immobilized BSPs under standard conditions of 8% water content and temperature of 40 °C. In addition, olive oil was found to be the best carbon source as well as lipase inducer amongst different oils (olive, palm and soybean oils). Further, to optimize the transesterification of waste cooking oil comprising fats, oil and grease and analyse the relationship of reaction variables and their influence on FAME yield using immobilized *A. niger*, response surface methodology (RSM) with a five level three factor central composite rotatable design was used by Xiao et al. (2010). Under optimized conditions, the validation experiments showed that the predicted value of maximum FAME yield that is 91.3% fell very close to the experimental value of 91.8%.

Hama et al. (2007) found that emulsification of the reaction mixture containing soybean oil and water improved the methanolysis reaction rate when *R. oryzae* was immobilized within cuboidal polyurethane foam biomass support particles (BSPs) for transesterification of soybean oil. Immobilization was carried out in 20 l air lift bioreactor, and 30 g/l olive oil was used as lipase inducer. First cycle of repeated batch methanolysis yielded 90% methyl ester content in the reaction mixture at a flow rate of 25 l/h and even after the tenth cycle a high value of 80% maintained. *R. oryzae* lipase, without any organic solvent, effectively catalysed the methanolysis in a water containing system (Hama et al. 2007).

In an industrial biodiesel production, it is desirable to prepare catalysts that are robust and stable over a long period of time and develop highly efficient and recyclable whole-cell biocatalysts immobilized on BSPs using a conventional biodiesel production system (Ban et al. 2001; 2002; Oda et al. 2005; Tamalampundi et al. 2007; Hama et al. 2007; 2008). Indeed, the long-time stability of biocatalysts should be crucial for industrial application. Keeping this in view, the observations with *A. flavus* was carried out by repeatedly using actively wet culture as well as immobilized PUF and luffa under optimized conditions over five cycles. The catalysis with immobilized PUF over five cycles showed a 17.5% decrease in yield of EE from

1st to 2nd cycle followed by a sharp decrease by 60% from 2nd cycle to 5th cycle. In case of immobilized Luffa, 23% decrease in the yield was observed from 1st cycle (81.5% EE) to 2nd cycle (67% EE), followed by only 38% decrease in the extent of transesterification from 2nd cycle (67% EE) to 5th cycle (28.5% EE). In contrast, the extent of decrease during the use of wet culture was not as significant.

Ban et al. (2002) previously reported that *R. oryzae* immobilized on BSPs could be significantly stabilized for biodiesel (alkyl ester) production, by cross-linking with glutaraldehyde, for use over number of cycles (Ban et al. 2002). Arai et al. (2010) studied the recyclability of the whole-cell biocatalysts, wild *R. oryzae* (w-ROL) which would maintain sufficient activity in the ionic liquid, after glutaraldehyde treatment. Koda et al. (2010) used wild *R. oryzae* for repeated cycles and observed that the FAEE production rate gradually decreased with repeated cycles, resulting in an FAEE content of about 60% on the fifth cycle and suggested that the decrease of the FAEE yield during repeated reactions could be caused by deactivation of enzyme in wild *R. oryzae*. It has been also reported that a glycerol layer formed on the surface of the BSPs could cause the loss of lipase activity during repeated use of whole-cell biocatalysts (Rodrigues et al. 2009). On the other hand, when similar studies were carried out with recombinant *F. heterosporum* as the catalyst, the FAEE content reached a high value of over 90% in the first cycle and retained its original productivity after five cycles.

The two factors that influence the stability of lipase activity during whole cell catalysed methanolysis as reported by Hama et al. (2007) are the physical damage to immobilized cells by the circulation flow of the reaction mixture and the inactivation of lipase. The immobilized lipase located on the exterior surface of the particles gets severely damaged due to high degree of shear stress (Arcos et al. 2000). The immobilized fungal cells located near the surface of the BSPs, as mentioned earlier, get exfoliated by fluid shear stress at high flow rate thereby leading to loss of lipase activity, however, it has also been assumed that inefficient mixing of the reaction mixture caused a hydrophilic layer to cover the BSPs leading to remarkable inactivation of the lipase by high alcohol concentration in the hydrophilic layer.

5.5 Observations of physico-chemical characteristics of ethyl ester obtained through biocatalyzed transesterification

Ethyl ester that was >98% during complete transesterification of oil was further examined for different physico-chemical properties as per standards outlined by ASTM for biodiesel. Results showed that the characteristics of the ethyl ester were significantly similar to biodiesel as per standard specified in ASTM D 6751 as well as Indian standard. The observations are of immense importance as this study demonstrates the use of whole-cell biocatalytic approach for generation of alkyl esters that are at par with those generated through any other approach, reported till date, for use as biodiesel. As-on-date there is no report available on the physico-chemical characterization of alkyl esters obtained by using whole cell as a catalyst.

5.6 Studies on esterification of fatty acids with wet/dry biomass

The studies on transesterification were further extended to examine the esterification of fatty acids by using the wet and dry biomass to determine the role of chain length of fatty acids on the yield of alkyl esters. Oleic acid was used as the fatty acid and the alcohols that were used as acyl acceptor ranged from methanol to decanol. With reference to the form of biomass used, esterification yielded better results using dried biomass as compared to active culture. Nearly complete conversion was obtained using ethanol as acyl acceptor in 36 h of reaction time (Aulakh et al. 2011) whereas under similar optimized conditions, rest of the alcohols resulted in incomplete conversion of fatty acid to alkyl ester. Extent of esterification increased from methanol (87.5% MO) to ethanol (98% EO) where as further increase in chain length of alcohol resulted in decrease in the yield from ethyl oleate (98% EO) to decyl oleate (77% DO). Alcohols with even number of carbons resulted in better yield as compared to alcohols with odd number of carbons. The yield of alkyl esters was better with dry biomass when compared to the wet culture wherein ethanol (32.8% EO) yielded maximum of alkyl ester as compared to the rest of the alcohols, however, trend of odd and even carbon chains was nearly same. To the best of the information available, there has been no study till date on effect of chain length of alcohol on the esterification process using a biocatalytic process. Li et al. (2007) utilized *R. oryzae* IFO4697 whole cell immobilized within biomass support particles (BSPs) catalysed biodiesel production from oleic acid in a *tert*-butanol system, *R. oryzae* whole cell

exhibited better methanol endurance and better stability than that in a solvent-free system, achieved biodiesel yield reached 90% at 48 h.

In summarizing the work carried out on the whole cell catalyzed transesterification 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 in isolation and characterization of seven fungi exhibiting potential to grow and utilize oil as carbon source to the extent of upto 90% supplementation in growth medium. One of the strains, Aspergillus flavus, was observed to catalyze transesterification of oils to alkyl esters, to the extent of ≥ 98% at 70% oil supplementation. The work presents the optimized conditions required for transesterification for wet, dry and immobilized forms of said strain with various oils. In addition, observations on esterification of fatty acids are also presented with dry biomass as catalyst. The work, thus carried out on various aspects of whole cell catalysis, is of importance in demonstrating the potential nature of fungus catalyzed transesterification for generation of alkyl esters of industrial and commercial importance.

Conclusion

Seven different fungal strains namely *Aspergillus flavus* (MTCC 5436), *Fusarium subglutians, Aspergillus sydowii* (MTCC 10397), *A. aculeatus, Curvularia pallescens* (MTCC 10390), *Penicillium citrinium* (MTCC 10398) and *Periconia sp* (MTCC 10391) isolated from contaminated butter and were observed to be oil tolerant in the growth medium. Among all the strains *A. flavus*, *F. subglutians*, *A. sydowii* showed maximum oil tolerance upto 80% oil in the growth medium, *A. flavus* exhibited maximum hydrolytic activity (77% FFA) with 40% oil supplementation in the growth media followed by *A. aculeatus* which resulted in 76.4% FFA with 50% oil. A notable observation in the study was the nature of *F. subglutians* to exhibit growth even at greater than 80% oil supplementation in the growth medium; however, the organism did not show any potential to facilitate hydrolytic activity.

A. flavus was observed to produces lipase exhibit maximum lipase activity of 21.8 U/ml with 50% (v/v) oil acting as the main carbon source and peptone (0.5% w/v) as nitrogen source. The optimum pH and temperature for enzymatic activity were observed to be 7.5 and 35 °C, respectively.

Intracellular/cell bound lipase production was carried out under standardized condition, it was observed that after 120 h of growth period, maximum 83% (with 70% oil in the growth medium) of FFA was produced which was significant. Dried biomass of *A. flavus* exhibited maximum of 1811 µmol/ml FFA was produced by 1 g biomass obtained from 70% oil in the growth medium.

A. flavus was observed to facilitate transesterification in an oil:minimal medium with the ratio of 70:30 resulting in nearly complete conversion of oil (used cottonseed, jatropha and karanj oils) to ethyl esters within 48 h at 28 °C and 120 rpm. It was observed that stepwise addition of ethanol at an interval of 12 h facilitate nearly complete conversion of oil to ester. Ethanol was proved to be better acyl acceptor with respect to methanol. Glycerol produced was easily separated and purified by a very simple technique and was without any contamination. Reusability of biomass was resulted in 50% decrease in the yield of 1^{st} cycle to 5^{th} cycle. The physico-chemical characteristics of the ethyl ester at 70% oil as carbon source were similar to the standards specified for biodiesel as per standards of American Society for Testing and Materials (ASTM) and Bureau of Indian Standards (BIS), India.

Among rest of the strains tested, *A. aculeatus* resulted in maximum of 77% of ethyl ester from 60% oil in the growth medium, followed by *A. sydowii* and *P. citrinium*, resulted in maximum of 61% and 25% ethyl ester with 20% and 30% oil supplementation respectively, *C. pallescens* and *Perconia sp*. yielding 22% ethyl ester from 10% oil in the growth medium.

Dried biomass of *A. flavus* resulted in maximum of 84% of ethyl ester, using 20% biomass, at 35 °C, with stepwise addition of ethanol at an interval of 8 h, total rection time of 36 h. With increase in moisture content in the reaction mixture decreses the extent of transesterification using dried biomass. Transesterification of frying oil collected at different time interval showed that with increase in time of frying, the extent of transesterification decreased due the formation of some unknown compounds during frying. Further, the transesterification by *A. flavus* was examined by immobilizing the biomass on polyurethane foam and luffa sponge. Polyurethane foam resulted in near-complete conversion (≥ 98%) of oil to ester where as luffa sponge resulted in 77% ethyl ester, with stepwise addition of ethanol at interval of 8 h, and total reaction time of 36 h indicating polyurethane foam to be a better matrix than luffa for use of immobilized biomass for transesterification reactions.

Esterification of oleic acid was carried out using wet biomass as well as dried biomass of *A. flavus* with different alcohols as acyl acceptor. It was observed that dried biomass resulted in complete conversion of fatty acid to ethyl oleate. Ethanol was observed to be better acyl acceptor than rest of the alcohols, as with increase in chain length of alcohol resulted in decrease in the yield from ethyl oleate (98% EO) to decyl oleate (77% DO), however, there were certain variation in decreasing order between wet and dried biomass, dried biomass resulted in better conversion than wet biomass. Further observed that alcohols with even carbons resulted in better yield as compared to alcohols with odd carbons.

The work, thus carried out on various aspects of whole cell catalysis, is of importance in demonstrating the potential nature of fungus catalyzed transesterification for generation of alkyl esters of industrial and commercial importance.

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Ethyl ester percentage calculated based on Ghesti et al. 2007

I_{TAG}	% C_{EE}	Reference
0.00	≥98%	Annexure II Fig 4.5.1.1 (I),(II) and (III) e, Annexure II 4.5.1.2 (I) (II) a, Annexure II 4.5.1.4 a, Annexure V Fig 4.7.2 (II)c, Annexure V Fig 4.7.2 (IV)d-e, Annexure V Fig 4.7.2 (VI)a, Annexure V Fig 4.7.2 (VIII)a
0.03	95.63	Annexure V Fig 4.7.2 (VIII)c
0.05	92.86	Annexure II Fig 4.5.1.1 (I) d
0.06	91.51	Annexure II Fig 4.5.1.1 (I) c
0.07	90.19	Annexure II Fig 4.5.1.1 (I) b
0.08	88.89	Annexure II 4.5.1.3 a,
0.11	85.14	Annexure V Fig 4.7.2 (VIII)b
0.12	83.93	Annexure II 4.5.1.3 b, Annexure IV Fig 4.6.5 (a), Annexure IV Fig 4.6.6 (a)
0.13	82.74	Annexure IV Fig 4.6.2 (e), Annexure IV Fig 4.6.5 (c), Annexure V Fig 4.7.2 (II)d
0.14	81.58	Annexure II Fig 4.5.1.1 (III) d, Annexure II 4.5.1.2 (I)b, Annexure IV Fig 4.6.4 (c), Annexure IV Fig 4.6.5 (a-b), Annexure V Fig 4.7.2 (V)a
0.15	80.43	Annexure II Fig 4.5.1.1 (II) d, Annexure IV Fig 4.6.3 (b), Annexure V Fig 4.7.2 (VI)b
0.16	79.31	Annexure IV Fig 4.6.1 (d), Annexure IV Fig 4.6.5 (b)
0.17	78.21	Annexure II Fig 4.5.1.1 (I) a, Annexure IV Fig 4.6.1 (c), Annexure IV Fig 4.6.6 (b), Annexure V Fig 4.7.2 (VII)a
0.18	77.12	Annexure III Fig 4.5.3 (I)f, Annexure V Fig 4.7.2 (III)d
0.19	76.05	Annexure IV Fig 4.6.5 (d)
0.20	75.00	Annexure II Fig 4.5.1.1 (II) c, Annexure III Fig 4.5.3 (I)d, e, Annexure IV Fig 4.6.5 (e), Annexure IV Fig 4.6.5 (c), Annexure IV Fig 4.6.6 (c), Annexure V Fig 4.7.2 (III)e, Annexure V Fig 4.7.2 (VII)c
0.21	73.97	Annexure V Fig 4.7.2 (I)c
0.22	72.95	Annexure V Fig 4.7.2 (I)e
0.23	71.95	Annexure II Fig 4.5.1.1 (II) b, Annexure II Fig 4.5.1.1 (III) c, Annexure IV Fig 4.6.4 (d)
0.24	70.97	Annexure IV Fig 4.6.2 (d), Annexure IV Fig 4.6.2 (f), Annexure IV Fig 4.6.4 (b)
0.25	70.00	Annexure II 4.5.1.4 b, Annexure V Fig 4.7.2 (I)d
0.28	67.19	Annexure II 4.5.1.4 c, Annexure V Fig 4.7.2 (V)b
0.29	66.28	Annexure V Fig 4.7.2 (III)c
0.31	64.50	Annexure IV Fig 4.6.5 (f), Annexure V Fig 4.7.2 (IV)c
0.34	61.94	Annexure II 4.5.1.2 (I) c, Annexure IV Fig 4.6.3 (c), Annexure V Fig 4.7.2 (VI)c, Annexure V Fig 4.7.2 (VII)b
0.35	61.11	Annexure III Fig 4.5.3 (I)g, Annexure III Fig 4.5.3 (II)b
0.39	57.91	Annexure II 4.5.1.2 (III)b, Annexure IV Fig 4.6.1 (b)
0.41	56.38	Annexure V Fig 4.7.2 (II)b
0.42	55.63	Annexure V Fig 4.7.2 (V)c

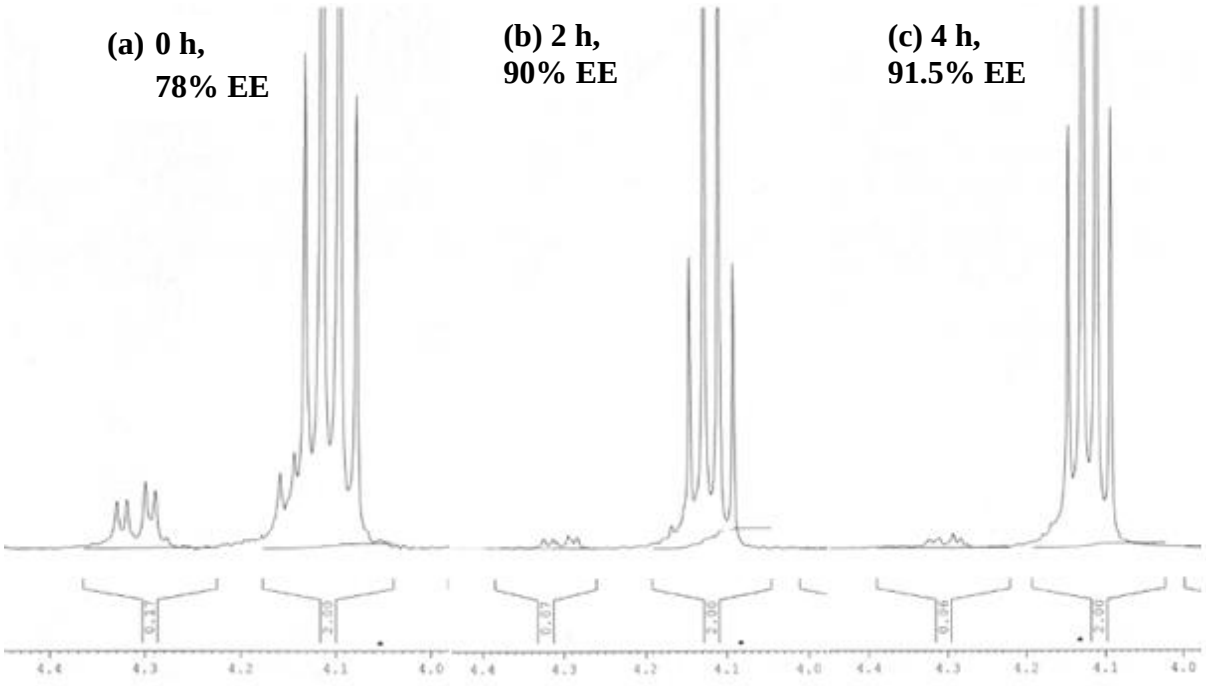
ITAG	% C EE	Reference
0.43	54.90	Annexure V Fig 4.7.2 (III)b, Annexure V Fig 4.7.2 (V)d
0.44	54.17	Annexure IV Fig 4.6.5 (g)
0.45	53.45	Annexure II 4.5.1.4 d
0.46	52.74	Annexure III Fig 4.5.3 (II)a
0.48	51.35	Annexure II Fig 4.5.1.1 (III) a, b, Annexure III Fig 4.5.3 (I)b
0.49	50.67	Annexure III Fig 4.5.3 (I)h, Annexure IV Fig 4.6.5 (d)
0.50	50.00	Annexure II 4.5.1.4 e
0.52	48.68	Annexure III Fig 4.5.3 (I)c, Annexure IV Fig 4.6.2 (c)
0.54	47.40	Annexure IV Fig 4.6.5 (e)
0.55	46.77	Annexure IV Fig 4.6.5 (h)
0.56	46.15	Annexure V Fig 4.7.2 (IV)b
0.59	44.34	Annexure IV Fig 4.6.2 (b)
0.60	43.75	Annexure V Fig 4.7.2 (I)a
0.62	42.59	Annexure II Fig 4.5.1.1 (II) a, Annexure III Fig 4.5.3 (II)c
0.64	41.46	Annexure IV Fig 4.6.5 (i). Annexure IV Fig 4.6.5 (f)
0.65	40.91	Annexure IV Fig 4.6.3 (a)
0.66	40.36	Annexure III Fig 4.5.3 (I)a
0.68	39.29	Annexure IV Fig 4.6.1 (e)
0.71	37.72	Annexure IV Fig 4.6.5 (j), Annexure V Fig 4.7.2 (I)f
0.72	37.21	Annexure IV Fig 4.6.2 (a), Annexure V Fig 4.7.2 (IV)a
0.75	35.71	4.5.1.2 (II)b, Annexure V Fig 4.7.2 (III)a
0.80	33.33	Annexure IV Fig 4.6.3 (d)
0.88	29.79	Annexure V Fig 4.7.2 (II)f
0.87	30.21	Annexure IV Fig 4.6.5 (g)
0.91	28.53	Annexure V Fig 4.7.2 (V)e
0.92	28.13	Annexure V Fig 4.7.2 (II)e
0.99	25.38	Annexure III Fig 4.5.3 (IV)c
1.01	24.63	Annexure IV Fig 4.6.5 (k)
1.02	24.26	4.5.1.2 (II)c,
1.03	23.89	Annexure V Fig 4.7.2 (II)a
1.06	22.82	Annexure III Fig 4.5.3 (v)
1.07	22.46	Annexure III Fig 4.5.3 (III)a
1.09	21.77	Annexure V Fig 4.7.2 (I)a
1.11	21.09	Annexure III Fig 4.5.3 (II)e
1.13	20.42	Annexure III Fig 4.5.3 (IV)b
1.20	18.18	4.5.1.2 (III)c, Annexure V Fig 4.7.2 (VI)d
1.21	17.87	Annexure IV Fig 4.6.3 (e)
1.24	16.96	Annexure III Fig 4.5.3 (III)b, Annexure V Fig 4.7.2 (VI)e
1.26	16.37	Annexure IV Fig 4.6.4 (a)
1.29	15.50	Annexure III Fig 4.5.3 (IV)a, Annexure IV Fig 4.6.1 (a)
1.31	14.94	Annexure III Fig 4.5.3 (II)d

DNA sequence of *Aspergillus flavus* (18s ribosomal RNA & Beta-tubulin gene)

>*Aspergillus flavus* isolate MTCC 5436, 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
tattgatatgcttaagttcagcgggtatccctacctgatccgaggtcaacctggaaaaagattgatttgcgttcggcaagcgcc
ggccgggcctacagagcgggtgacaaagccccatacgctcgaggatcggacgcggtgccgccgctgcctttggggccc
gtccccccggagaggggacgacgacccaacacacaagccgtgcttgatgggcagcaatgacgctcggacaggcatgc
ccccggaataccaggggcgcaatgtgcgttcaaagactcgatgattcacggaattctgcaattcacactagttatcgcatt
tcgctgcgttcttcatcgatgccggaaccaagagatccattgttgaaagttttaactgattgcgatacaatcaactcagacttca
ctagatcagacagagttcgtggtgtctccggcgggcgcgggcccggggctgagagcccccggcggccatgaatggcgg
gcccgccgaagcaactaaggtacagtaaacacgggtgggaggttgggctcgctaggaaccctacactcggtaatgatcc

>*Aspergillus flavus* isolate MTCC 5436 beta-tubulin gene, partial cds
tggtaaccaaatcggtgctgctttctggtatgtctcaatgccttcgagttagtatgctttggaccaaggaactcctcaaaagcat
gatctcggatgtgtcctgttatatctgccacatgtttgctaacaactttgcaggcaaaccatctctggcgagcacggccttgac
ggctccggtgtgtaagtacagcctgtatacacctcgaacgaacgacgaccatatggcattagaagttggaatggatctgacg
gcaaggatagttacaatggctcctccgatctccagctggagcgtatgaacgtctacttcaacgaggtgcgtacctcaaaattt
cagcatctatgaaaacgctttgcaactcctgaccgcttctccaggccagcggaaacaagtatgtccctcgtgccgtcctcgtt
gatcttgagcctggtaccatggacgccgtccgtgccggtcccttcggtcagctcttccgtcccgacaacttcgttttcggcca
gtccggtgctggtaacaactgggccaagggtcactacactgagggta

Fig. 4.5.1.1 (I) Effect of time interval of alcohol addition on the extent of transesterification of used cottonseed oil ethyl ester (EE)



