APPLICATION OF HETEROGENOUS BIOCATALYST FOR TRANSESTERIFICATION

A Thesis Submitted

in partial fulfillment of the requirements for the degree of

Master of Science (Chemistry)

Submitted by

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June 2009

Dedicated to My Parents

CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled **"Role of Heterogeneous Biocatalyst in Transesterification of Oil"** in partial fulfillment of the requirements for the award of degree of **Masters of Science (Chemistry)** in the School of Chemistry and Biochemistry, Thapar University, Patiala is an authentic record to my own work during a period of 5 months from January 2009 to May 2009, under the supervision of Dr. Ranjana Prakash, Lecturer, School of Chemistry and Biochemistry, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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This is to certify that the project entitled **"Role of Bio-Catalyst in Transesterification of Oil "** in partial fulfillment of the requirements for the award of degree of **Masters of Science (Chemistry)** in the School of Chemistry and Biochemistry, Thapar University, Patiala is a bonafide work carried out under my guidance and supervision and that no part of this project has been submitted for the award of any other degree or diploma.

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Ruchika Thakur

RUCHIKA THAKUR

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Summary

The ever increasing costs of energy generation and consumption has resulted in focus towards technological routes to alternatives energy resources. Transesterification of oils has been in vogue in recent past as an approach towards generation of biodiesel.

Biodiesel production is gaining interest amongst researchers due to the relevance due to the ever increasing price of crude oil and environmental concerns. Among the various approaches, biocatalytic processes have shown certain promises over the conventional methods.

The conventional methods including acid catalyzed and alkali catalyzed transesterification of oil and others all of which have benefits and drawback, drawing attention towards biochemical processes like enzymatic processes.Most important being is the use of lipase enzyme for transesterification of oil which act as an important biocatalyst carrying out novel reactions in aqueous and non- aqueous media including important reactions such as transesterification. Whole cell catalysis is a recently approach being used to catalyze transesterification reactions for production of biodiesel that facilitates easy separation of catalyst and glycerol as a bioproduct.

The present study was carried out to examine (a) the extent of transesterification using short chain alcohols and used cotton seed oil for the production of biodiesel and (b) study the effects of various parameters such as reaction time, incubation time, types of alcohol used on the extent of transesterification.

1.0 *Introduction*

Fatty acid alkyl esters, which are derived from triglycerides by transesterification with alcohol, have attracted considerable attention during the past decade as a renewable, biodegradable, and non-toxic fuel. Biodiesel, defined as "a substitute for /or an additive to diesel fuel, is derived from the oils and fats of plants and animals" or "as mono-alkyl esters of long chain fatty acids derived from a renewable lipid feedstock, such as vegetable oil or animal fat", has become popular as alternatives [1]. As on date, fossil diesel blended with 20% of bio-diesel produced by soybean oil is available in the US market. The European Union has set an objective to secure a market share of 20% of total motor fuel consumption by 2020 for motor bio-fuels [2].

Historically, it is believed that Rudolf Diesel himself started research with respect to the use of vegetable oils as fuel for diesel engines [3]. In the following decades, the studies became more systematic towards properties and application culminating in its use as alternative fuel in the present day. Despite obvious advantages, the direct use of vegetable oils in fuel engines is problematic. Due to the high viscosity and low volatility, they undergo incomplete combustion and form deposits in the diesel fuel injector of diesel engine. Furthermore, acrolein known for its toxicity gets generated through thermal decomposition of glycerol [4-7]. Different ways have been considered to reduce the high viscosity of vegetable oils:

- Dilution of 25 parts of vegetable oil with 75 part of diesel fuel. [5]
- Microemulsions with short chain alcohols (e.g. ethanol or methanol).[5]
- Thermal decomposition, which produces alkanes, alkenes, carboxylic acid and aromatic compounds [6]
- Catalytic cracking, which produces alkanes, cycloalkanes and alkylbenzenes; [7] and
- Transesterification with ethanol or methanol [8]

Among, all these alternatives, transesterification has been consider to be a good choice, as the physical characteristics of fatty acid esters generated through this process are very close to those of diesel fuel and the process is relatively simple [5]. The methyl or ethyl ester of fatty acids can be burned directly in unmodified diesel engines, with low deposit formation [9-11]. This product is popularly termed as biodiesel; a fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats [12].