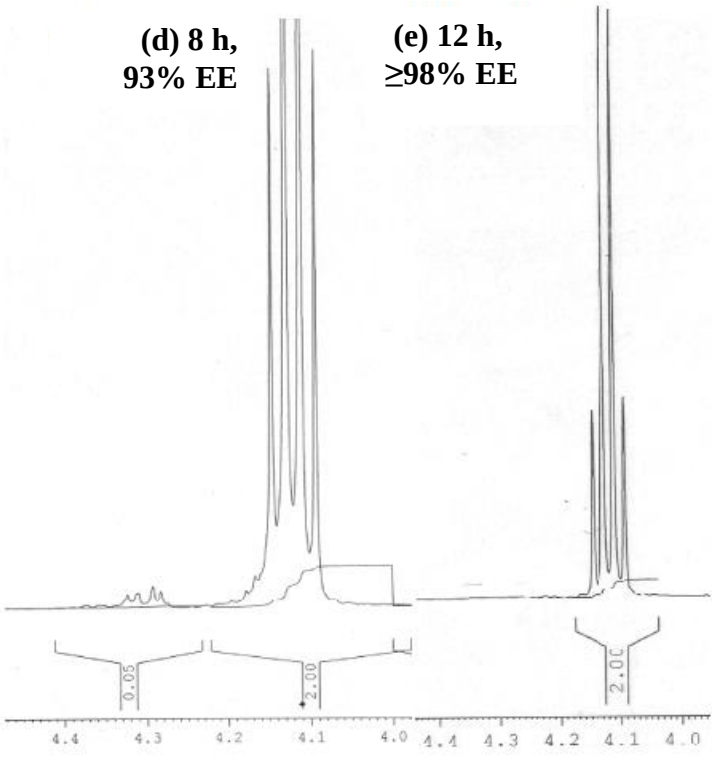


Fig. 4.5.1.1 (II) Effect of time interval of alcohol addition on the extent of transesterification of Karanj oil ethyl ester (EE)

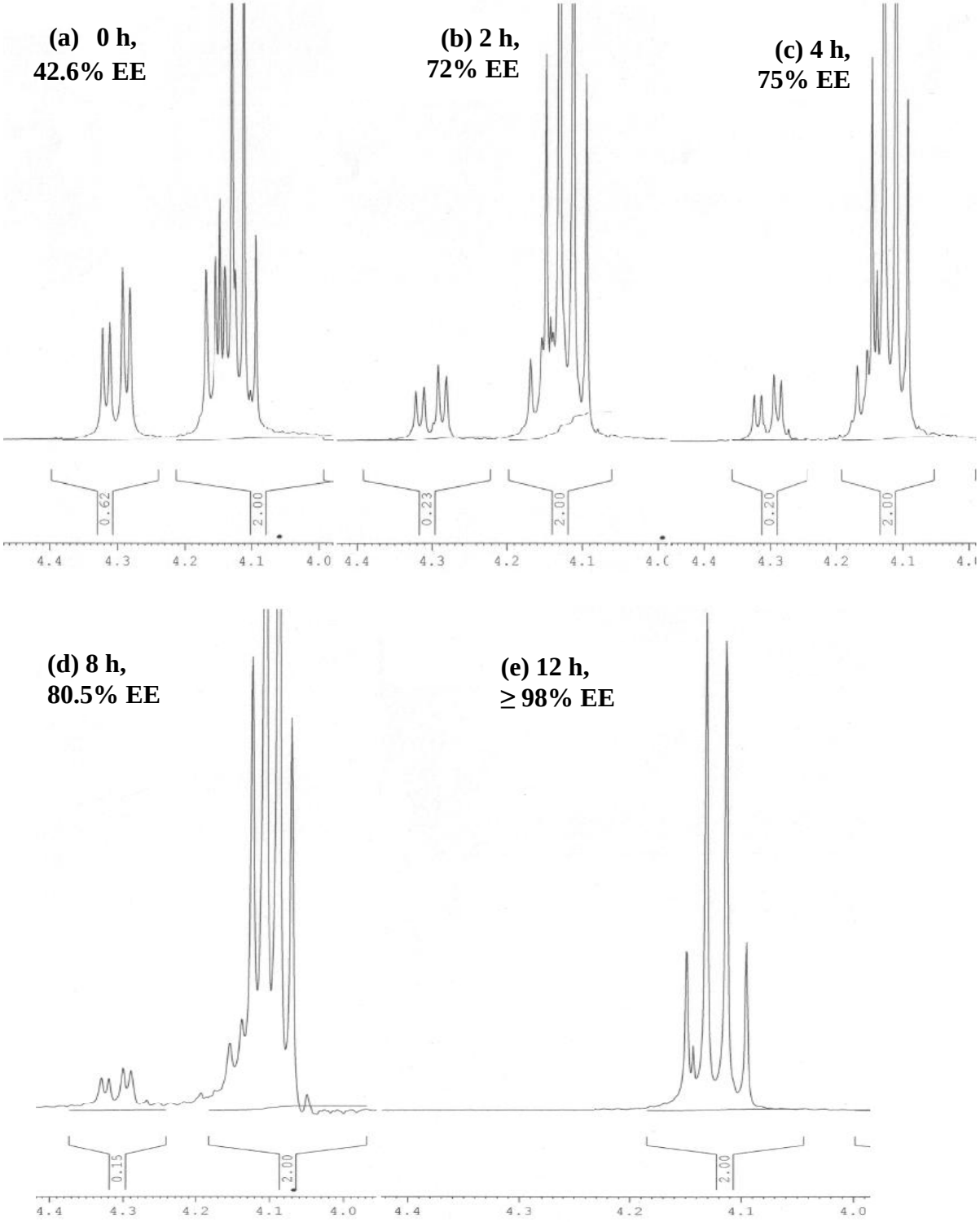


Fig. 4.5.1.1 (III) Effect of time interval of alcohol addition on the extent of transesterification of Jatropha oil ethyl ester (EE)

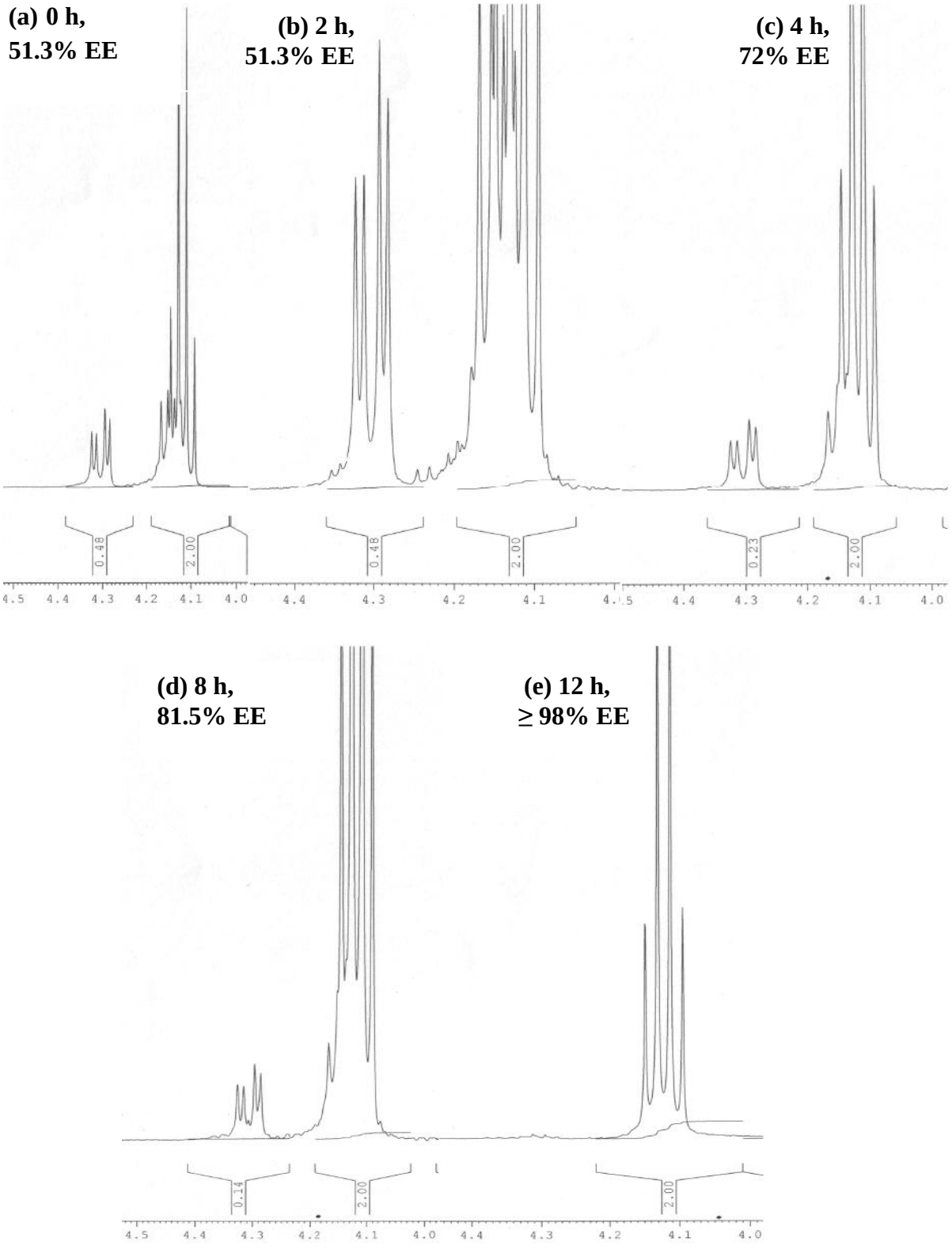


Fig. 4.5.1.2 (I) Ethyl ester (EE) yield from used cottonseed oil with different oil concentration in the reaction media

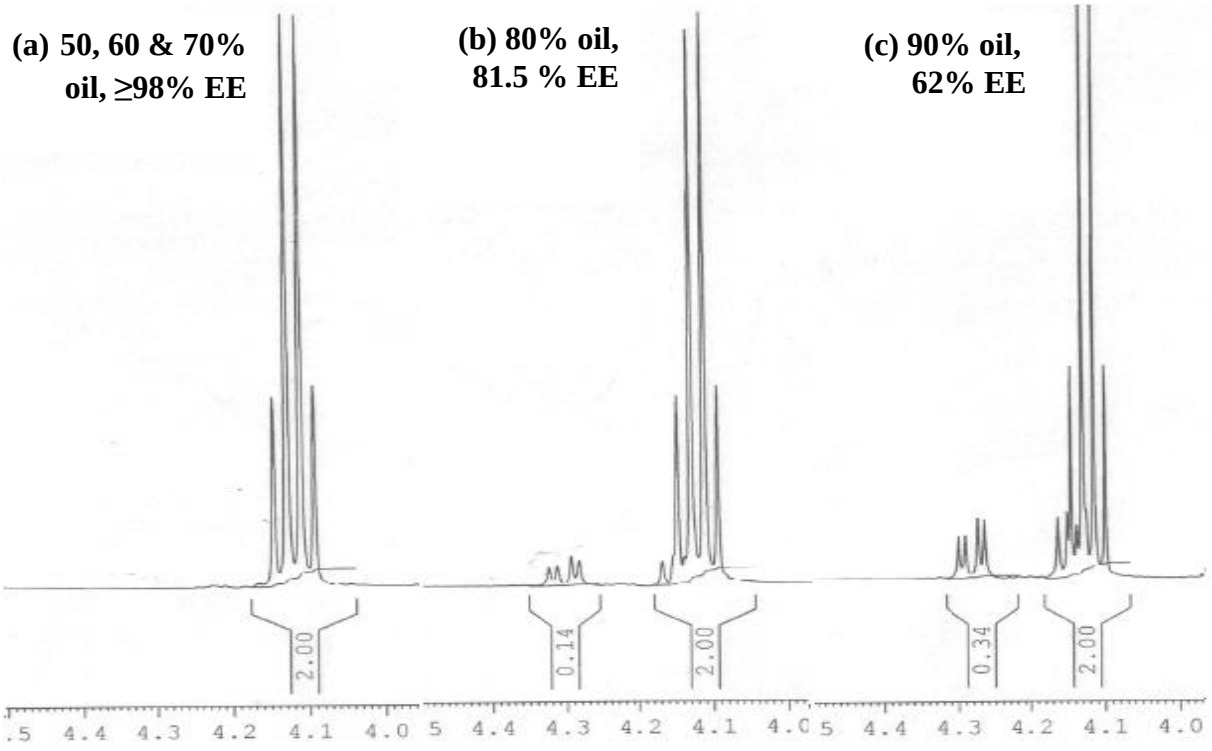


Fig. 4.5.1.2 (II) Ethyl ester production from used jatropha oil with different oil concentration in the reaction media

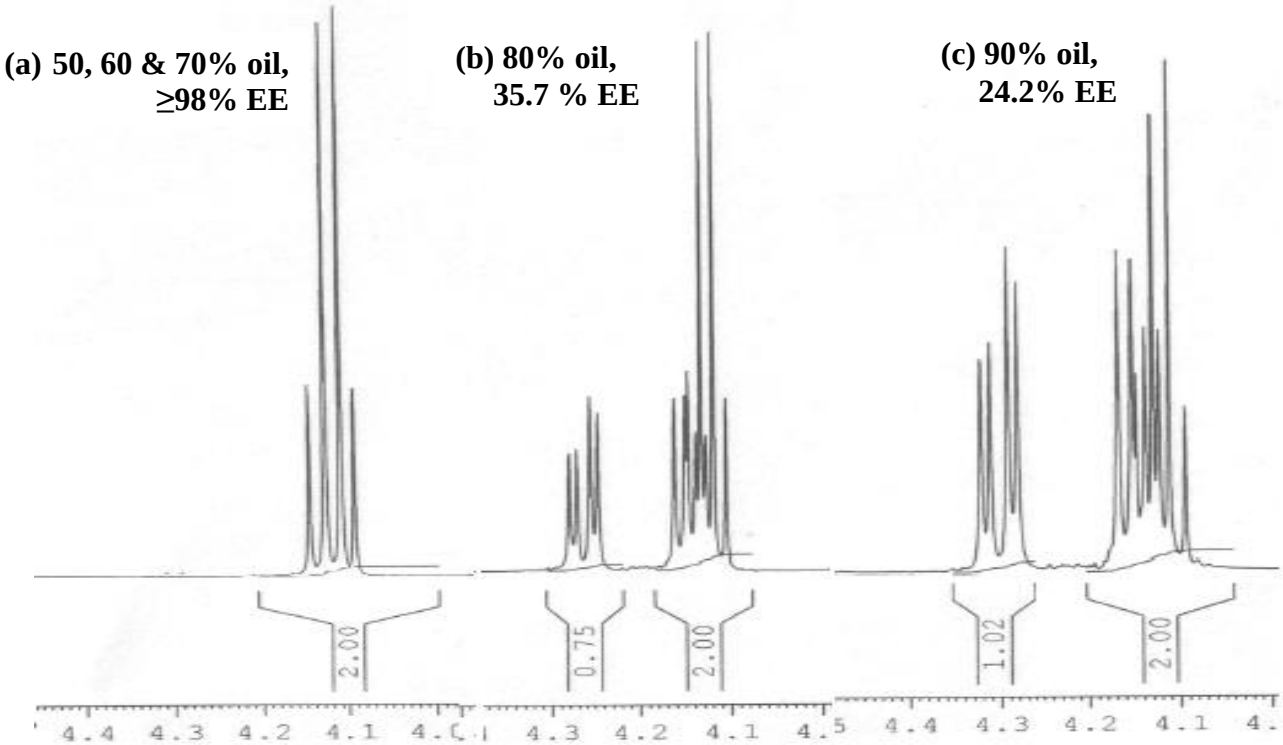


Fig. 4.5.1.2 (III) Ethyl ester (EE) from karanj oil with different oil concentration in reaction media

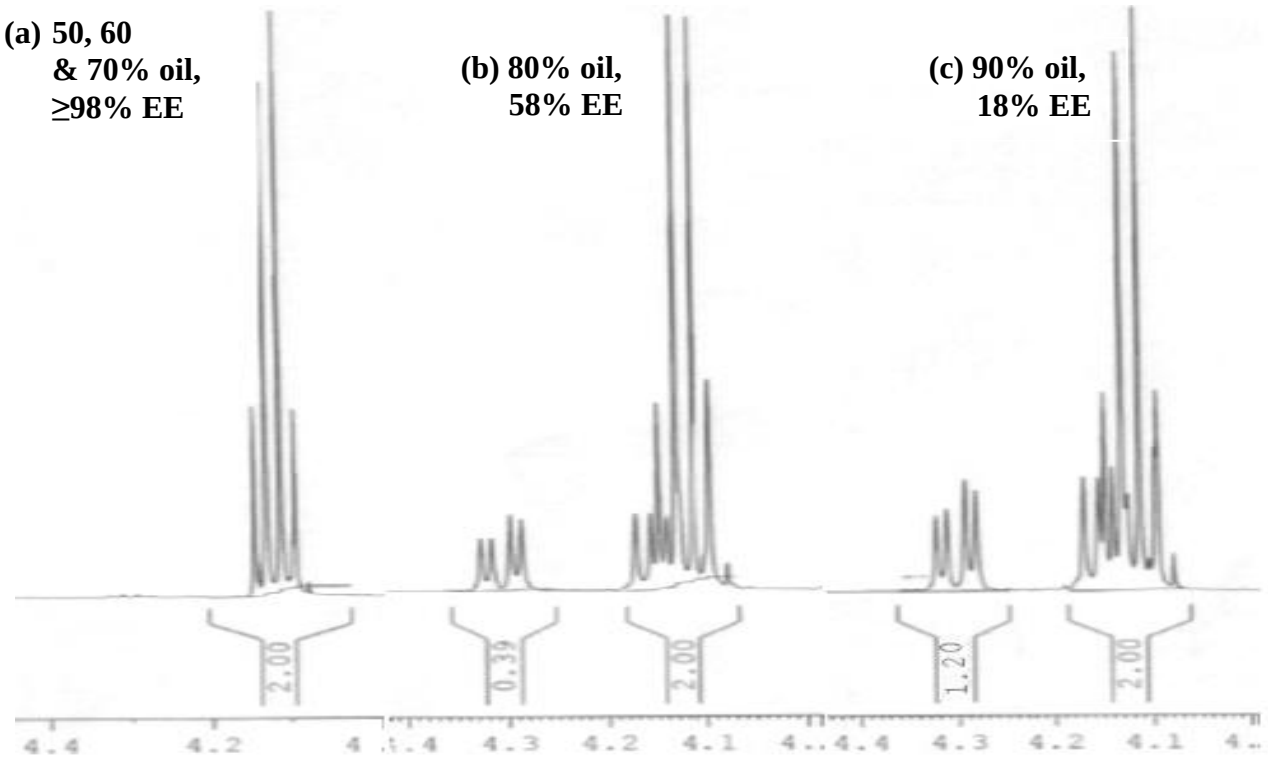


Fig. 4.5.1.3 Transesterification (EE) of different oils (at higher supplementation) by varying the percentage of peptone as nitrogen source

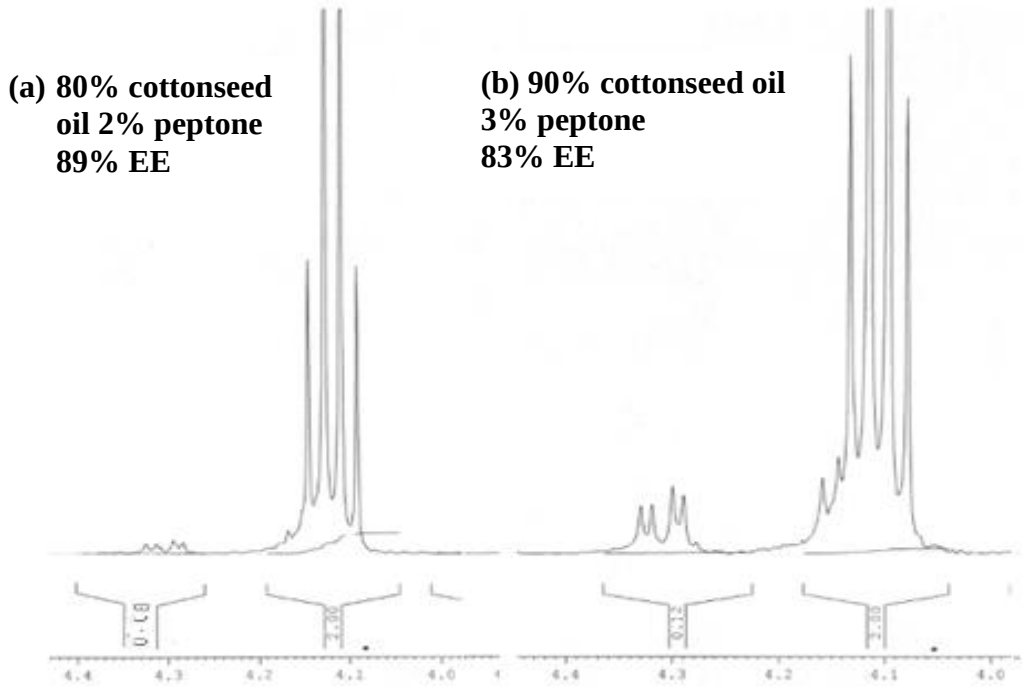


Fig. 4.5.1.4 Reusability potential of *A. flavus* biomass for transesterificaion (EE) of 70% cottonseed oil in the growth medium