For the production of biodiesel fuel, in general alkali-catalysis process is employed that gives high conversion levels of oils to alkyl esters, during biodiesel production.

However, it has several drawbacks, including the difficulty of recovering pure glycerol and the need for removal of the catalyst through effluent treatment. In addition, presence of free fatty acids and moisture content makes alkali catalysis unsuitable for transesterification of used oils [13].

To overcome these drawbacks, which may limit the availability of biodiesel fuel, enzymatic processes using both extracellular and intracellular lipase have been developed [13], which also facilitated recovery of pure glycerol. However, the high cost of lipase production has been a main hurdle for commercialization of the lipase-catalyzed process. As an alternative to use of extracellular pure lipase in catalyzing the transesterification reaction, the present study is proposed to examine the use of dried biomass as a whole cell biocatalyst for biodiesel production.

2.0 *Literature review*

Vegetable oils and their derivatives (especially methyl esters), commonly referred to as "biodiesel," are prominent candidates as alternative diesel fuels. They have advanced from being purely experimental fuels to initial stages of commercialization. These fuels are technically competitive and offer technical advantages over conventional diesel fuel. Besides being a renewable and domestic resource, biodiesel reduces most emissions while engine performance and fuel economies are nearly identical compared to conventional fuels [14].

The term bio-diesel has no unambiguous definition. It stands for vegetable oils used as diesel fuel as well as methyl esters prepared from vegetable oils or animal fats and blends of conventional diesel fuel with vegetable oils or methyl esters. With increasing emphasis on the use of esters as diesel fuel, however, the term "biodiesel" increasingly refers to alkyl esters of vegetable oils and animal fats and not the oils or fats themselves [14]. Bio-diesel is defined as a fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats, designated as B100. A blend of 20% bio-diesel with 80% petrodiesel, by volume, is termed B20 [12].

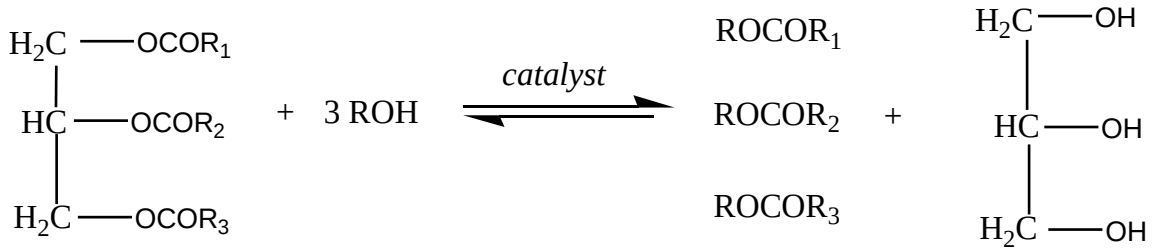
Mono-alkyl esters are the product of the reaction of a straight chain alcohol, such ethanol or methanol, with a fat or oil to form glycerol and the esters of long chain fatty acids.

Among the attractive features of bio-diesel fuels are [15-19]

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

2.1 Transesterification

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



Triglyceride *Alcohol* *Fatty acid ester* *Glycerol*

Alcohols suitable for transesterification reaction include methanol, ethanol, propanol, butanol and amyl-alcohol. Methanol and ethanol are utilized more frequently, especially methanol because of its low cost and favourable physical and chemical properties. This process has been widely used to reduce the viscosity of triglcerides, thereby enhancing the physical properties [20].

Two approaches for transesterification of vegetable oils for production of biodiesel have been explored extensively [21]. 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 [13].