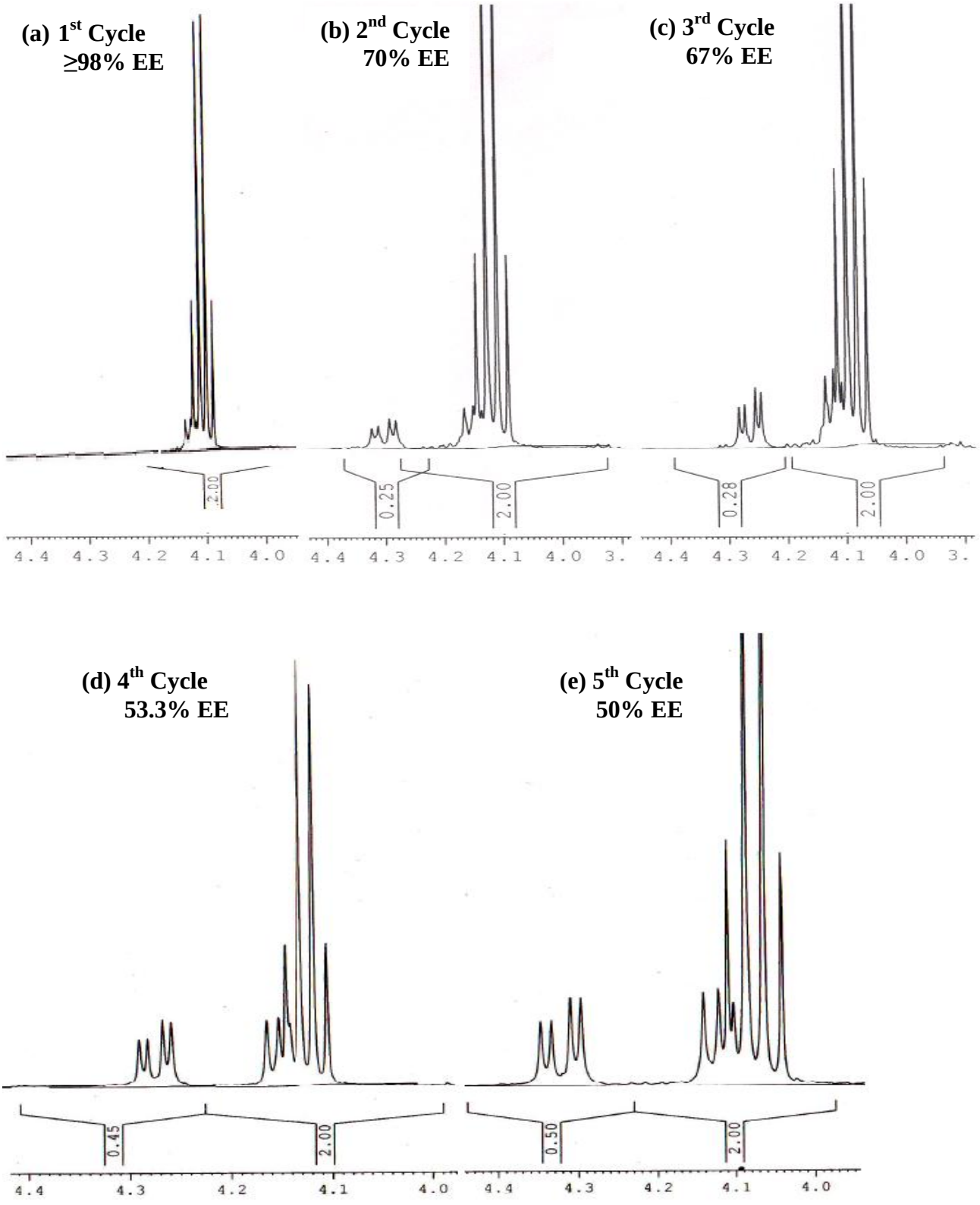
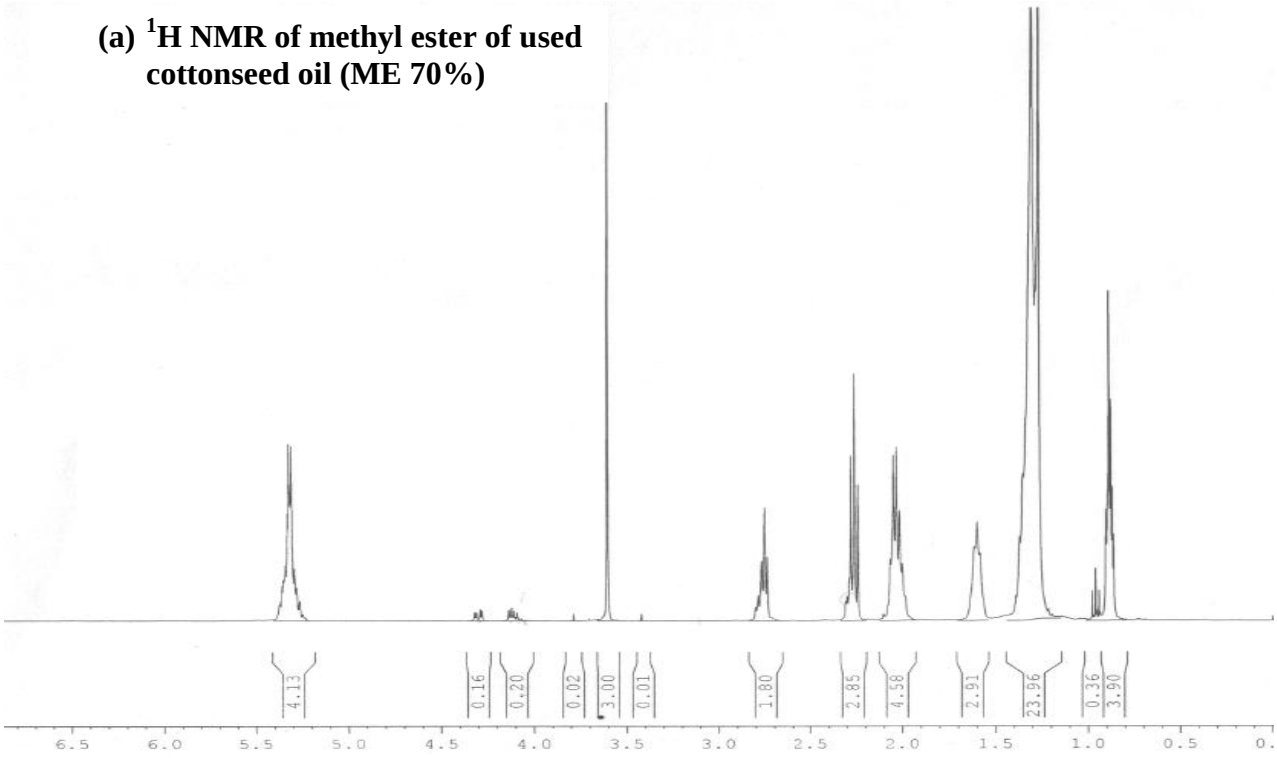
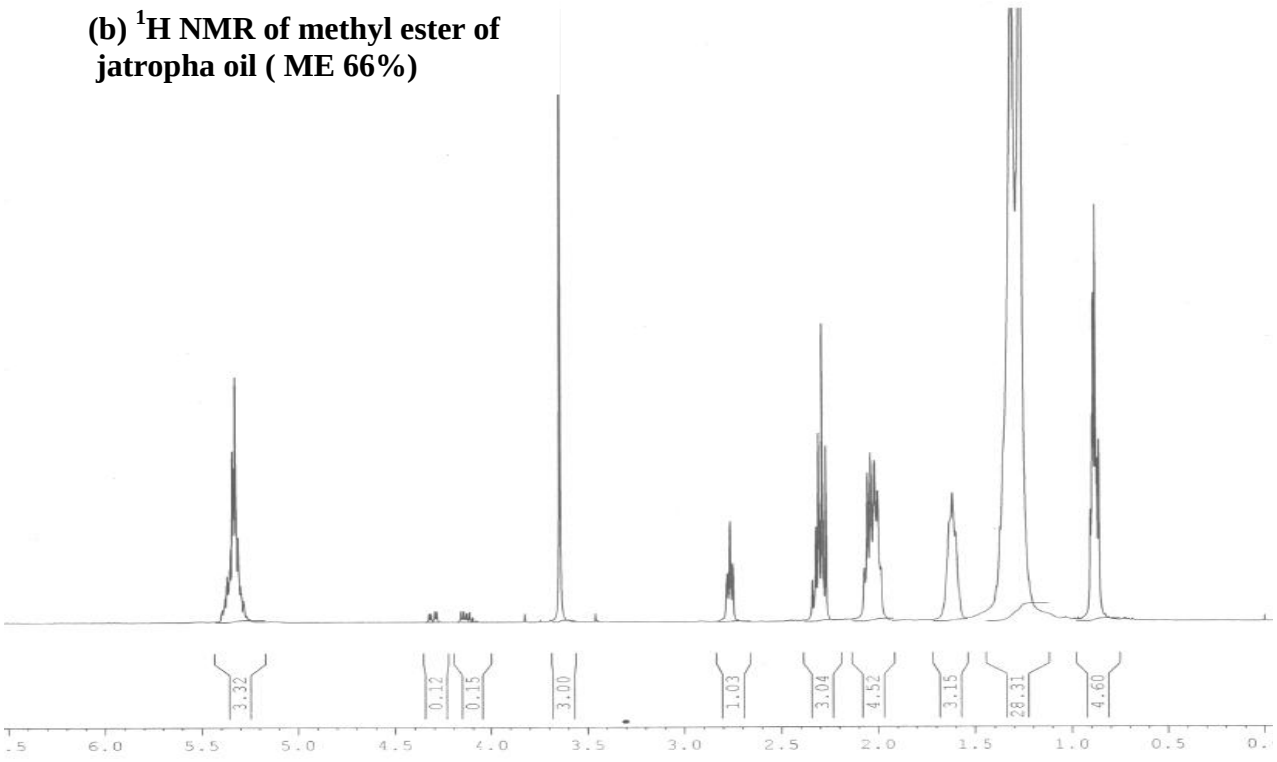


Fig. 4.5.2 Methyl ester (ME) production from 70% of different oils: (a) Used cottonseed, (b) Jatropha oil (c) Karanj oil

(a) ^{1}H NMR of methyl ester of used cottonseed oil (ME 70%)



(b) ^{1}H NMR of methyl ester of jatropha oil (ME 66%)



(c) ^{1}H NMR of methyl ester of karanj oil (ME 62.3%)

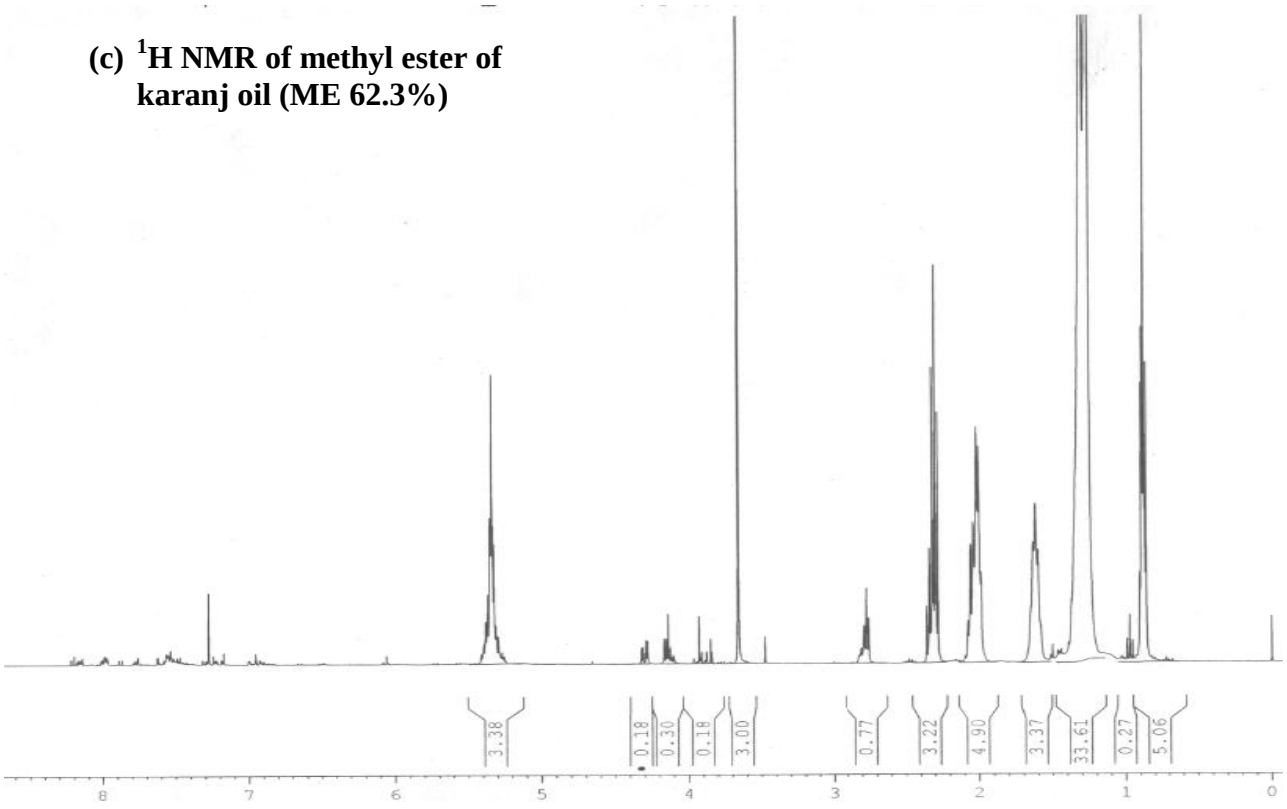
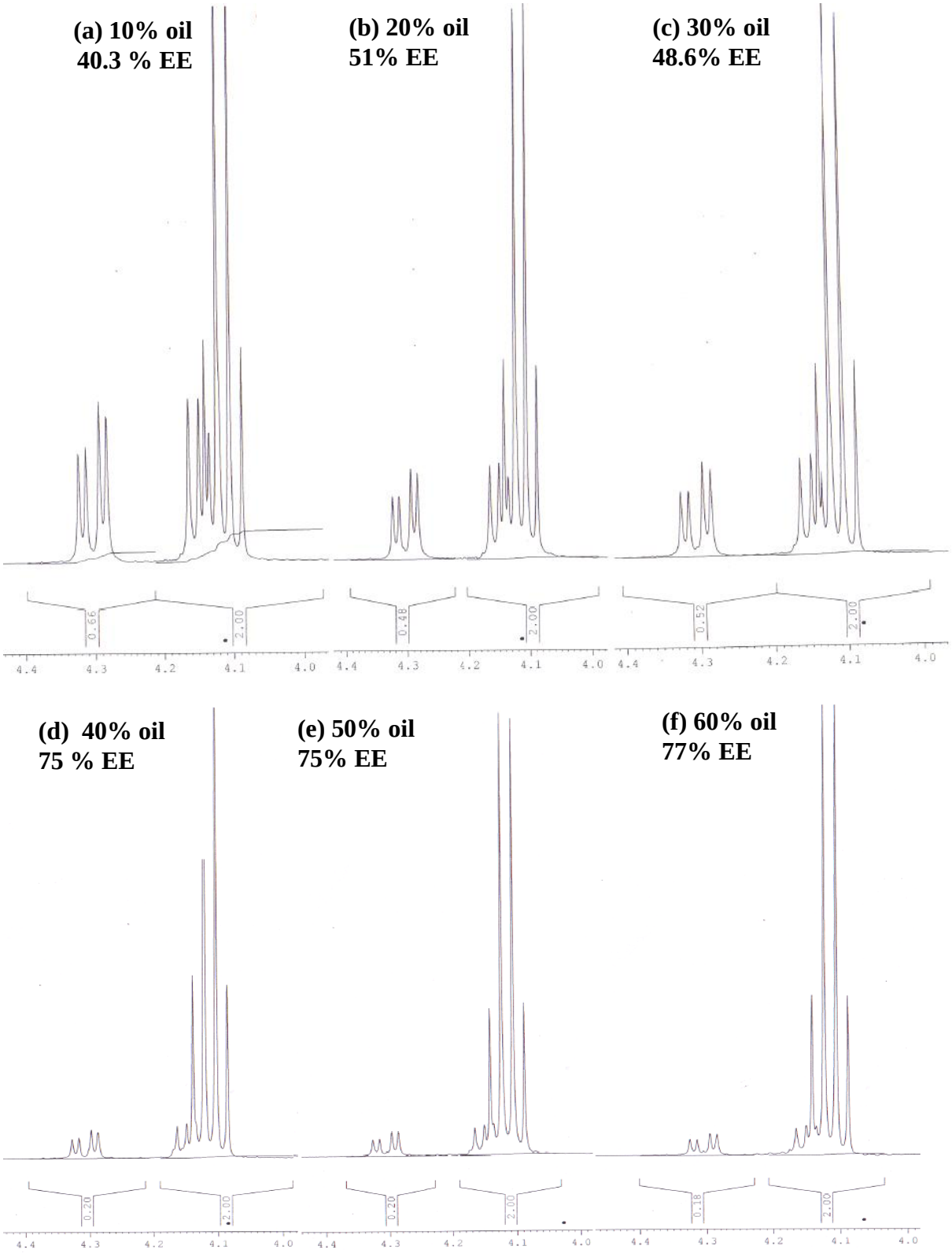


Fig 4.5.3 (I) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by *Aspergillus aculeatus* with varying percentage of cottonseed oil in the growth medium



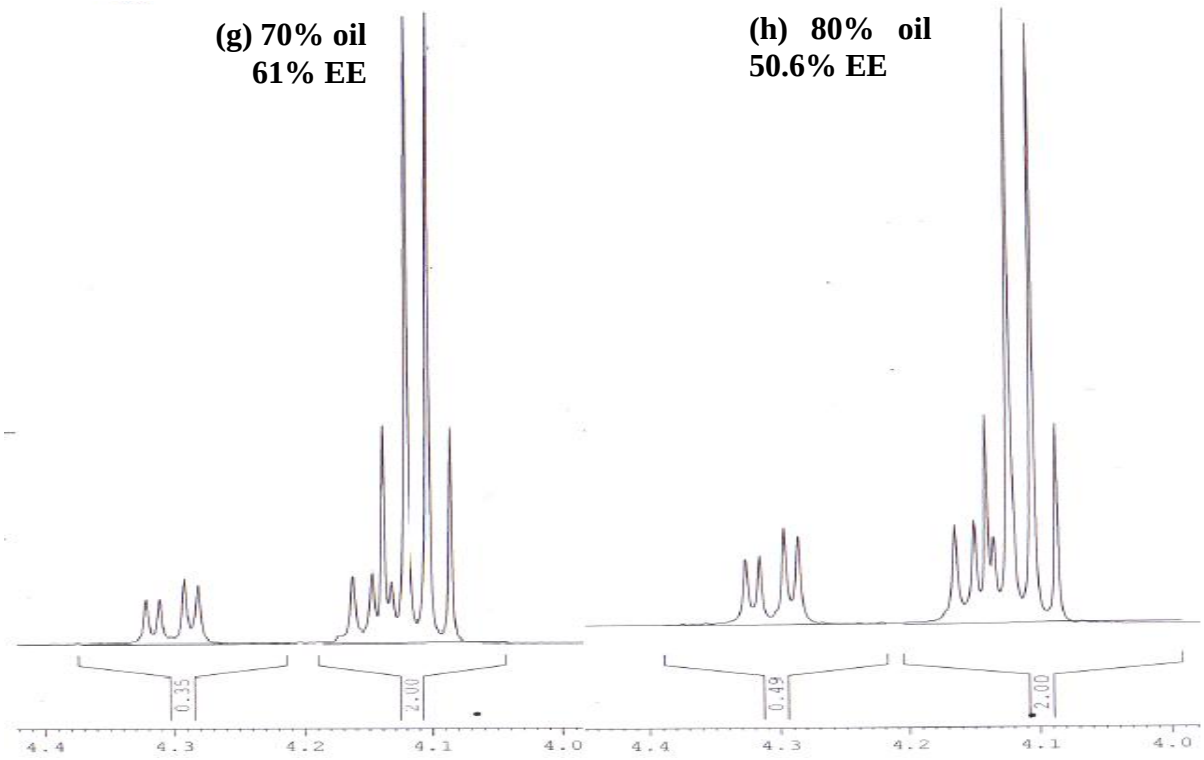
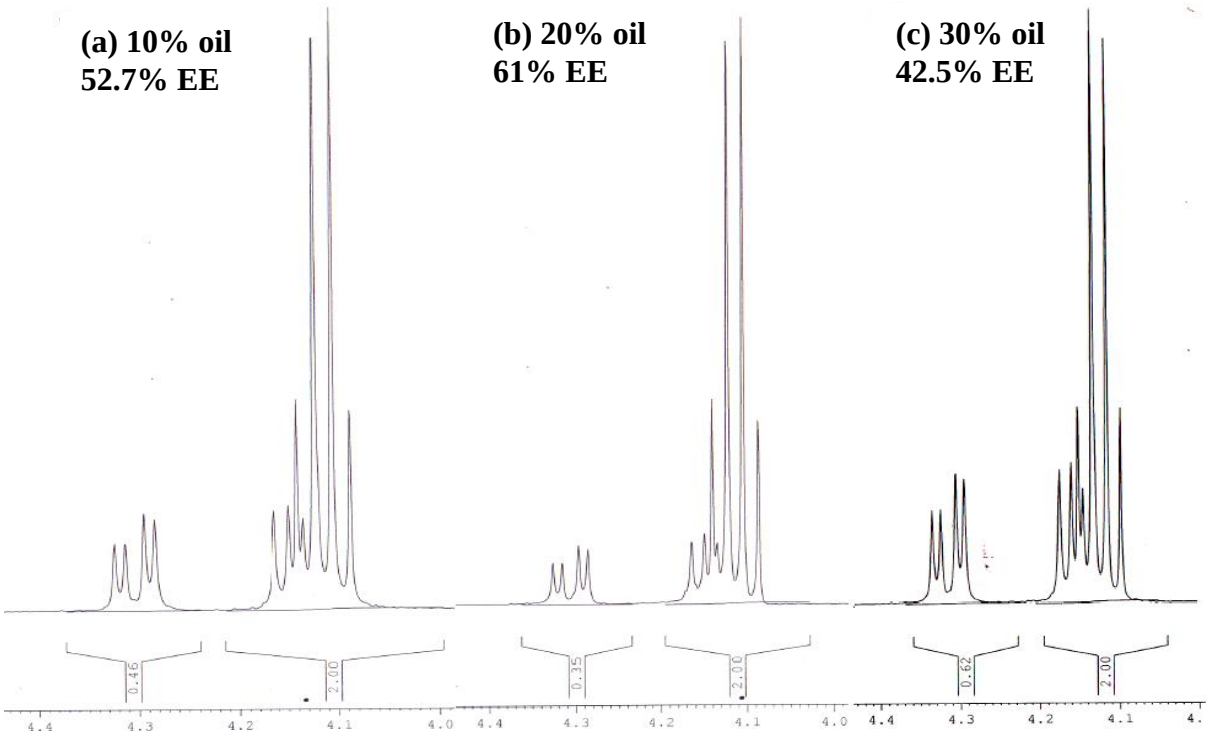


Fig. 4.5.3 (II) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by *Aspergillus sydowii* by varying the percentage of cottonseed oil in the growth medium



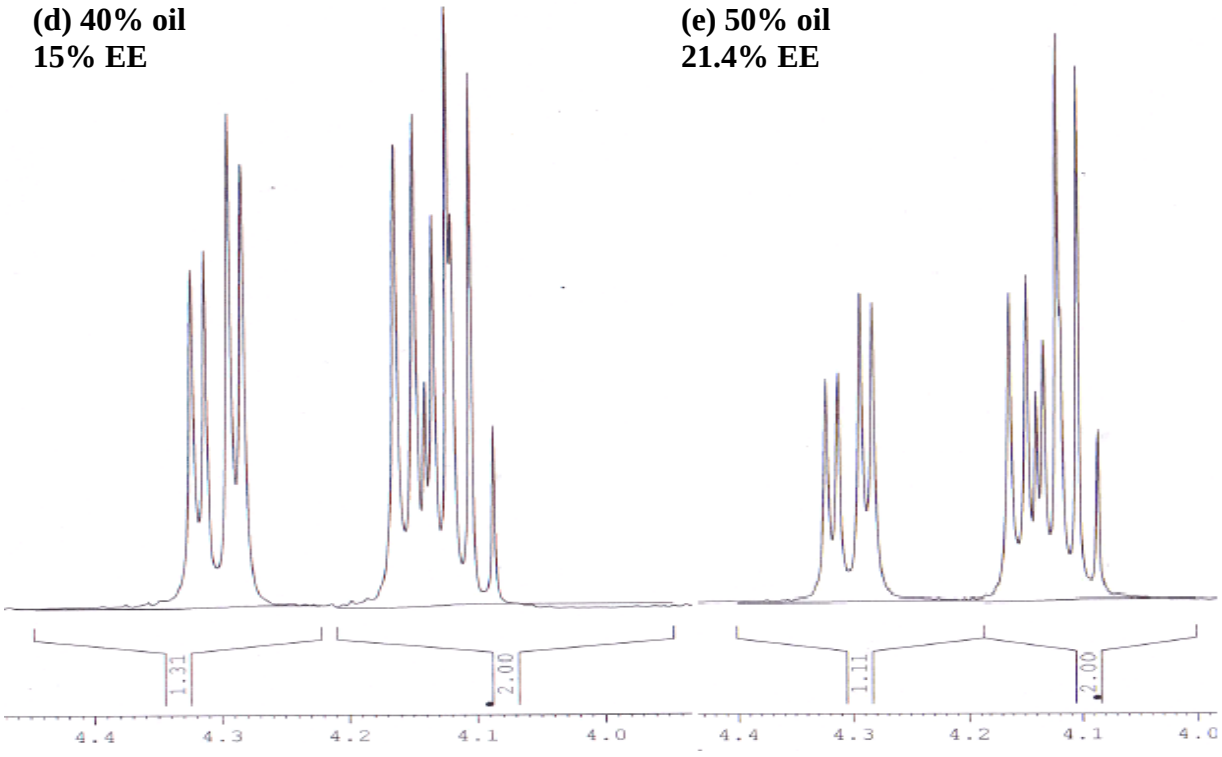


Fig. 4.5.3 (III) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by *Curvularia pallescens* by varying the percentage of cottonseed oil in the growth medium