2.1.1 Chemically Catalyzed Transesterification

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 [22]

$$\text{Triglyceride(TG)} + \text{ROH} \rightleftharpoons \text{Diglyceride(DG)} + \text{RCOOR}_I$$

$$\text{Diglyceride(DG)} + \text{ROH} \rightleftharpoons \text{Monoglyceride(MG)} + \text{RCOOR}_2$$

$$\text{Monoglyceride(MG)} + \text{ROH} \rightleftharpoons \text{Glycerol(GL)} + \text{RCOOR}_3$$

Transesterification reaction can be catalyzed chemically either by acid or alkalies. Diverse varieties of acid have been used for transesterification which include sulfuric, phosphoric, hydrochloric and organic sulfonic acids. [1,23]. Acid catalysis facilitated very high yield of esters but the reaction is very slow [24]. Although excess of alcohol can be used in the process to obtain better conversion of triglycerides, recovering glycerol becomes more difficult. On the other hand serious environmental and corrosion related problems, make their use non-practical for biodiesel production at industrial scale [25].

Alkali commonly used for transesterification includes sodium hydroxide, potassium hydroxide, carbonates, and alkoxides such as sodium methoxide, sodium ethoxide, sodium

propoxide and sodium butoxide. For alkali transesterification, the glycerides and alcohol must necessarily be anhydrous as presence of water result in saponification[26]. The soap thus reduces the catalytic efficiency, and increases the viscosity through the formation of gels, resulting in difficult separation of glycerol[13]. In addition, alkali catalyzed reaction necessitate low free fatty acid content in oil (≤ 0.5%) [27]. Ester yields were observed to significantly reduce if the reactants did not meet these requirements.

A variety of heterogeneous catalysts have been examined in recent past for biodiesel production through transesterification. This include sulphated zirconia tin compounds supported in ion-exchange resins [28] alkyl guanidines heterogenized on organic polymers [29] immobilized enzymes [30-33], calcium carbonate [34] 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 [35].

2.2 Enzyme Catalyzed Transesterification

Biocatalysts allow synthesis of specific alkyl esters, easy recovery of glycerol, and transesterification of glycerides with high free fatty acid content [30]. Extensive studies have been carried out on the lipase-catalyzed transesterification of triglyceride [36]. Enzyme catalyzed procedures using lipase as catalyst does not produce side reactions [37], but use of lipases at industrial scale is cost-intensive. The cost is further added due to involvement of a three-step process for 95% conversion of oil [31]. 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 [30] (b) Prevention of glycerol contamination and ease in separation of product [13] and (c) transesterification in normal temperature (30-40°C) and reaction conditions[13]. Most importantly process is possible with crude enzyme formulation.

However 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 molecules and lack of uniformity in performance of support system available till date[38].

2.3 Whole Cell Catalyzed Transesterification

In addition to use of pure lipase, microbial strains exhibiting potential to synthesize lipases have been exploited as whole cell catalysts for transesterification reaction, by various research groups. Transesterification reaction was carried out using Jatropha oil with methanol using immobilized cell of *Pseudomonas fluorescens*. The various parameters affecting biodiesel yield were studied and the maximum yield of 72% was obtained at optimum

condition of 40°C, pH 7, reaction time of 48h, 3g of beads containing immobilized enzymes and 1:4 molar ratio of oil to alcohol [39].

Utilizing *Rhizopus oryzae* cells immobilized within biomass supporting particles (BSPs) as a whole cell biocatalyst, Ban *et al.* investigated the culture conditions for lipase production and on methanolysis[40]. *R. oryzae* cells were effectively immobilized within the polyurethane foam BSPs during batch cultivation. When methanolysis was carried out with stepwise addition of methanol using BSP-immobilized cells, in the presence of 10-20% water, methyl ester content in the reaction mixture was observed to reach 80-90% without any organic solvent pretreatment. This level of methyl ester production was observed to almost same as that achieved using extra-cellular lipase[41]. Further, studies on immobilization of *R.oryzae* cells, through cross-linking treatment with 0.1% gluteraldehyde solution was examined indicated high efficiency in methyl ester generation during six batch cycles, with the methyl ester content reaching 70-83% within 72h in each cycle [40]. Such reports indicate promising means of biodiesel fuel production for industrial application because of the simplicity of the lipase production process as well as the stability of lipase activity over a long period.