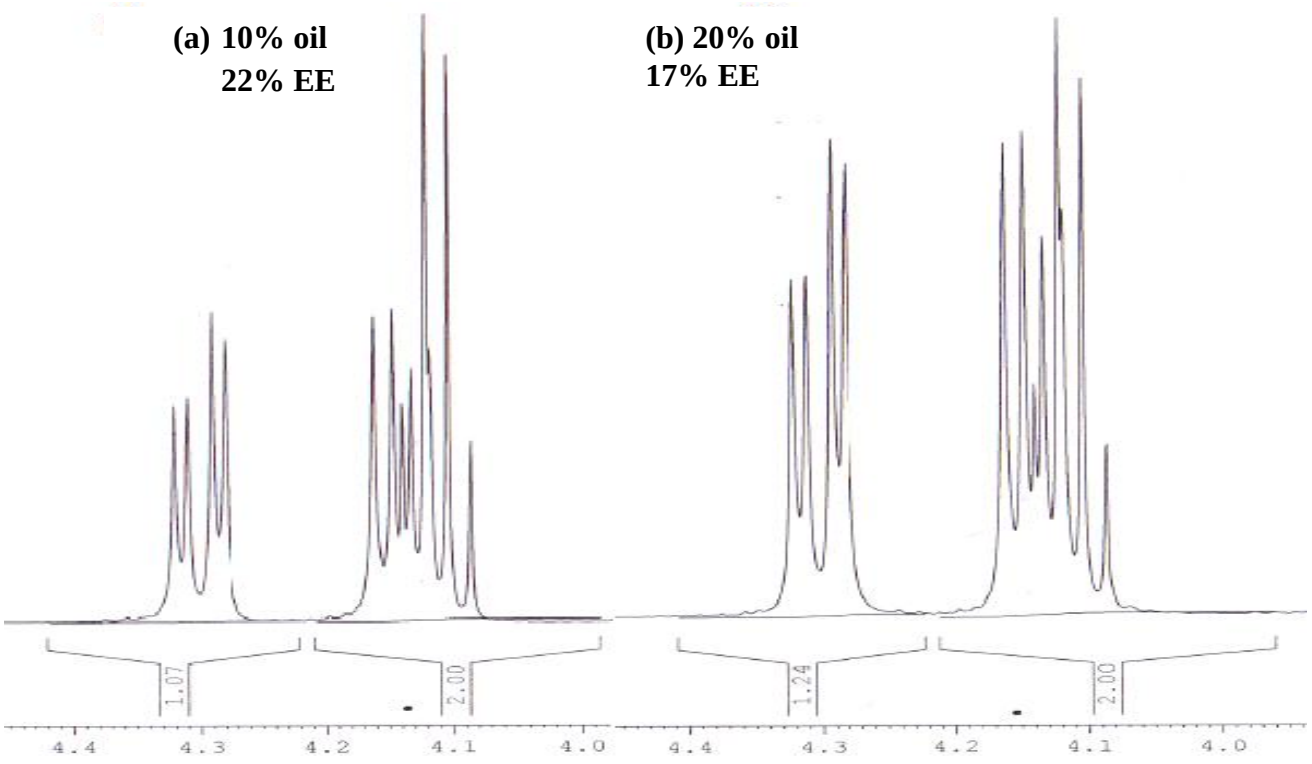


Fig. 4.5.3 (IV) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by *Penicillium citrinium* with varying percentage of cottonseed oil in the growth medium

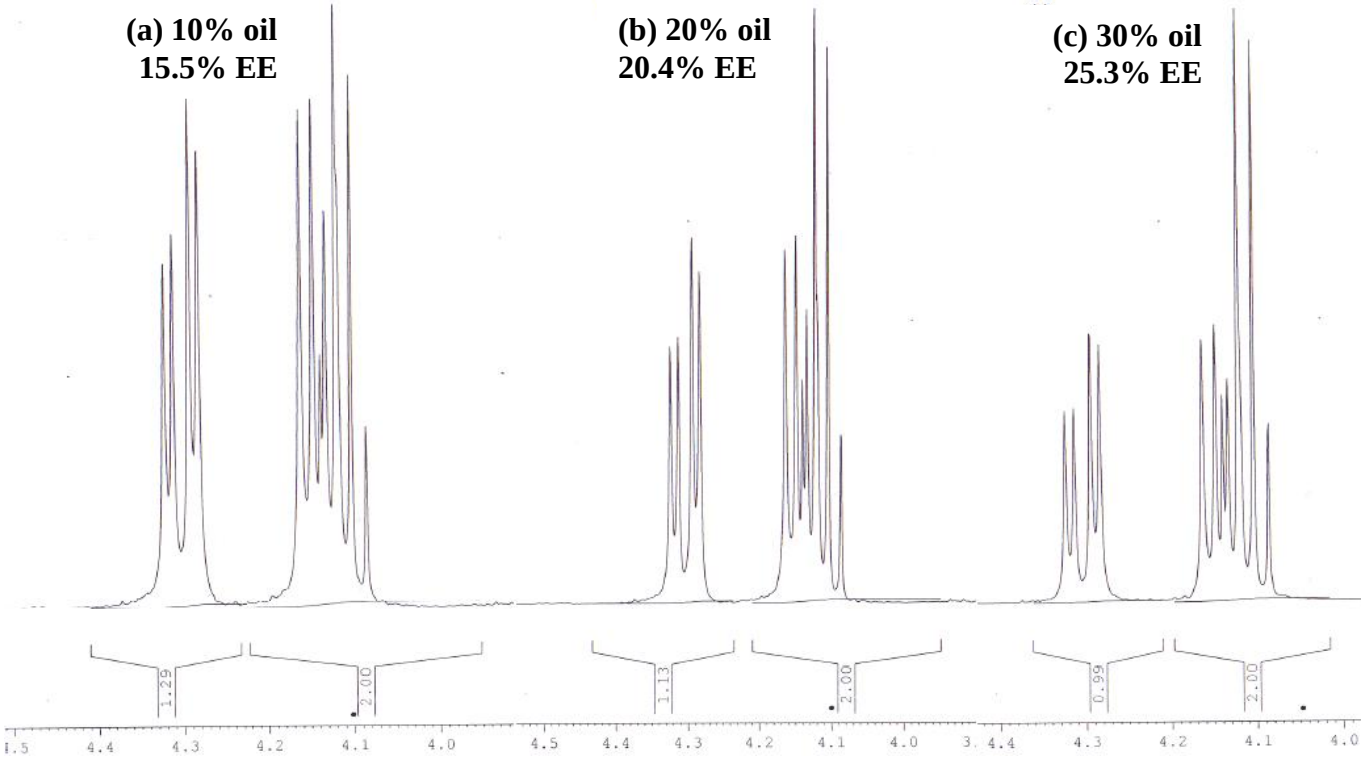


Fig. 4.5.3 (V) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by *Perconia sp*.

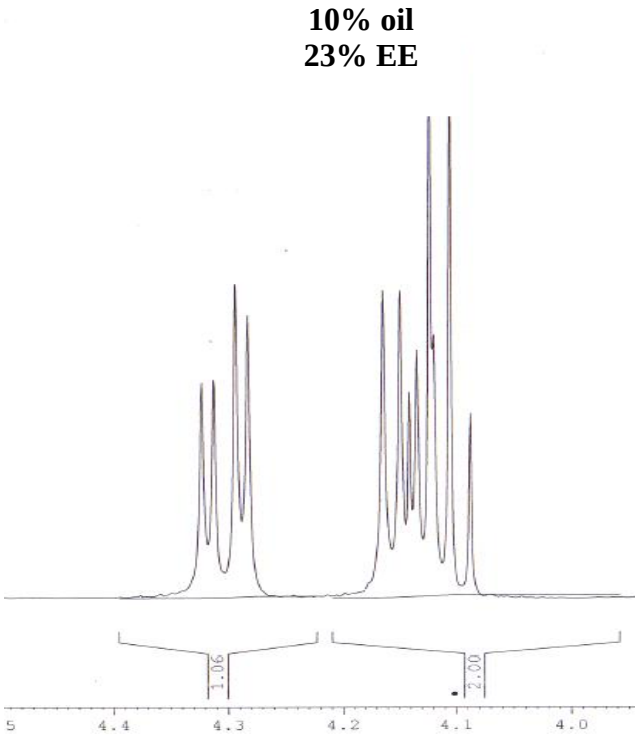
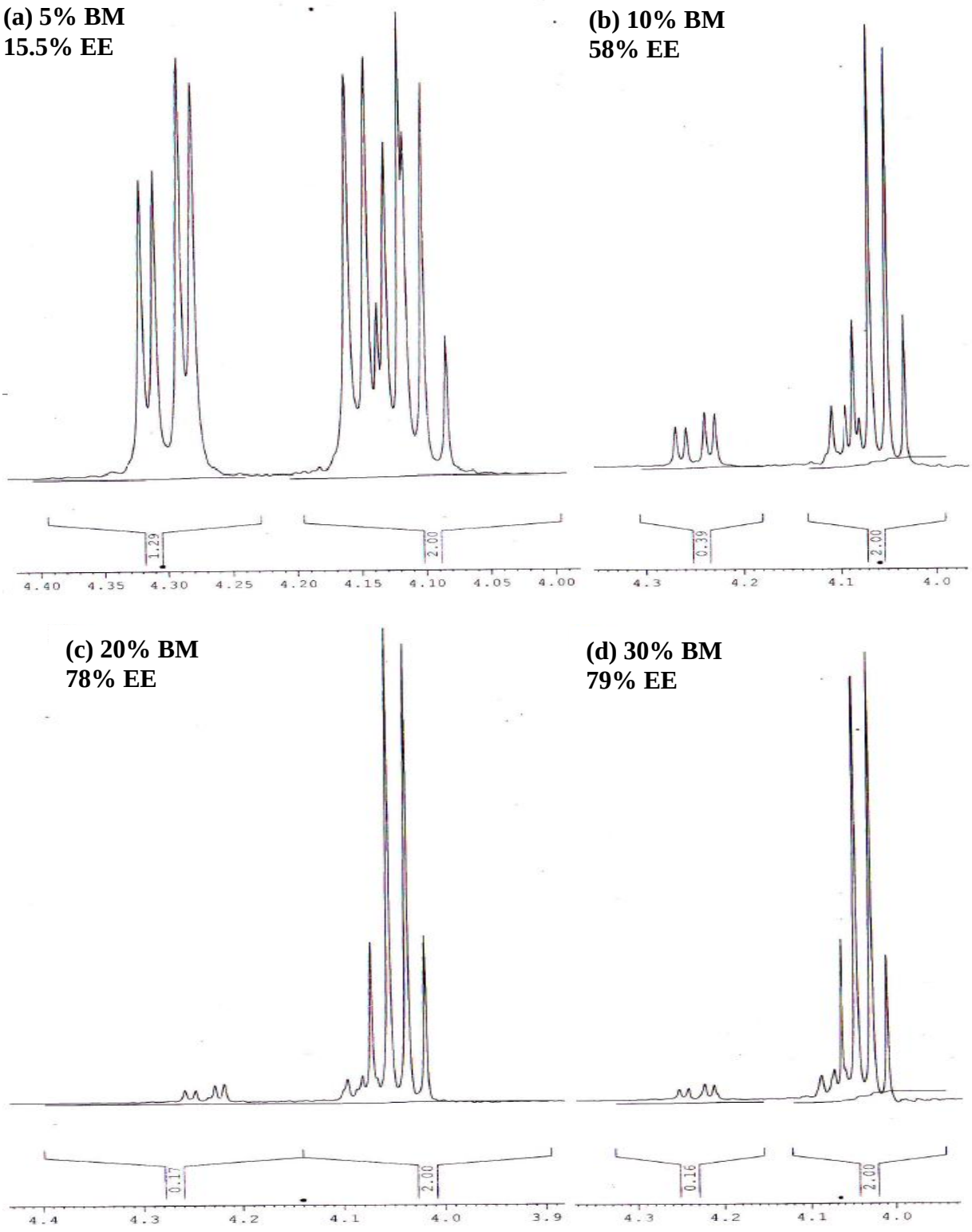


Fig. 4.6.1 Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by varying the percentage of dried biomass (BM) in reaction



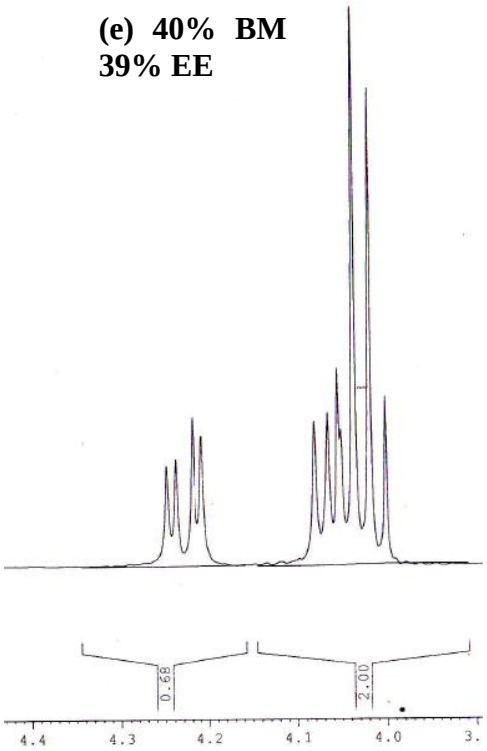
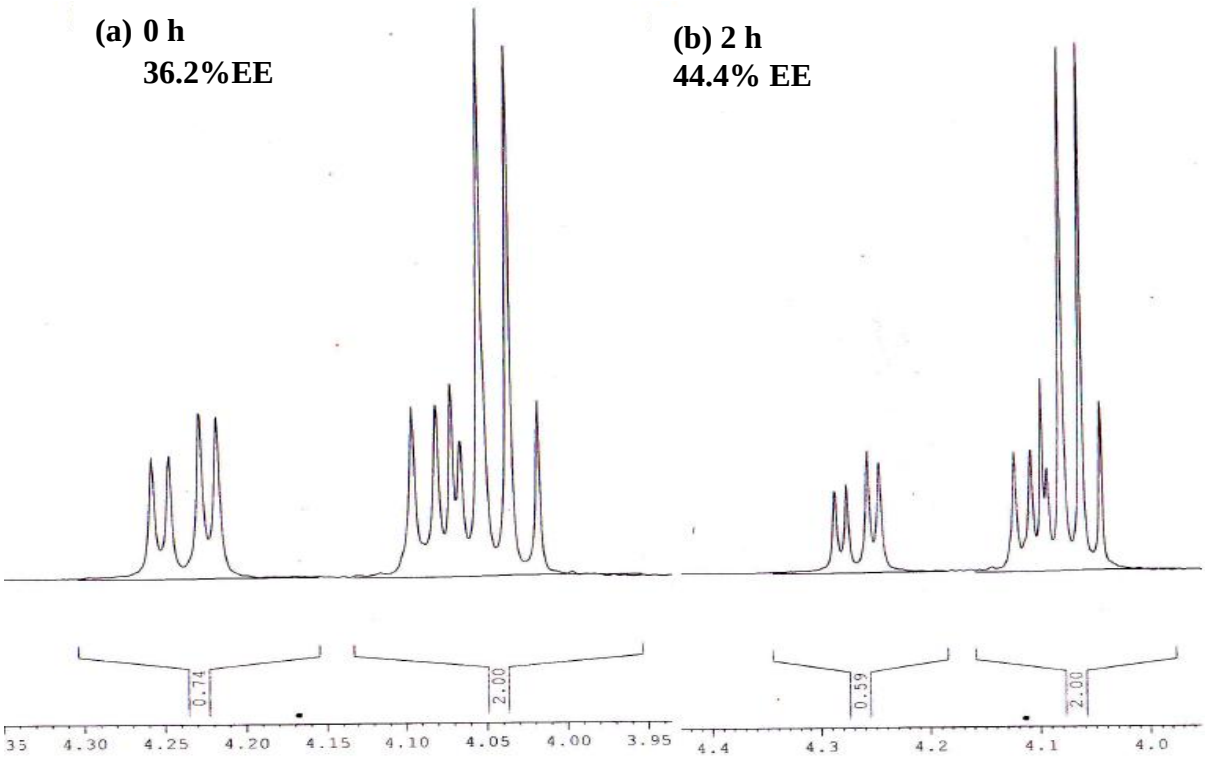


Fig. 4.6.2 Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by adding alcohol at different time interval



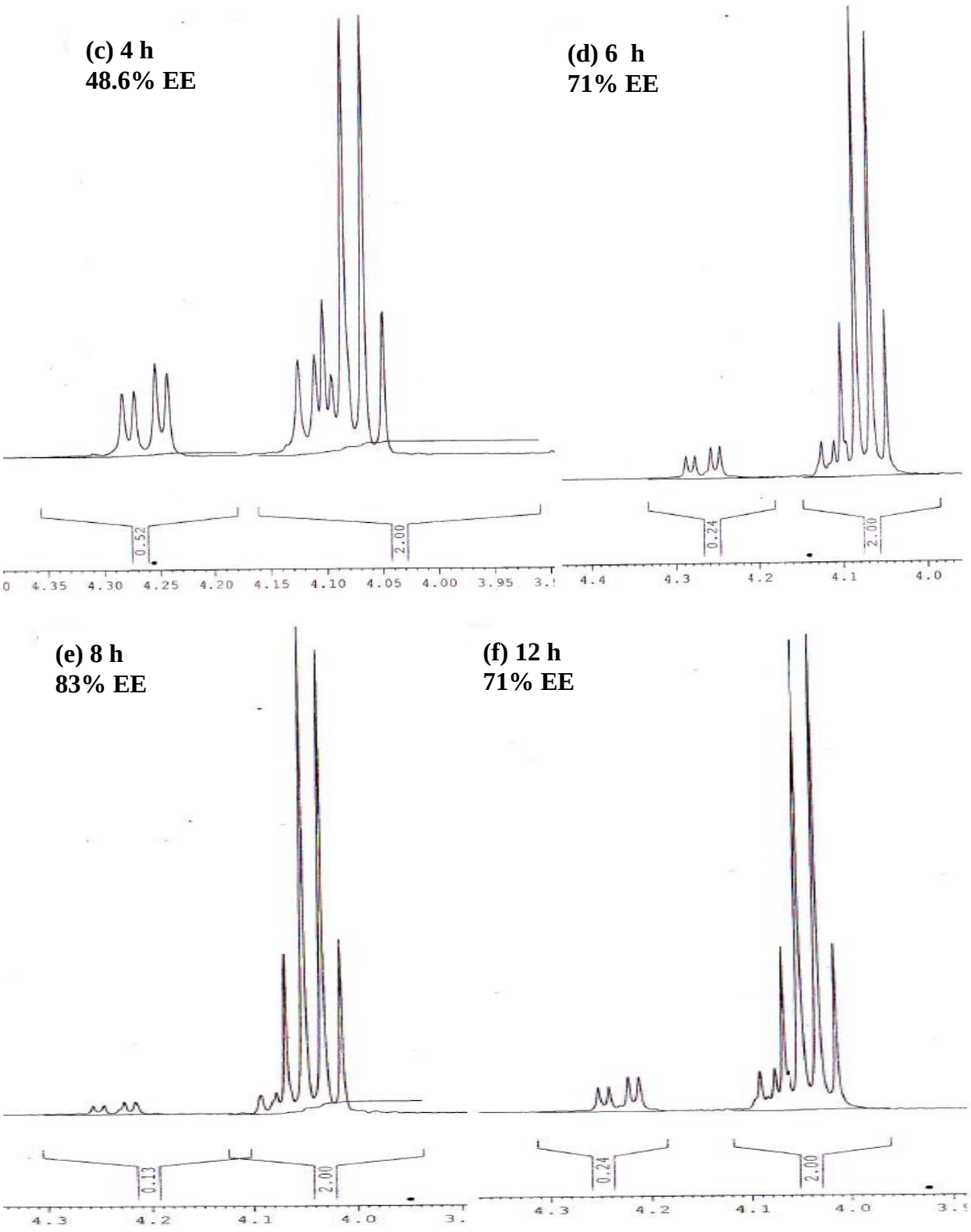
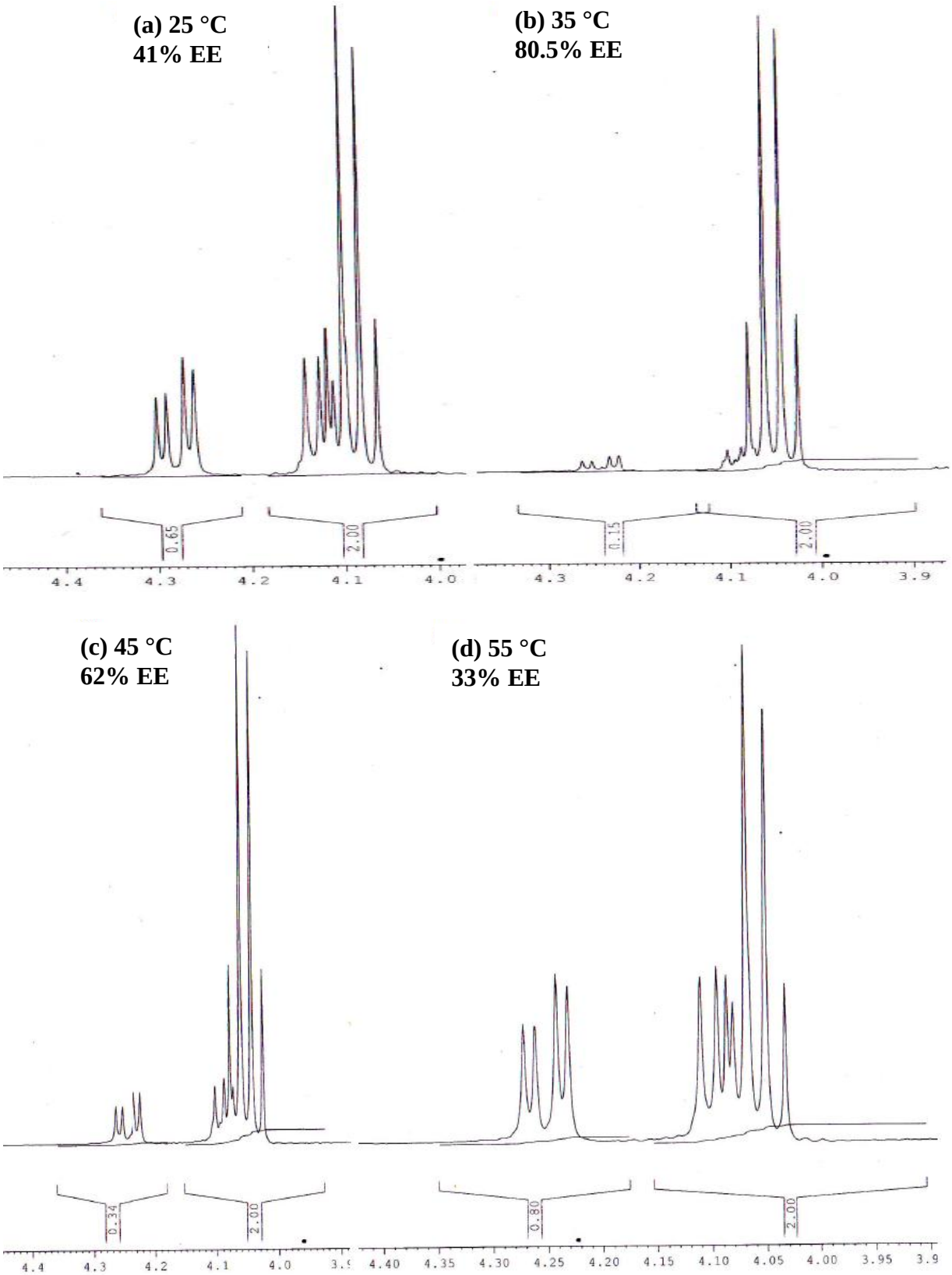

(c) 4 h
48.6% EE
0.52
2.00
4.35 4.30 4.25 4.20 4.15 4.10 4.05 4.00 3.95
(d) 6 h
71% EE
0.24
2.00
4.4 4.3 4.2 4.1 4.0
(e) 8 h
83% EE
0.13
2.00
4.3 4.2 4.1 4.0
(f) 12 h
71% EE
0.24
2.00
4.3 4.2 4.1 4.0

Fig. 4.6.3 Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced at different temperatures



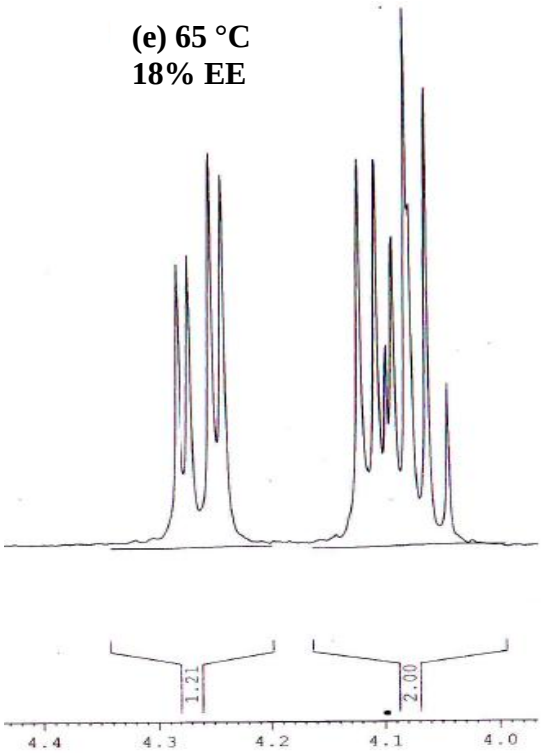
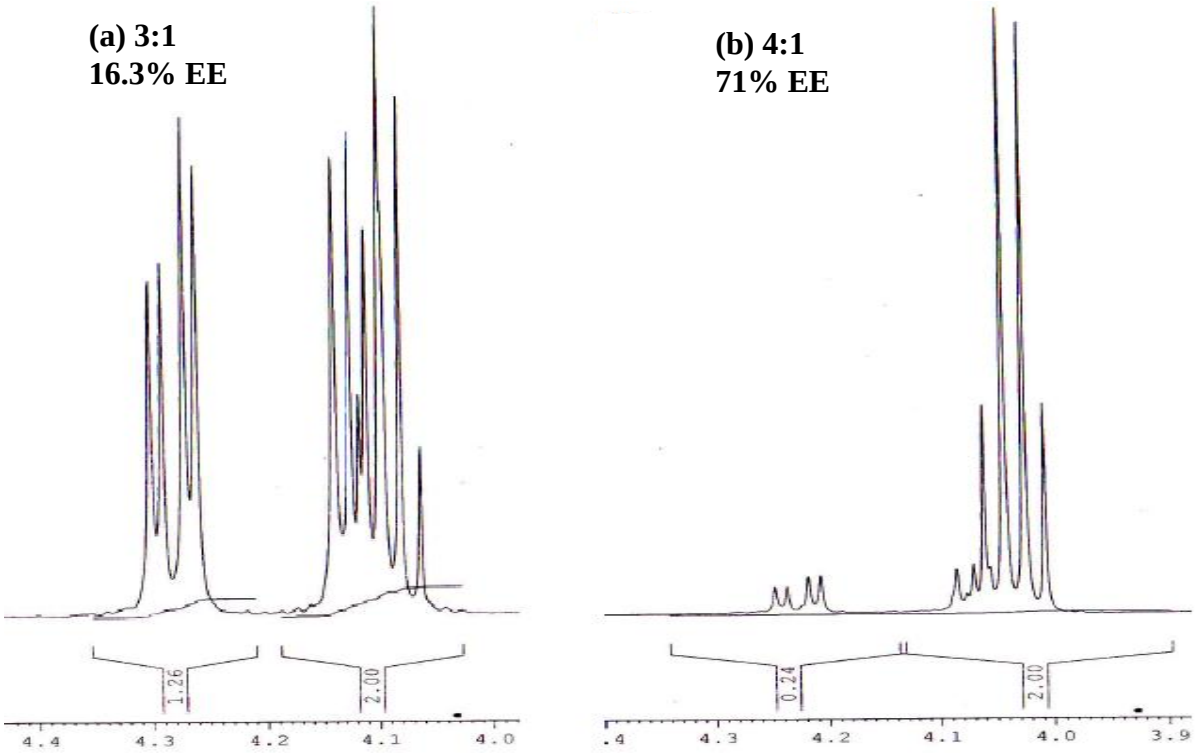


Fig. 4.6.4 Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by adding different molar ration of alcohol to oil



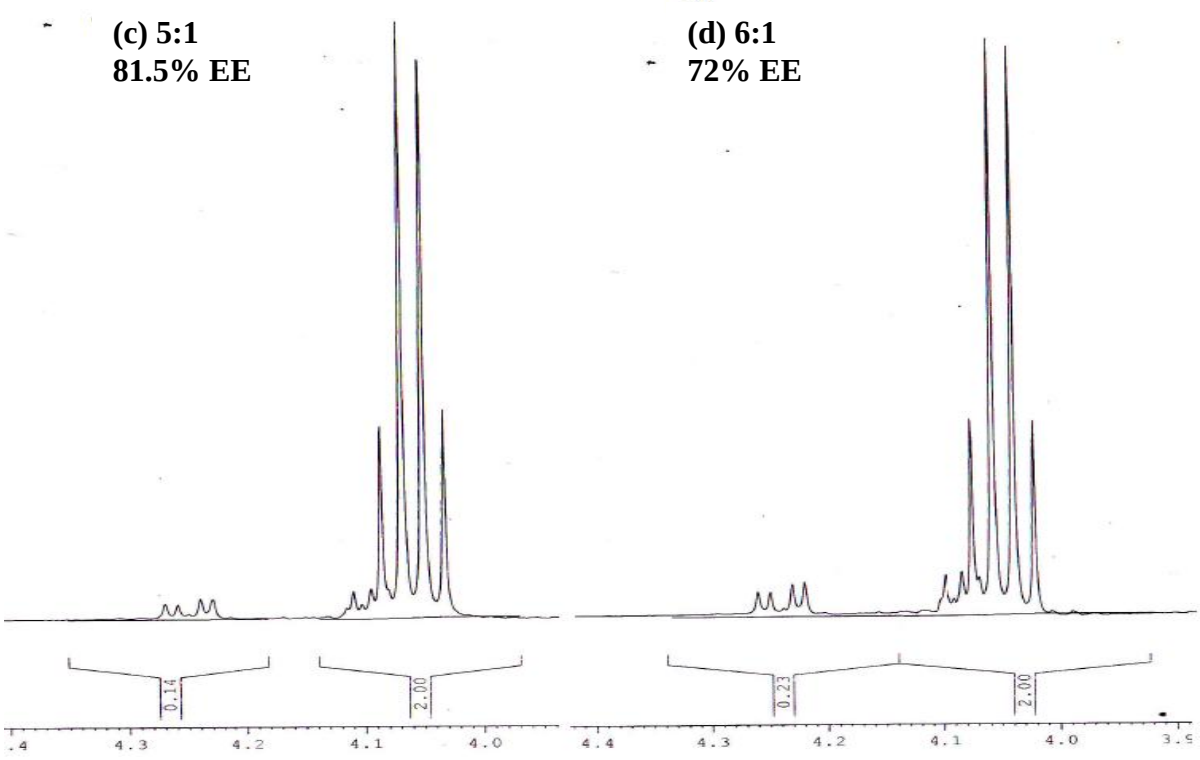
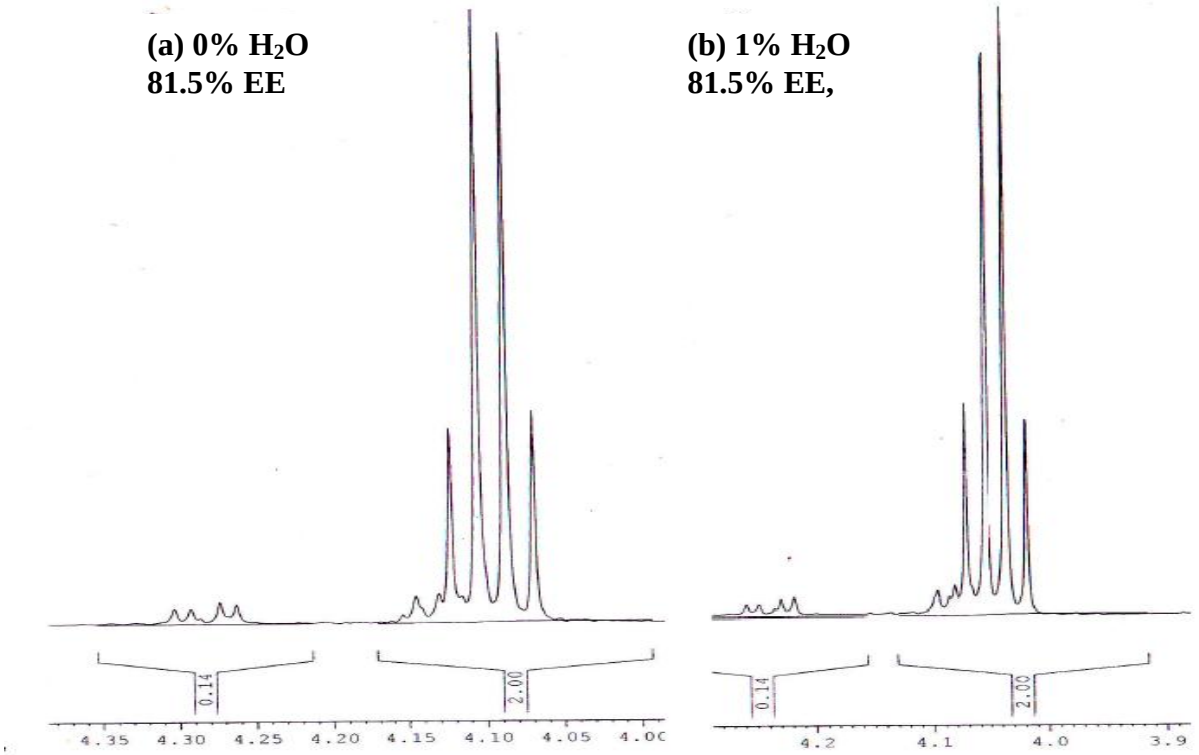
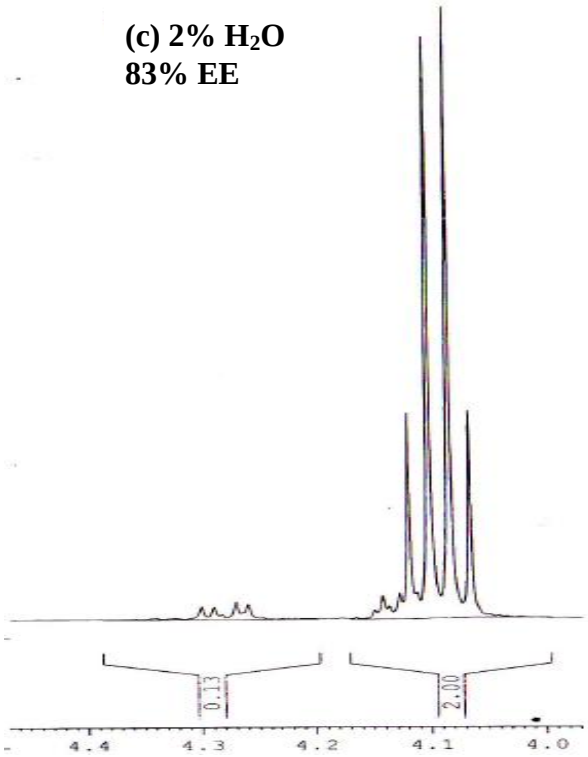


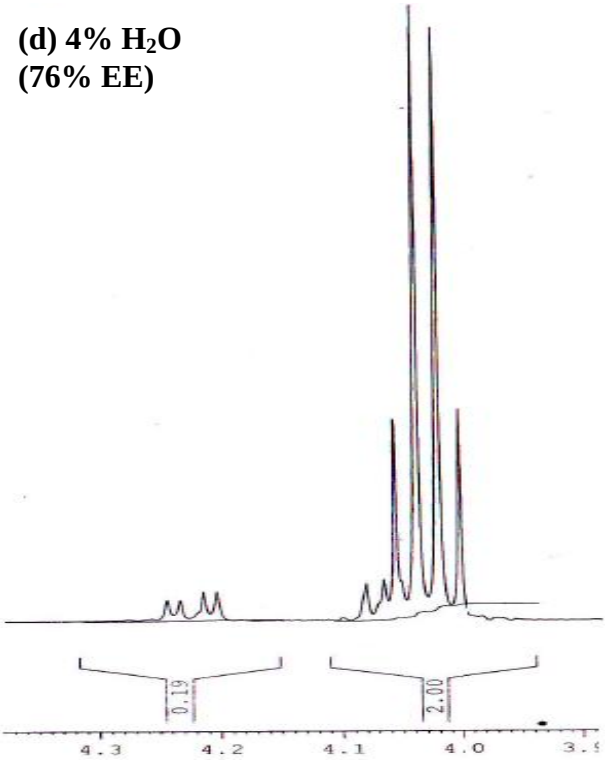
Fig. 4.6.5 Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced during reaction in the presence of different water percentage in the reaction mixture



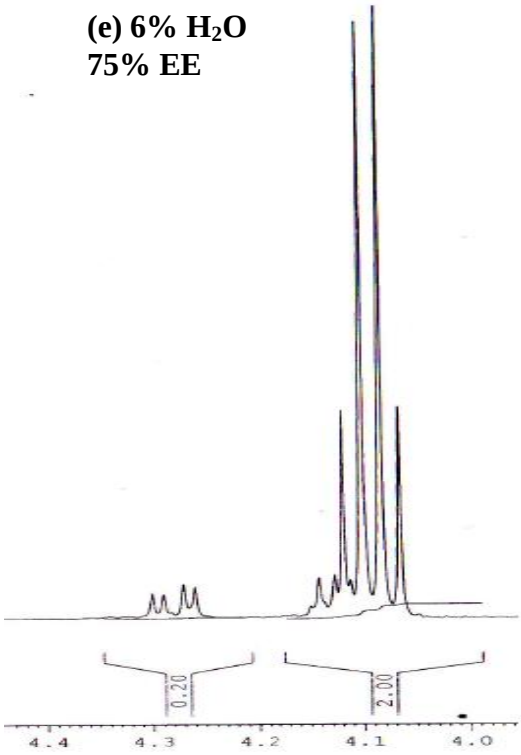
(c) 2% H_2O
83% EE



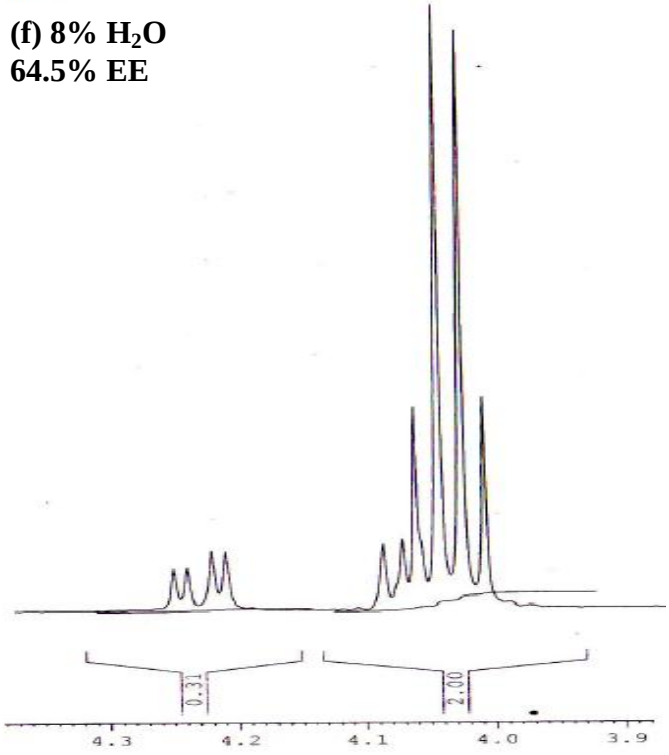
(d) 4% H_2O
(76% EE)