Keeping in the view that there are limited reports on whole cell bio-catalyzed transesterification most of which are associated with *Rhizopus* sp., There are limited studies on alcoholysis of oils with whole cell systems as biocatalyst. Keeping this in view, the present study is focused on the application of heterogeneous biocatalytic system for transesterification.

3.0 ***Objectives***

The following objectives were framed focusing on the application of dry biocatalyst for transesterification of used cooking oil

1) **Transesterification reaction using dried fungal biomass.**
2) **Optimization of the various parameters to increase the extent of transesterification reaction.**

4.0 *Material and Methods*

Following series of experiments were carried out to study (a) the catalytic potential of whole cell biomass and (b) effects of various parameters on the extent of transesterification reaction.

4.1 Materials

Waste cooking oil (sourced from Jaggi Sweets, Thapar University, Patiala), ethanol and methanol (SD Fine Chem) were used as substrates for the reaction. Dry biomass of fungus *Aspergillus sp*. prepared by the research group earlier was used as biocatalyst in the study.

4.2 Methodology

4.2.1 Determination of catalytic potential of the dry whole cell biomass

The dry biomass of *Aspergillus* sp. prepared earlier by the research group was used for examining its potential to catalyze transesterification of used cooking oil to ethyl/methyl esters. 3g of biomass was introduced in a 100 ml Erlenmeyer flask containing 2 g waste cooking oil and 5 ml distilled water. This reaction mixture was incubated for 24h at 33^{o}C and 120 rpm. Following incubation, 2ml of ethanol was added at an interval of 0.5ml for 2h. Reaction mixture was further incubated for 24 h. 1ml hexane was added to separate the reactants and the products from the catalytic mixture through further incubation for 24h. The total reaction time including the primary incubation was 76 h.

4.2.2 Optimization of reaction conditions for transesterification

A series of experiments were carried out to determine the potential of the dried fungal biomass as a catalyst and optimize parameters to obtain maximum transesterfication. The parameters considered under the study were (i) presence/absence of water; (ii) type of alcohol (ethanol/methanol); (iii) amount of biomass (4g / 6g); (iv) incubation time (2h / 12h); (v) periodicity of alcohol addition; and (vi) reaction time. The reactions conditions set across the study are outlined in Table 4.1 – 4.3.

4.2.2 Time dependent kinetics

Following the optimization of the reactions conditions (biomass: 2g; waste cooking oil: 5ml; incubation at 120 rpm and 33^{o}C; Alcohol addition: 0.5 ml/h for 2h), time dependent kinetics was done with reference to (a) incubation time (2h and 12h) and (b) reaction time (2h to 7h) to examine the extent of transesterification over time.

Table 4.1: Reactions conditions altering type of alcohol along with presence or absence of water

Parameter	Reaction Conditions			
Biomass (gm)	2 gm	2 gm	2 gm	2 gm
Oil (ml)	3 ml	3 ml	3 ml	3 ml
water	3 ml	NIL	3 ml	NIL
Incubation with shaking (h)	24 hrs	24 hrs	24 hrs	24 hrs
Alcohol	Ethanol	Ethanol	Methanol	Methanol
Alcohol addition	0.5 ml/2h	0.5 ml/2h	0.5 ml/2h	0.5 ml/2h
Incubation with shaking (h) after addition of ethanol	1.5 h	1.5 h	1.5 h	1.5 h
Hexane addition	10 ml	10 ml	10 ml	10 ml
Incubation after the addition of hexane	24 h	24 h	24 h	24 h