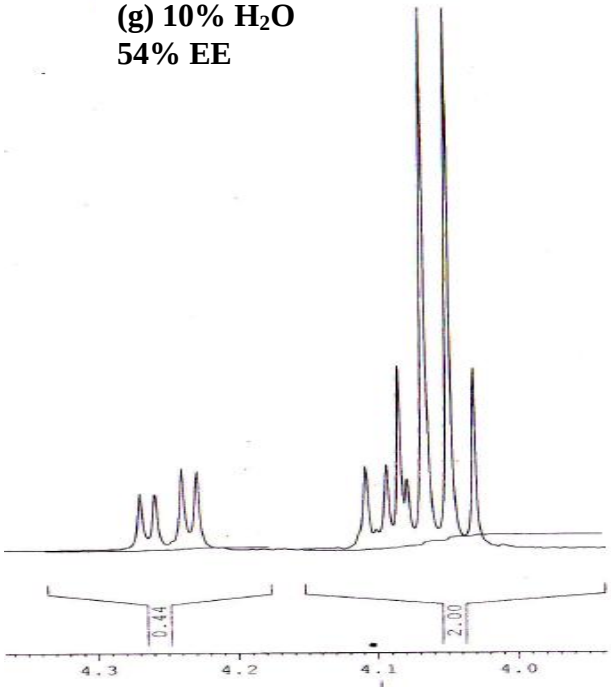
(e) 6% H_2O
75% EE



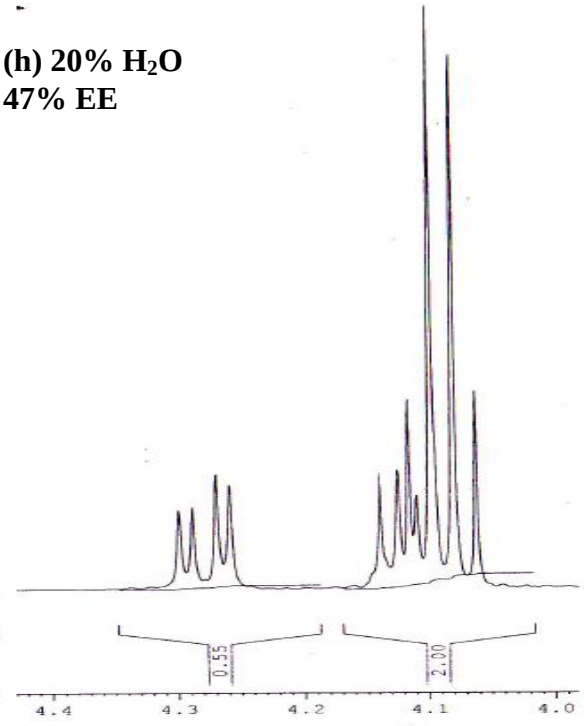
(f) 8% H_2O
64.5% EE



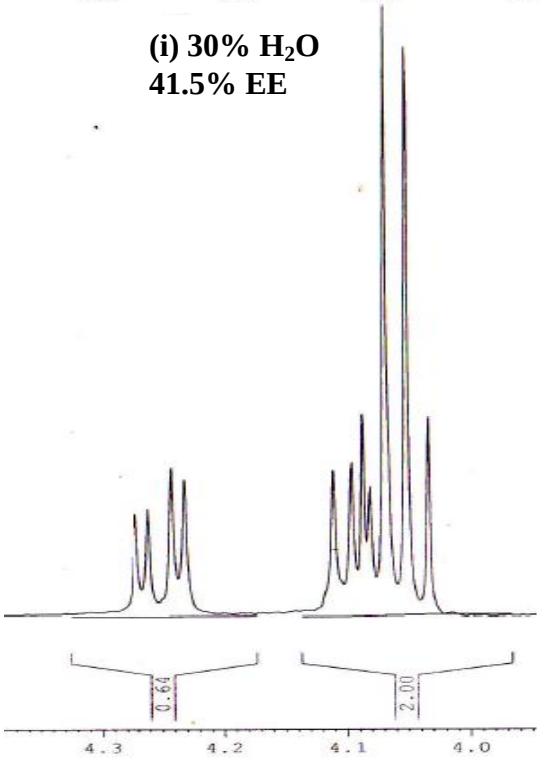
(g) 10% H_2O
54% EE



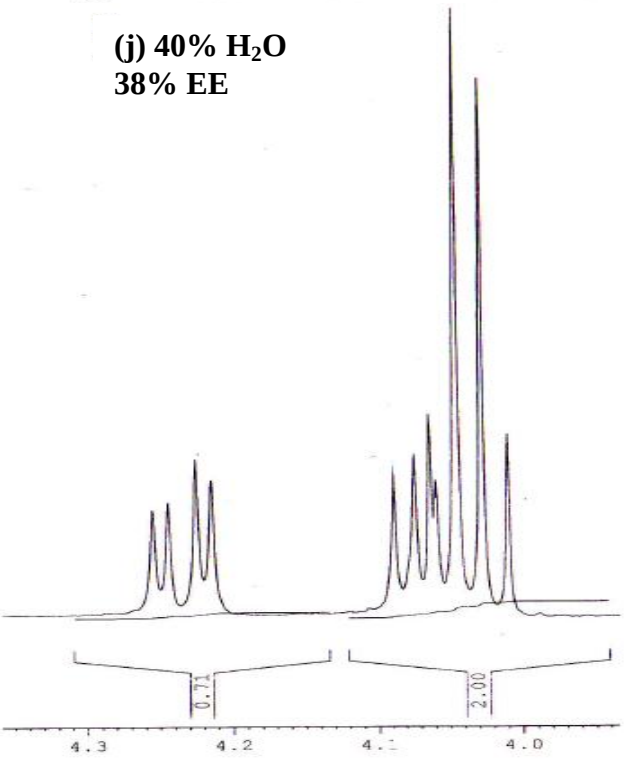
(h) 20% H_2O
47% EE



(i) 30% H_2O
41.5% EE



(j) 40% H_2O
38% EE



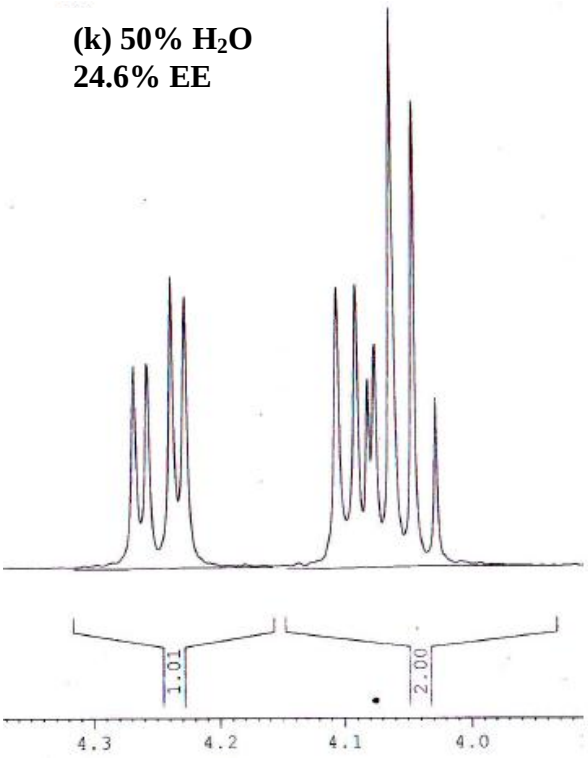
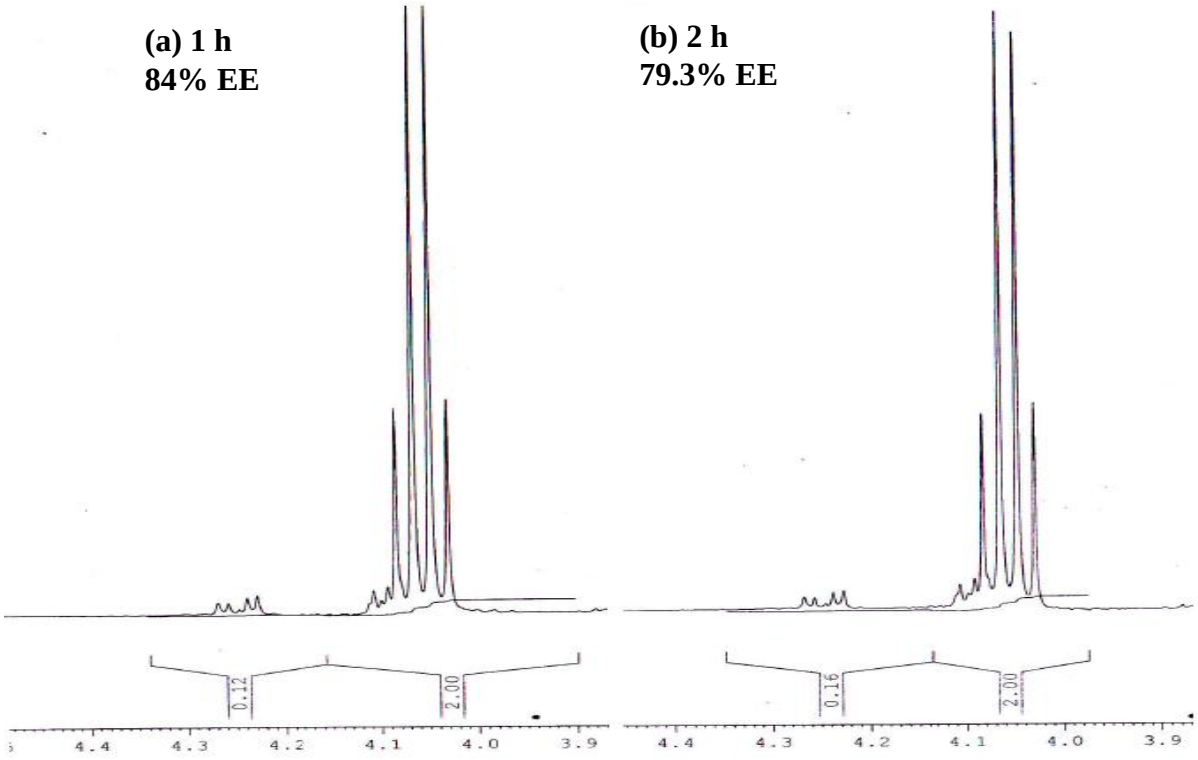
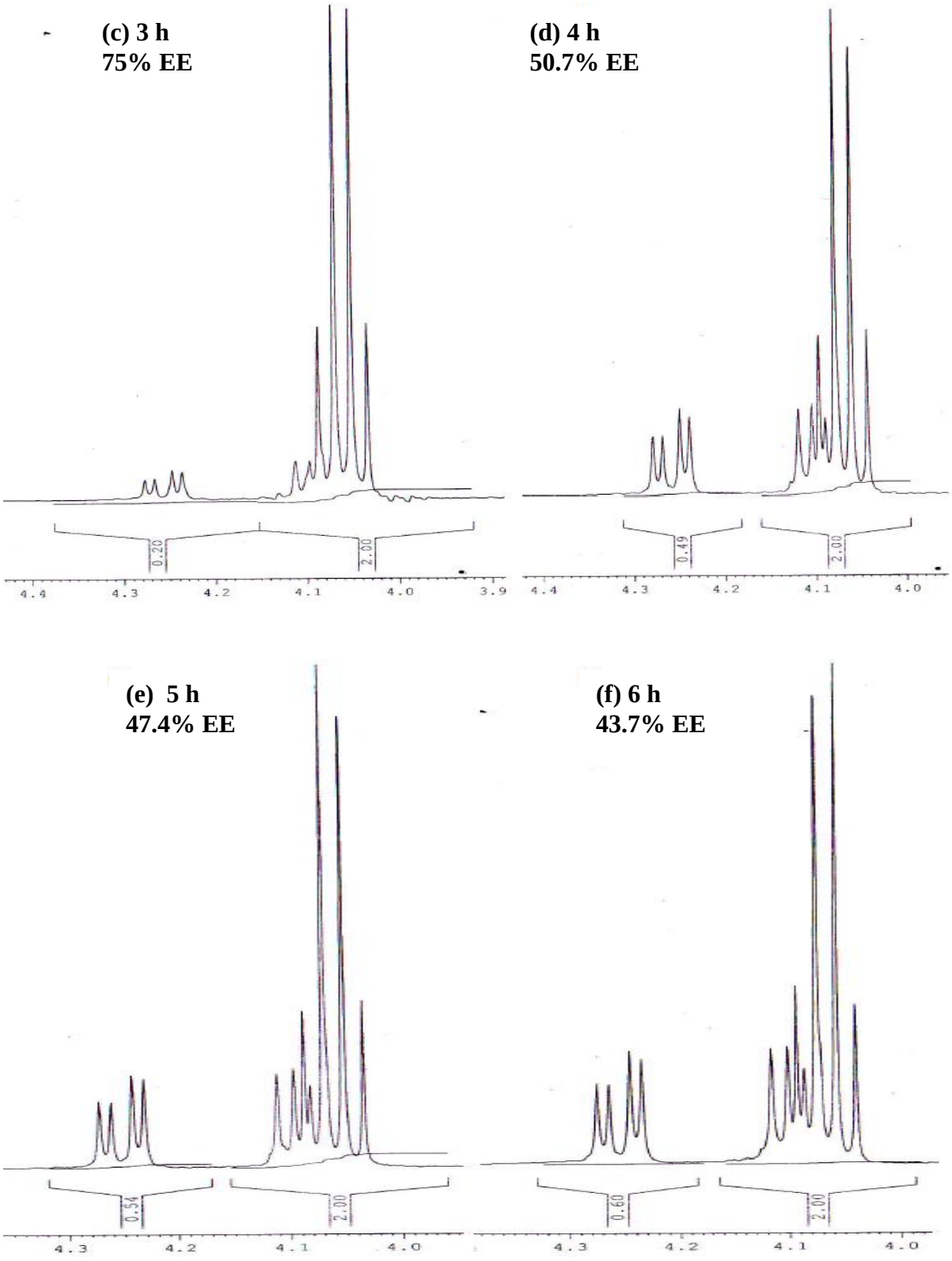


Fig. 4.6.6 Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by using oil with different frying time




(c) 3 h
75% EE
(d) 4 h
50.7% EE
(e) 5 h
47.4% EE
(f) 6 h
43.7% EE

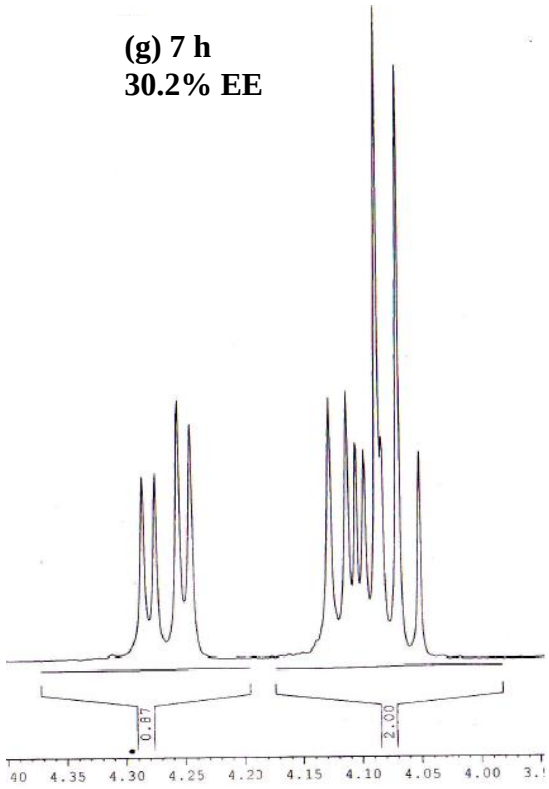


Fig. 4.6.7 Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by using different oil under standardized conditions

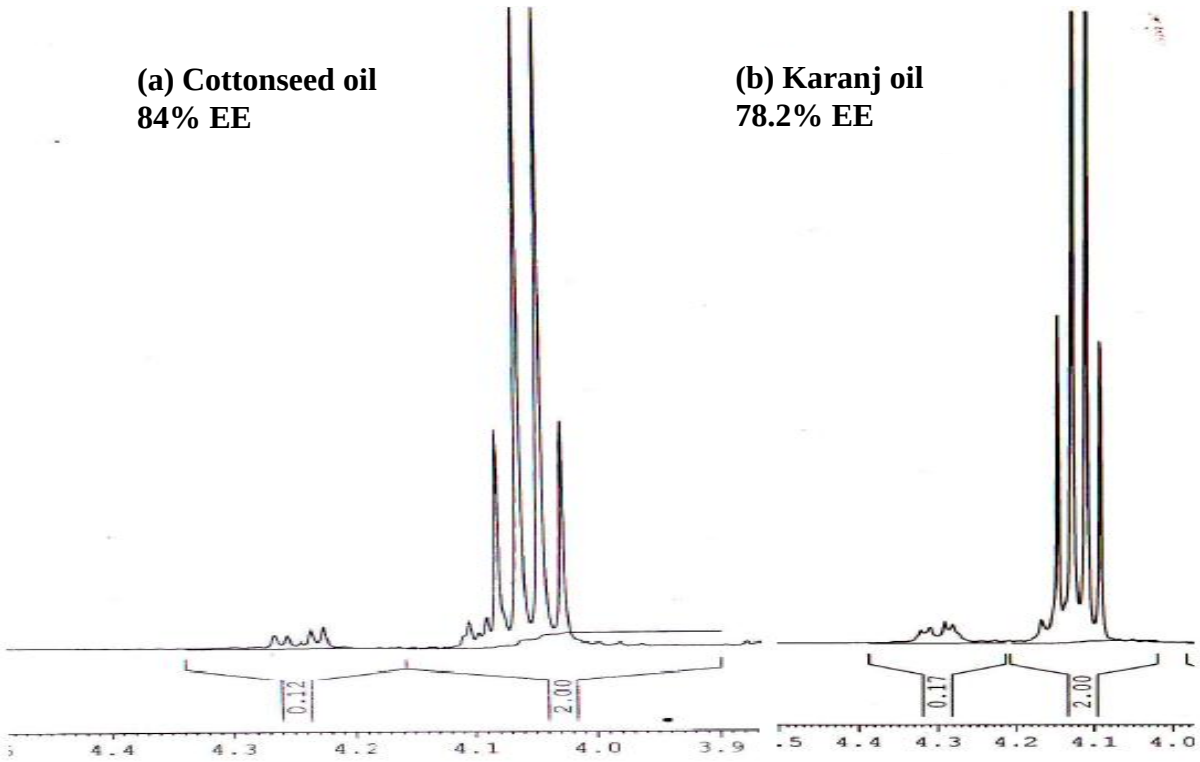
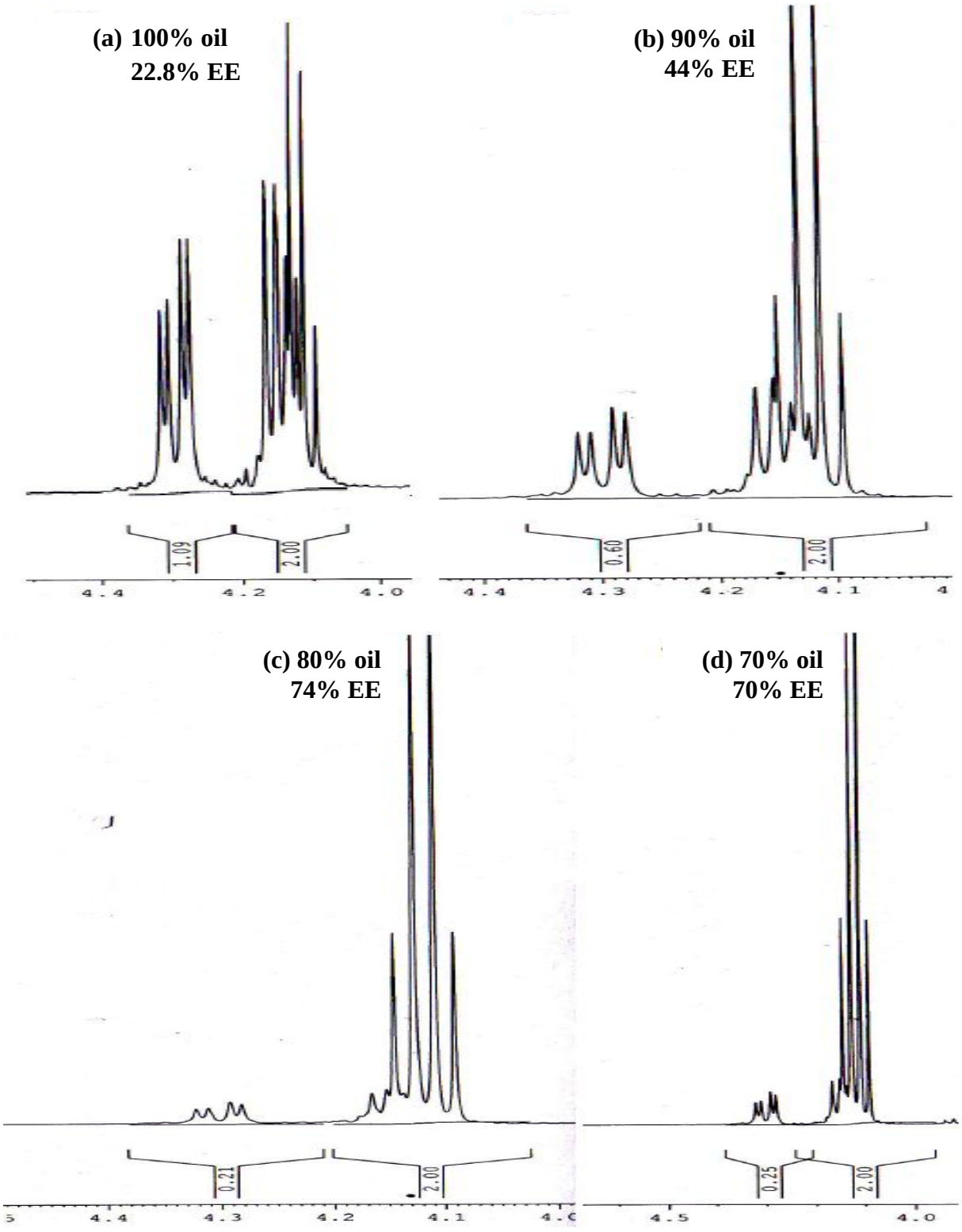


Fig. 4.7.2 (I) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced from cottonseed oil using immobilized luffa as a whole cell catalyst with varying percentage of oil in the reaction media



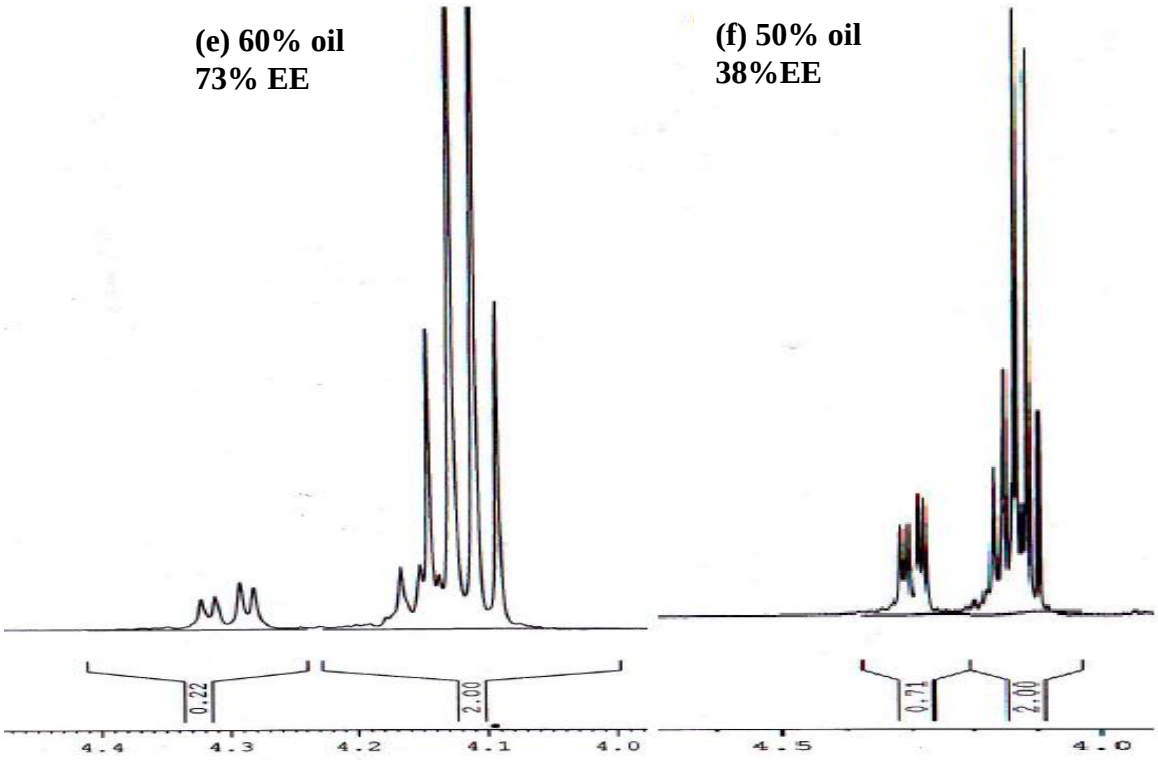
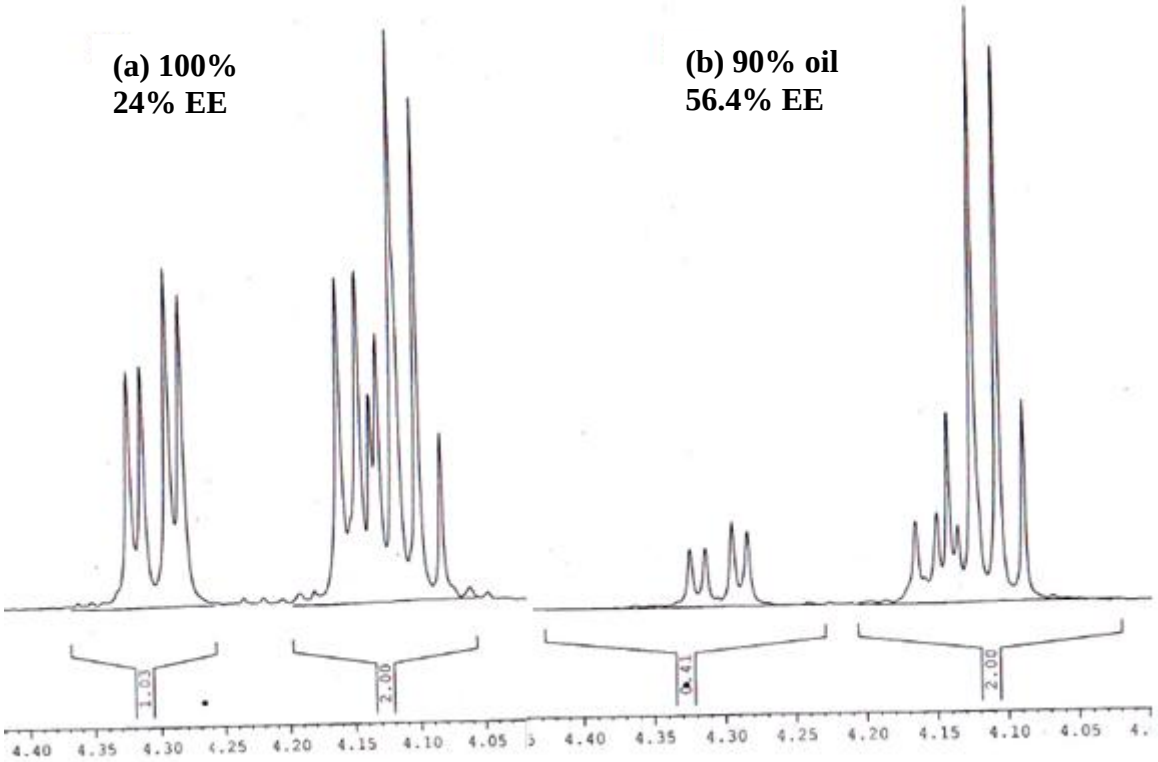
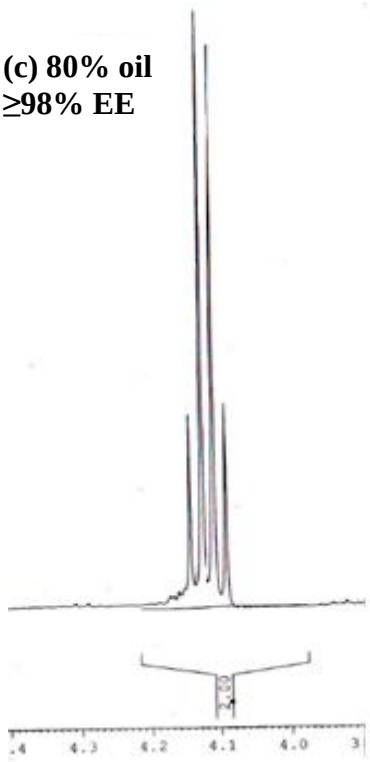


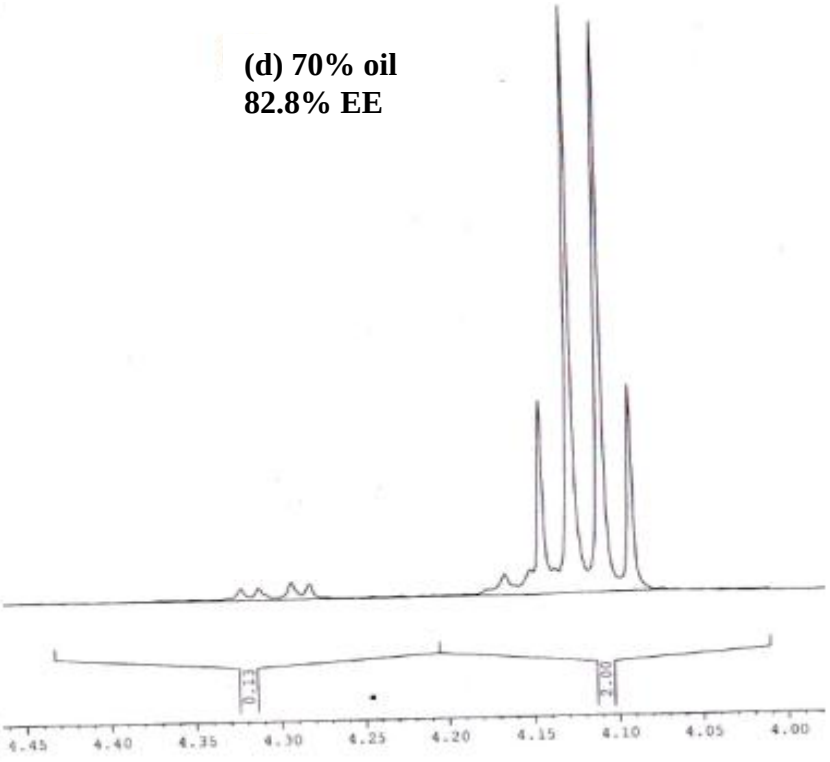
Fig. 4.7.2 (II) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced from cottonseed oil using immobilized polyurethane foam as a whole cell catalyst with varying percentage of oil in the reaction media