Table 4.2: Reaction conditions altering amount of biomass

Parameter	Reaction conditions	
Biomass (gm)	4 gm	6 gm
Oil (ml)	10 ml	10 ml
Incubation with shaking (h)	14 hrs	14 hrs
Ethanol addition	0.5ml/30 min	0.5ml/30 min
Incubation with shaking (h) after addition of ethanol	52 h	52 h

Table 4.3: Reaction conditions altering periodicity of alcohol (ethanol) addition

Parameter	Reaction conditions	
Biomass (gm)	2 gms	2 gms
Oil (ml)	5 ml	5 ml
Incubation with shaking (h)	2 hrs	2 hrs
Alcohol addition	0.5 ml/1hr	1.5 ml (one time addition)
Incubation with shaking (h) after addition of ethanol	5.30 h	5.30 h
Total time	9.30 h	9.30 h

In the study where the incubation time was 2h, the sample was collected up to 7h, at hourly interval after the first addition of 0.5ml ethanol. In the case of incubation time of 12h, the sample was collected at hourly interval after complete addition (1.5ml) of ethanol and reaction time of 2h.

4.2.3 Agitation dependent kinetics

Kinetic studies were carried out to examine the effect of stirring vs shaking condition of reaction mixture on the extent of transesterification. Keeping the reaction conditions constant (biomass: 2g; waste cooking oil: 5ml; incubation at 33^{o}C; alcohol addition: 0.5 ml/h for 2h), the samples were kept either on a magnetic stirrer set at approximately 33^{o}C or orbital shaker set at 120 rpm and 33^{o}C.

4.3 Calculation of methyl ester content

Following equation, derived by Neto *et al.* based on ^{1}H NMR spectroscopy, was used to monitor the yield of methyl esters[42]

$$\mathbf{C = 100 \times (2\ A_{ME} / 3A\alpha - CH_2)} \qquad \mathbf{(1)}$$

Where

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

$\mathbf{A_{ME}}$ =integration value of the protons of the methyl esters at 3.6 ppm (the strong singlet peak).

$\mathbf{A\alpha\text{-}CH_2}$ =integration value of the methylene protons at 2.3ppm.

The factors **2** and **3** derived from the fact that the methylene carbon possesses two protons and the alcohol (methanol- derived) carbon has three attached protons.

4.4 Calculation of Ethyl ester content

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 -OCH_2 from ethyl ester in biodiesel. Ghesti *et al*.proposed a new equation in which the numbers **4** and **6** are related to glyceryl methylenic hydrogens present in TAG molecules and to six hydrogens formed in three ethyl ester products. This equation was used for further quantification of ethyl esters[43].

$$\mathbf{\%CEE = 100(4ITAG+EE - ITAG)/4(ITAG+EE - ITAG) + 6(2ITAG)} \qquad \mathbf{(2)}$$

Where

***I*TAG** = integration of glyceryl methylenic hydrogens at 4.25-4.35 ppm;

***I*TAG+EE =** integration of glyceryl methylenic hydrogens and –OCH_2 of ethoxy hydrogens superimposed at 4.10-4.20 ppm; and

$\mathbf{IRCH_2}$ = integration of R-acyl methylenic hydrogens in soybean oil and ethyl esters at 2.20 - 2.40 ppm.

5.0 *Result and Discussion*

5.1 *Dry biomass as catalyst for transesterification*

The present study was firstly focused on examining the potential of *Aspergillus* sp. to tranesterify used cooking oil. The product, alkyl ester, was identified through thin layer chromatogram with chemically synthesized alkyl ester as standard (Figure 1). The product was further quantified using ^{1}H-NMR and the yield was observed to be >95%, in the case of ethyl ester and >62% in the case of methyl ester. Figure 2 a & b are the NMR spectra of pure oil and its ethyl ester after catalysis.