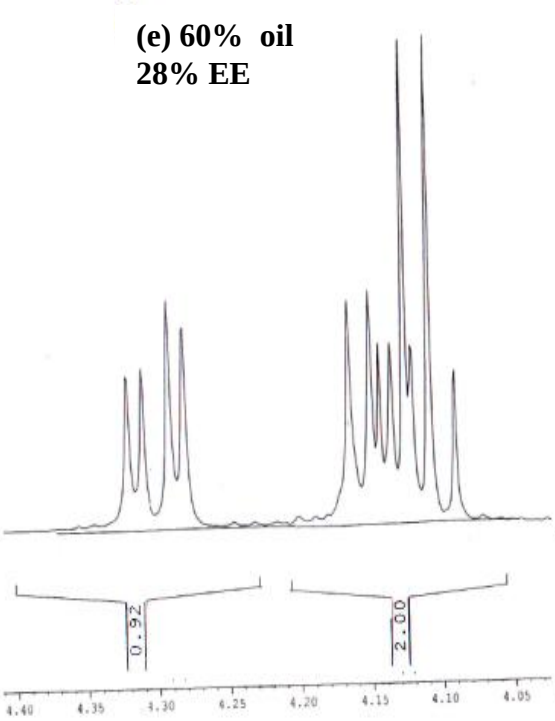
(c) 80% oil
≥98% EE



(d) 70% oil
82.8% EE



(e) 60% oil
28% EE



(f) 50% oil
30% EE

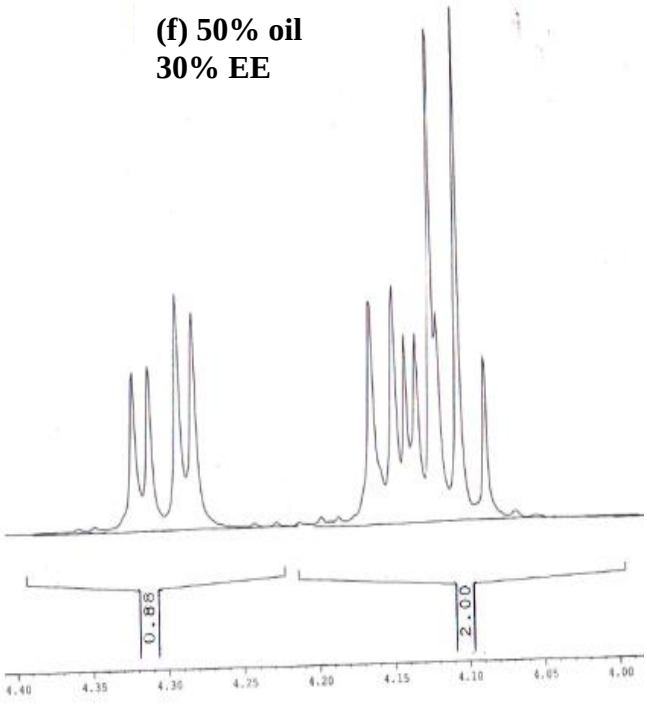
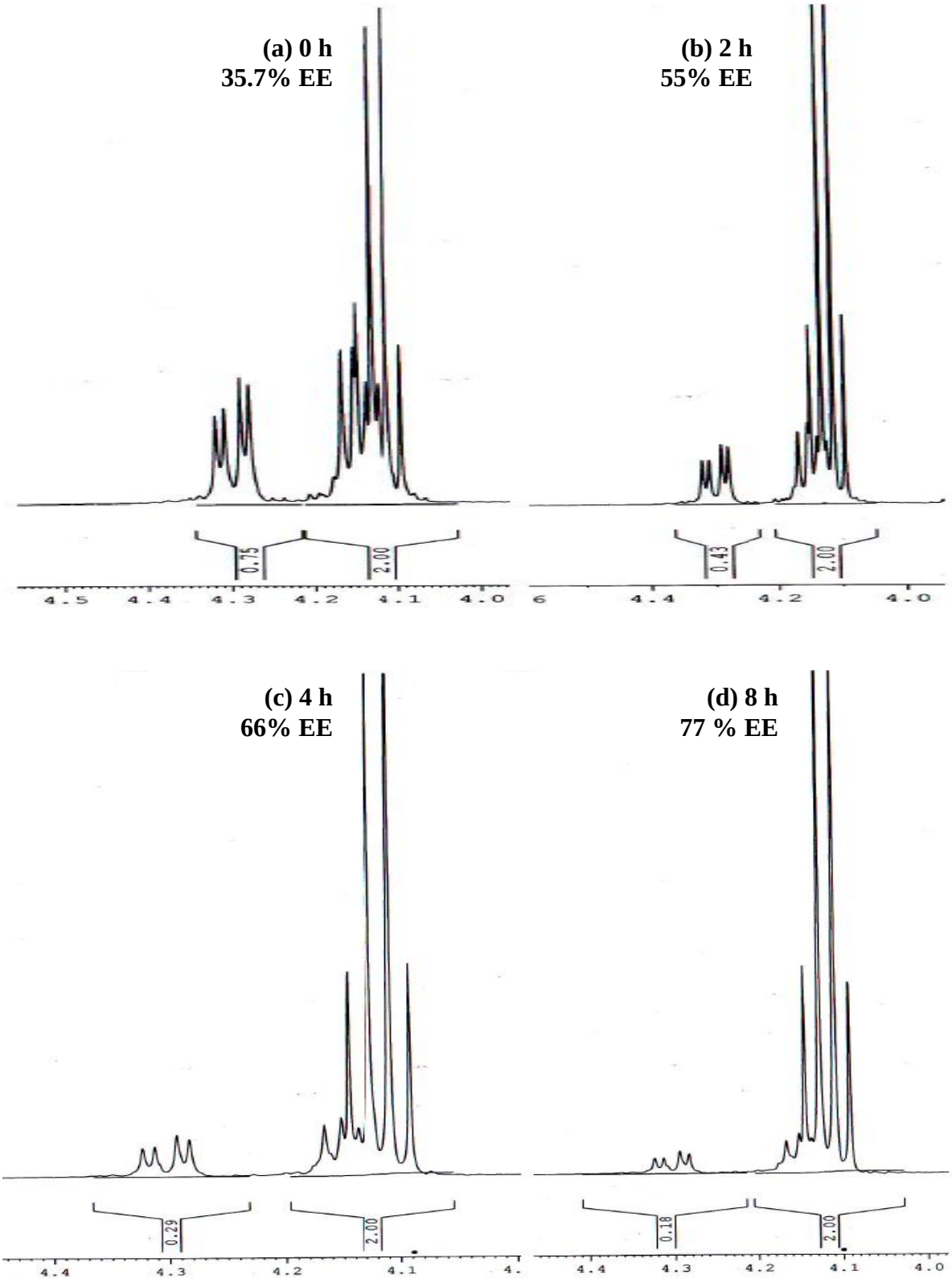


Fig. 4.7.2 (III) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by immobilized luffa as a whole cell catalyst from cottonseed oil, with ethanol addition at different time interval



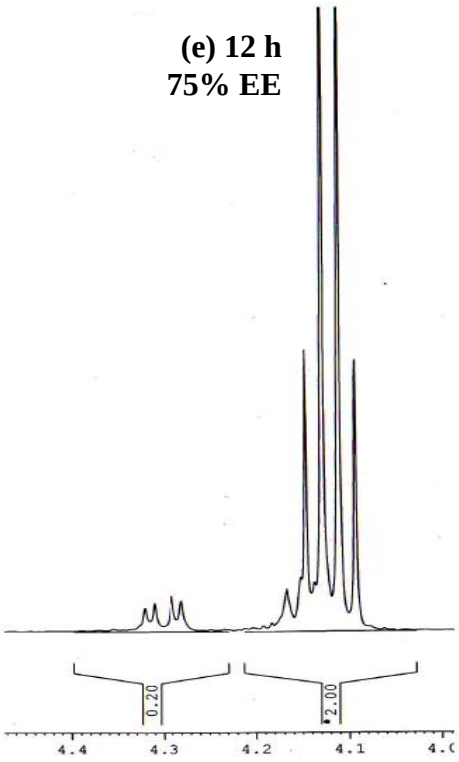
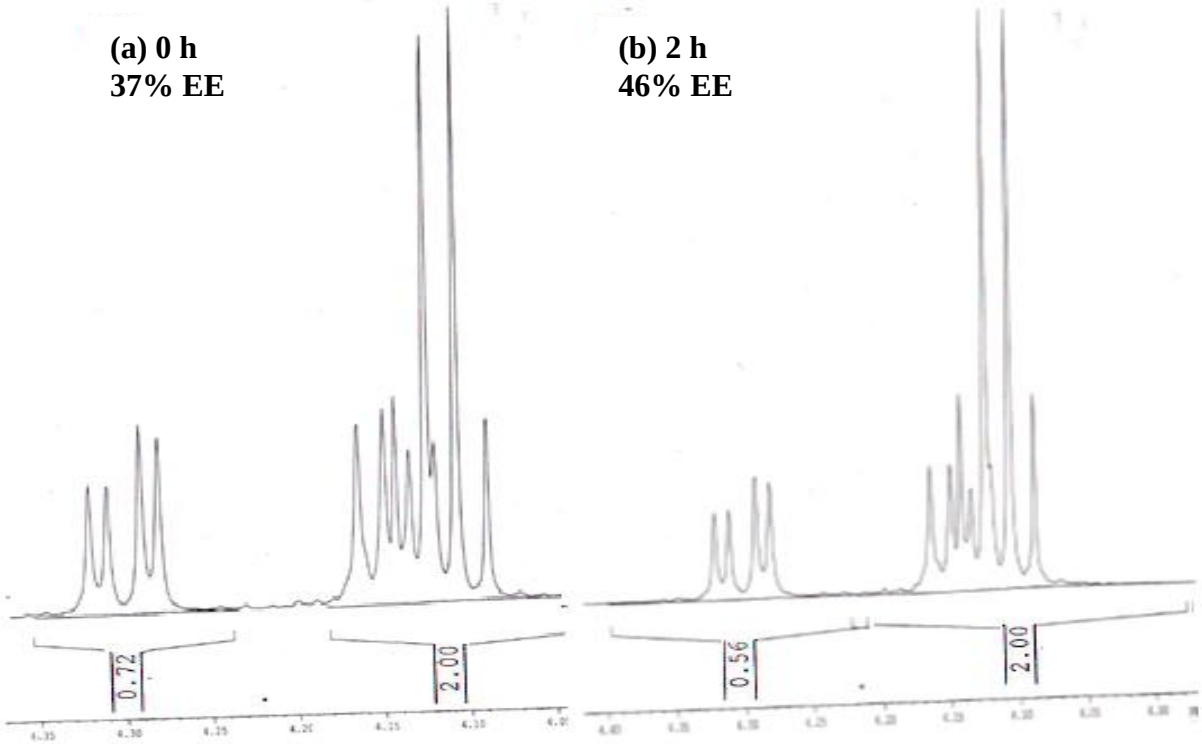
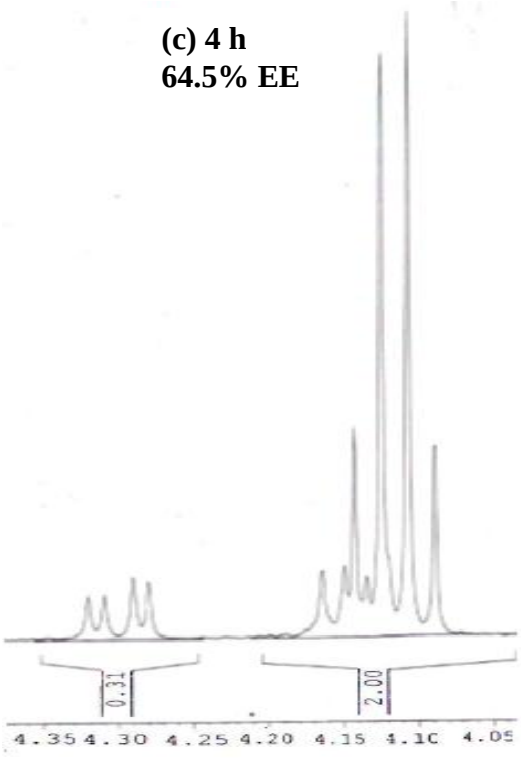


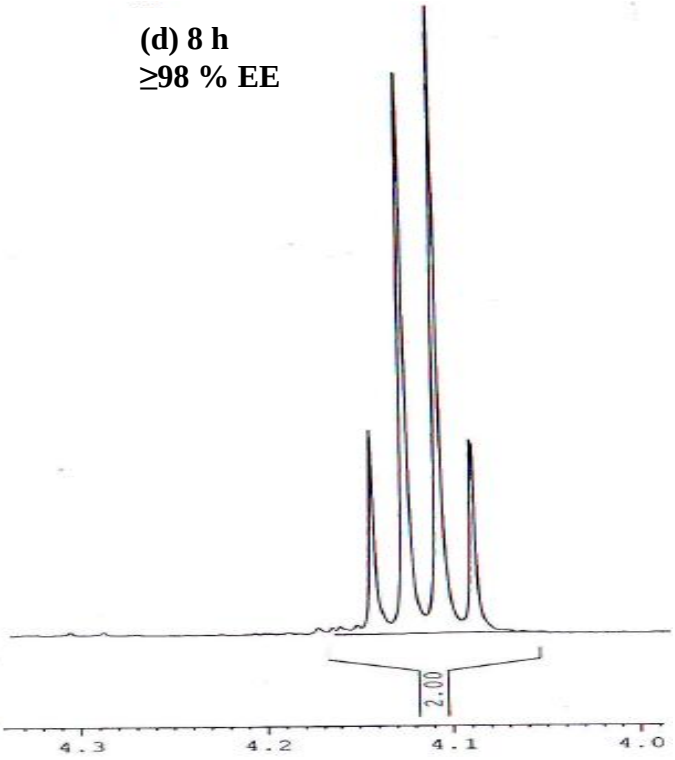
Fig. IV Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by immobilized polyurethane foam as a whole cell catalyst from cottonseed oil by adding alcohol stepwise at different time intervals



(c) 4 h
64.5% EE



(d) 8 h
≥98 % EE



(e) 12 h
≥98% EE

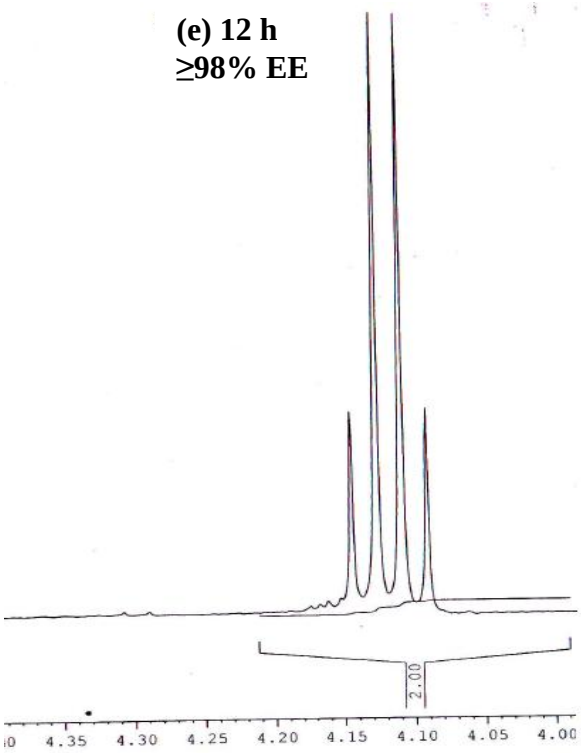
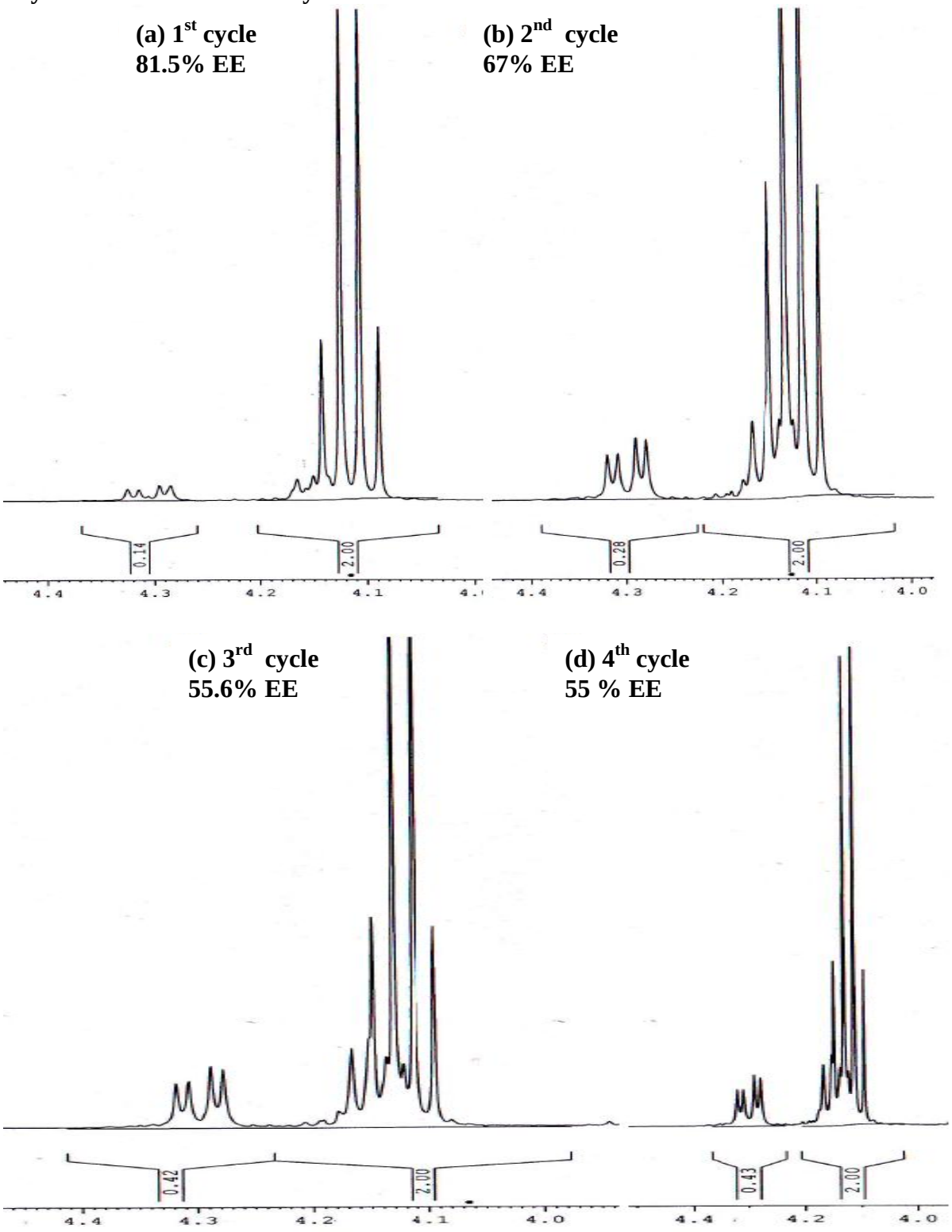


Fig. 4.7.2 (V) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced from cottonseed oil using immobilized luffa at different cycles as a whole cell catalyst with at different time interval of alcohol addition



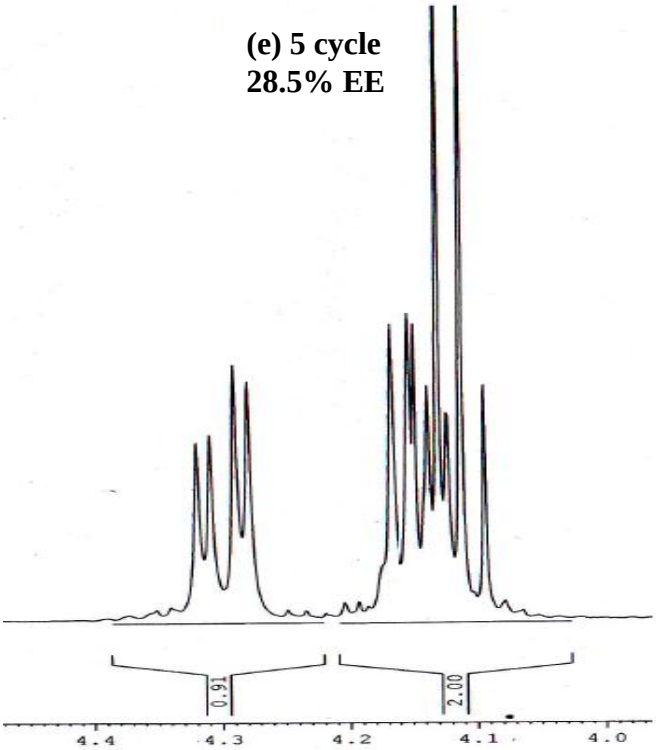
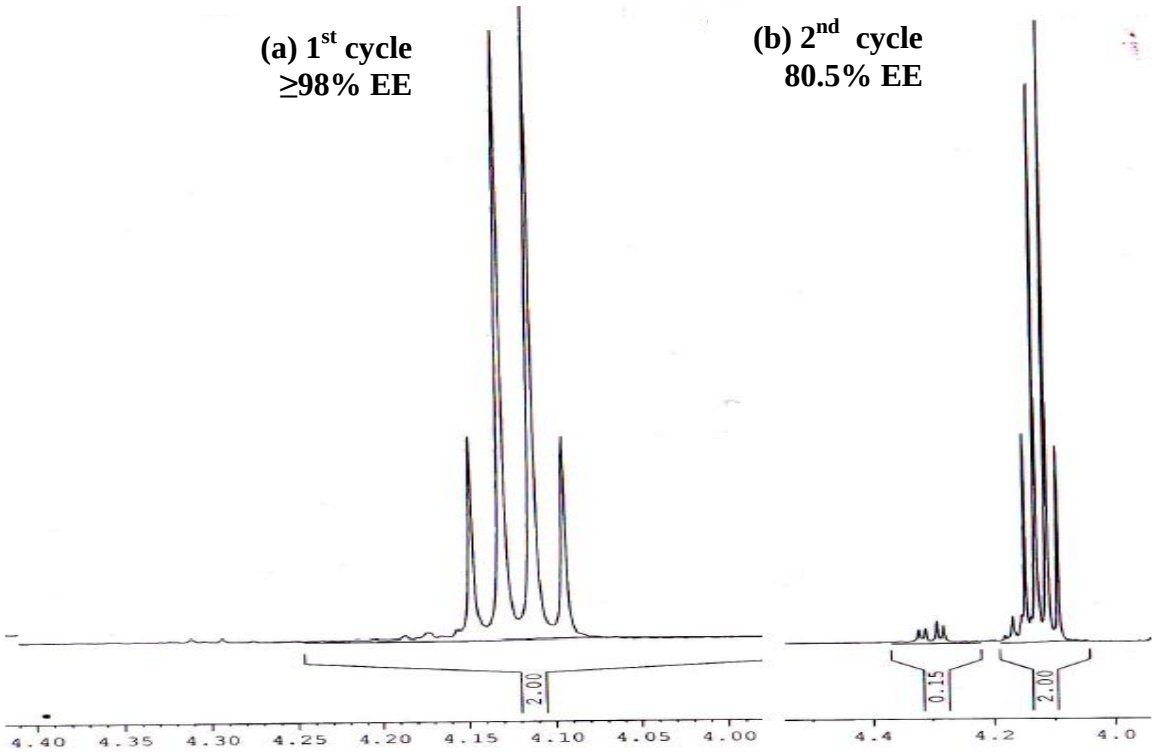
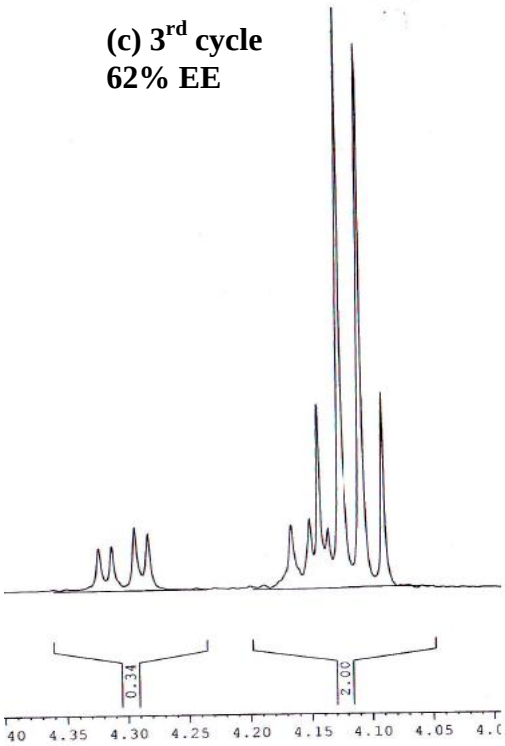


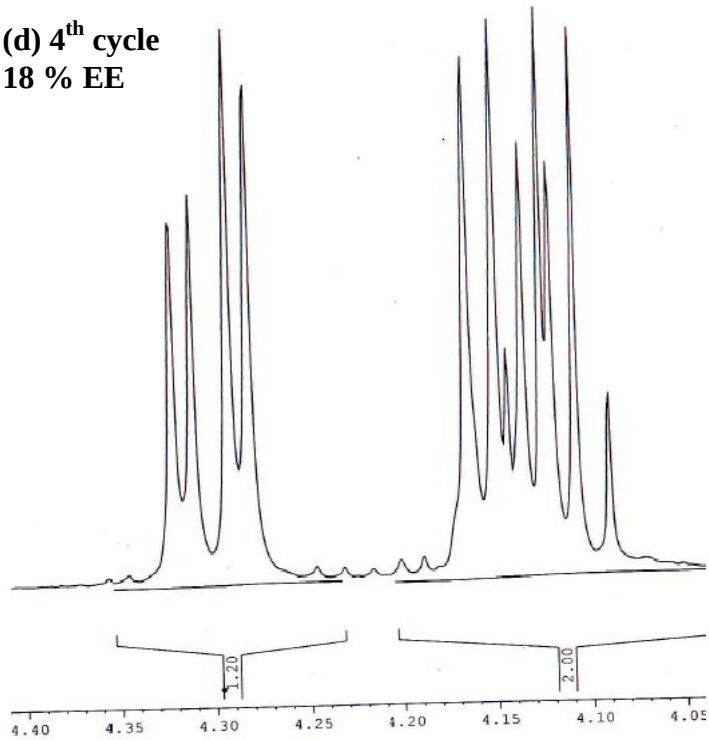
Fig. 4.7.2 (VI) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced from cottonseed oil using immobilized polyurethane foam at different cycles as a whole cell catalyst



(c) 3rd cycle
62% EE



(d) 4th cycle
18 % EE



(e) 5th cycle
17% EE

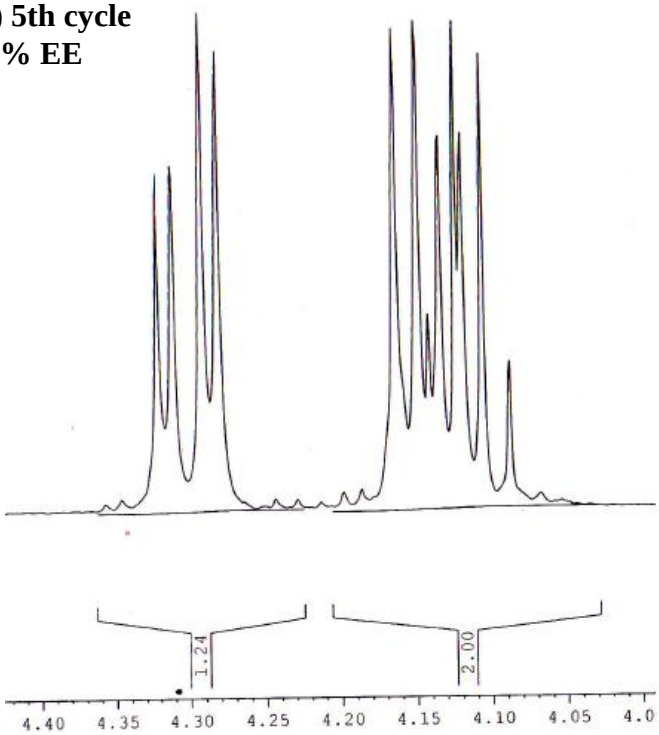


Fig. 4.7.2 (VII) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) from different oils using immobilized luffa as catalyst

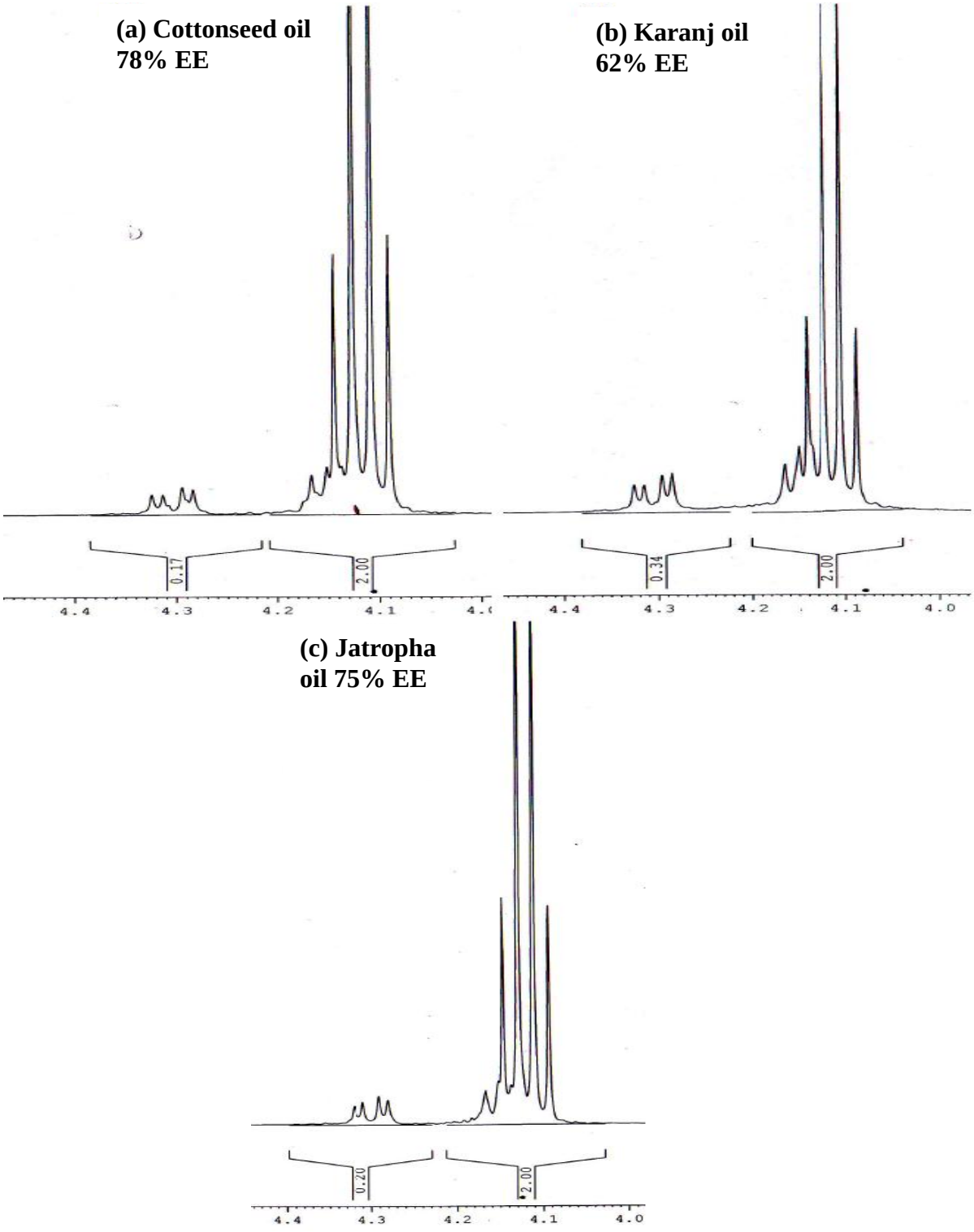
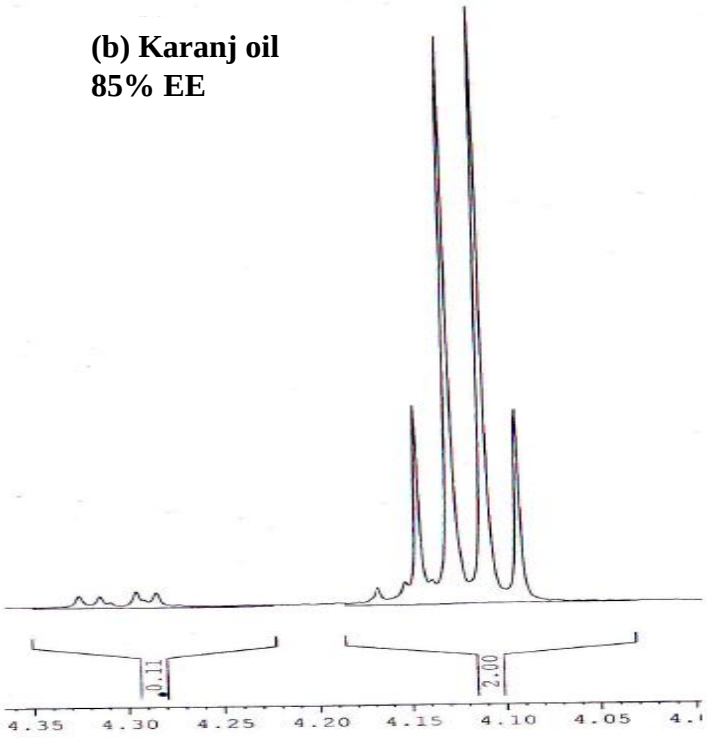


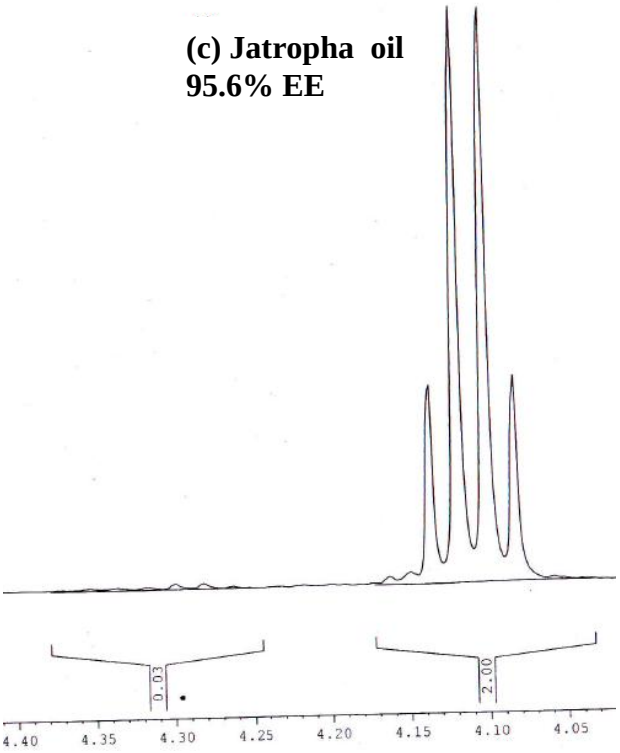
Fig. 4.7.2 (VIII) Extended region of ^{1}H NMR from 4.00 ppm to 4.40 ppm of ethyl ester (EE) produced by from different oils using polyurethane foam as catalyst

(a) Cottonseed oil 98% EE

(b) Karanj oil 85% EE



(c) Jatropha oil 95.6% EE



Annexure VI: Transesterification with different alcohols as acyl acceptor

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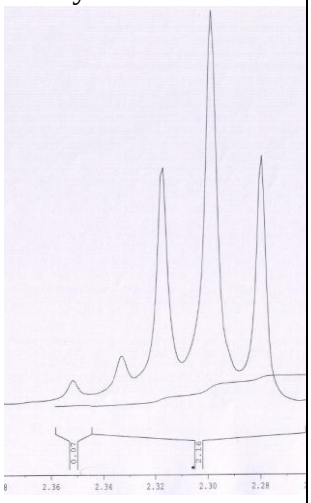
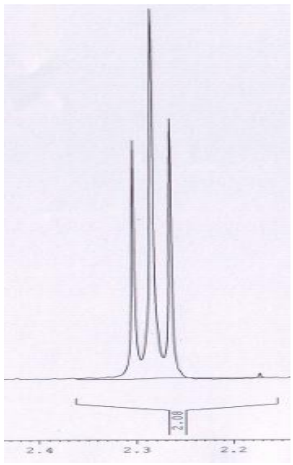
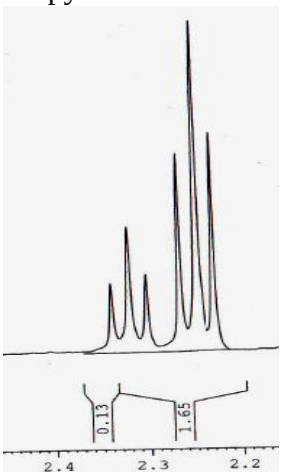
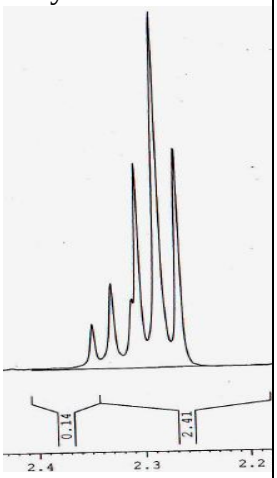
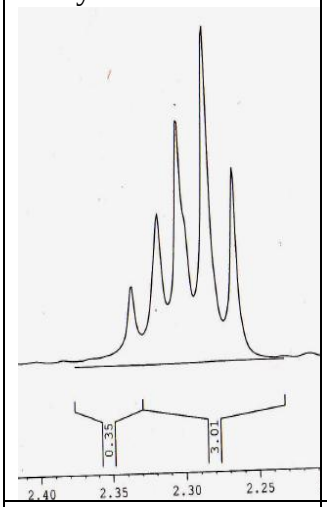
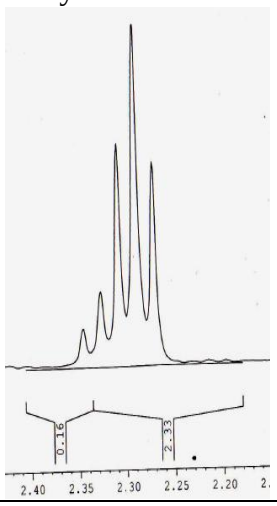
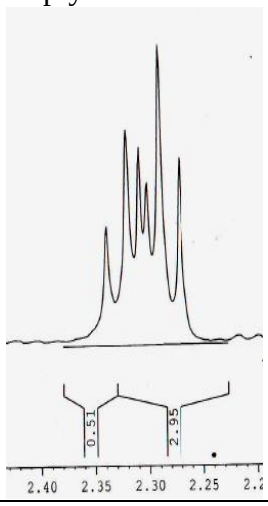
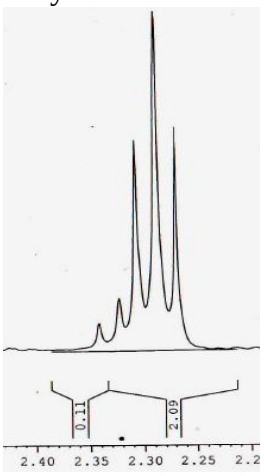
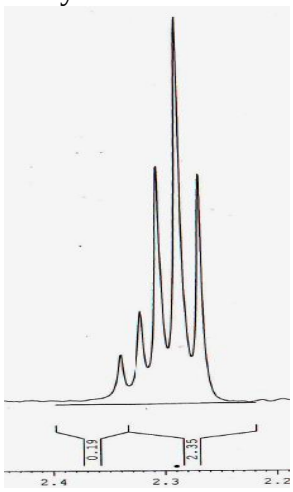
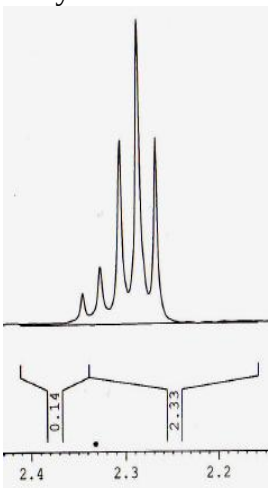
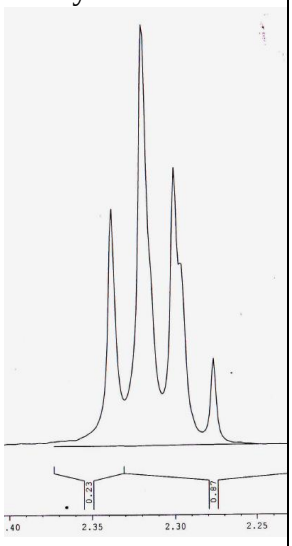
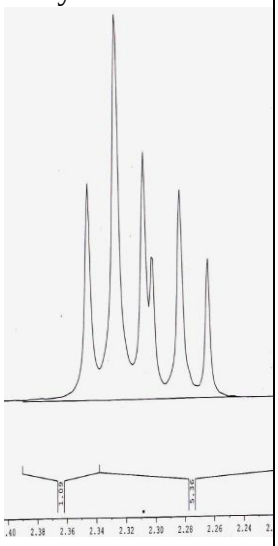
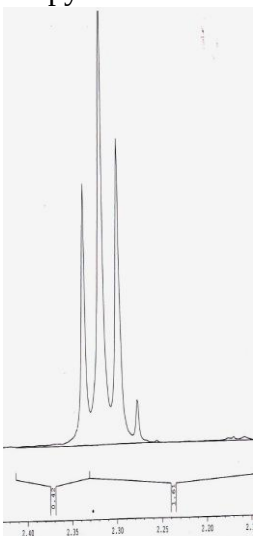
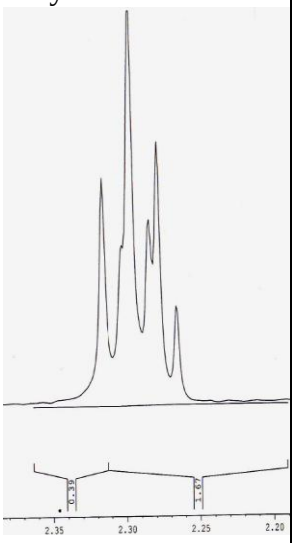
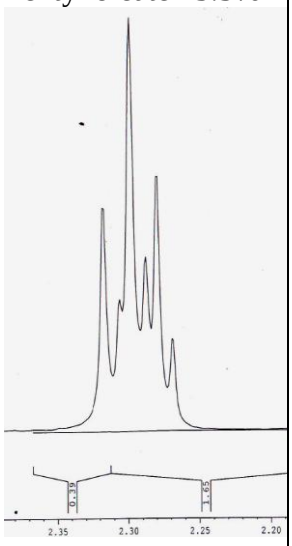
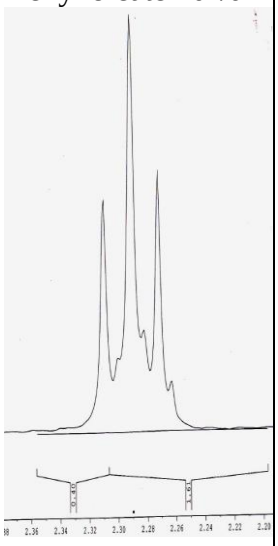
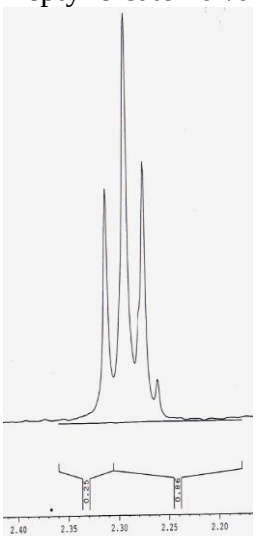
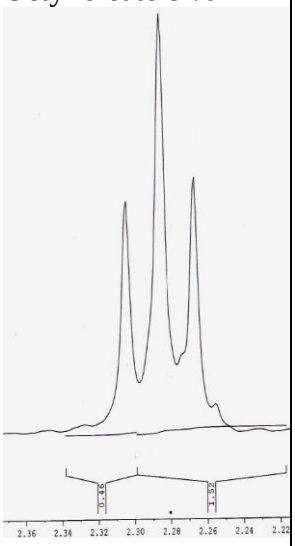
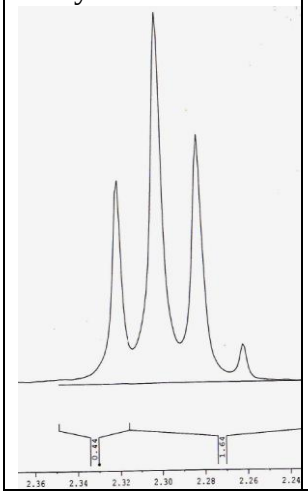
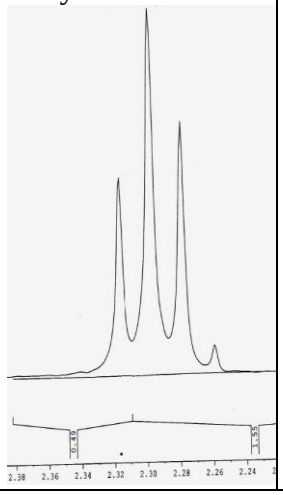
<tr><td>Methyl Oleate 87.5%
  </td><td>Ethyl Oleate 98%
  </td><td>Propyl Oleate 70%
  </td><td>Butyl Oleate 78%
  </td></tr>
<tr><td>Pentyl Oleate 58%
  </td><td>Hexyl Oleate 75%
  </td><td>Heptyl Oleate 41%
  </td><td>Octyl Oleate 80%
  </td></tr>
<tr><td>Nonyl Oleate 70%
  </td><td>Decyl Oleate 77%
  </td><td colspan="2">(I)
Extended region of ^{1}H NMR from 2.20 ppm to 2.40 ppm of alkyl Oleates produced from oleic acid using dried biomass as whole cell catalyst and different alcohols as acyl acceptor</td></tr>
</table>

Methyl oleate 16.3% 	Ethyl oleate 32.8% 	Propyl oleate 17.3% 	Butyl oleate 24.3% 
Pentyl oleate 23.5% 	Hexyl oleate 20 % 	Heptyl oleate 10 % 	Octyl oleate 8 % 
Nonyl oleate 15 % 	Decyl oleate 4% 	(II) Extended region of ^{1}H NMR from 2.20 ppm to 2.40 ppm of alkyl Oleate produced from oleic acid using **wet culture** as whole cell catalyst and different alcohols as acyl acceptor	

1. Prakash, R., Aulakh, S.S., 2011, Transesterification of used edible and non-edible oils to alkyl esters by *Aspergillus* sp. as a whole cell catalyst. *Journal of Basic Microbiology, 51: 1-7.*
2. Aulakh, S.S., Chhibber, M., Mantri, R., Prakash, R., 2011, Whole cell catalyzed esterification of fatty acids to biodiesel using *Aspergillus* sp. *Biocatalysis and Biotransformation, 29: 354-358.*
3. Aulakh SS, Prakash, R. 2010, Optimization of medium and process parameters for production of lipase from an oil tolerant *Aspergillus sp. Journal of Basic Microbiology, 50: 37-42.*
4. Prakash, R., Aulakh, S.S., Kalra, R., 2010, Effect of frying time on free fatty acid generation and esterification rate in Aspergillus sp. catalysed transesterification of cottonseed oil. *Biocatalysis and Biotransformation, 28: 403-407.*