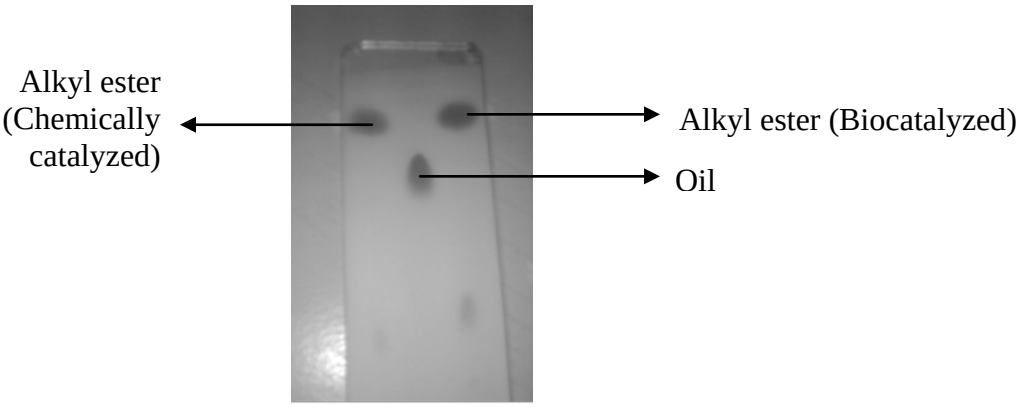
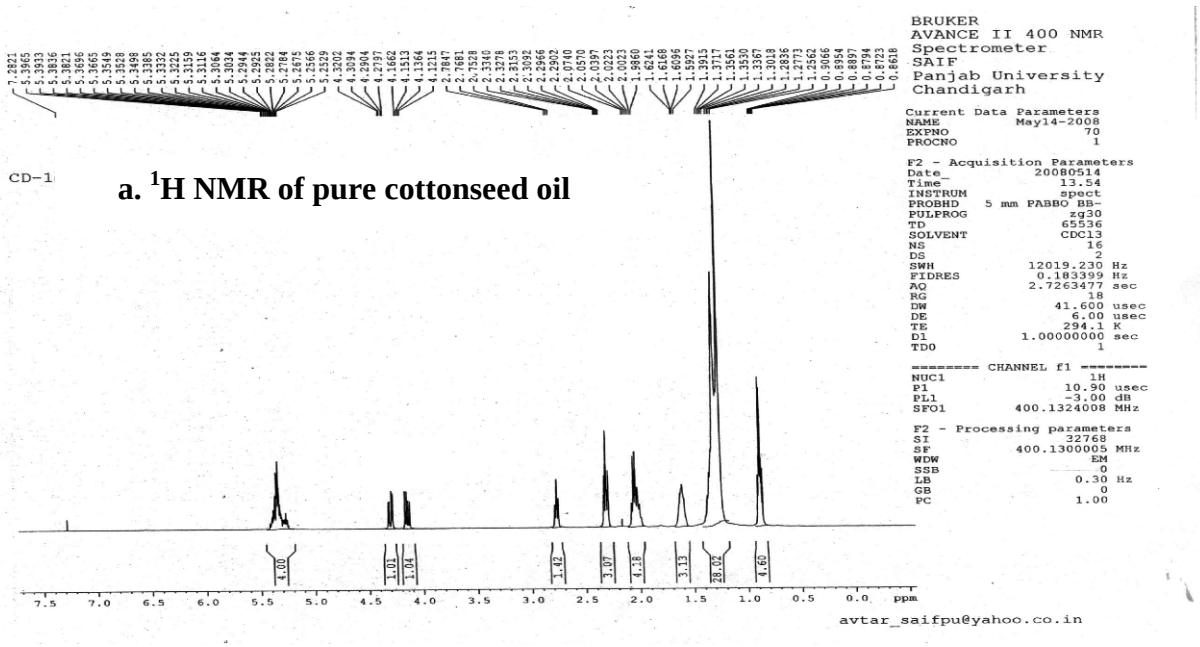


Figure 1: Thin layer chromatogram of biocatalyzed alkyl ester



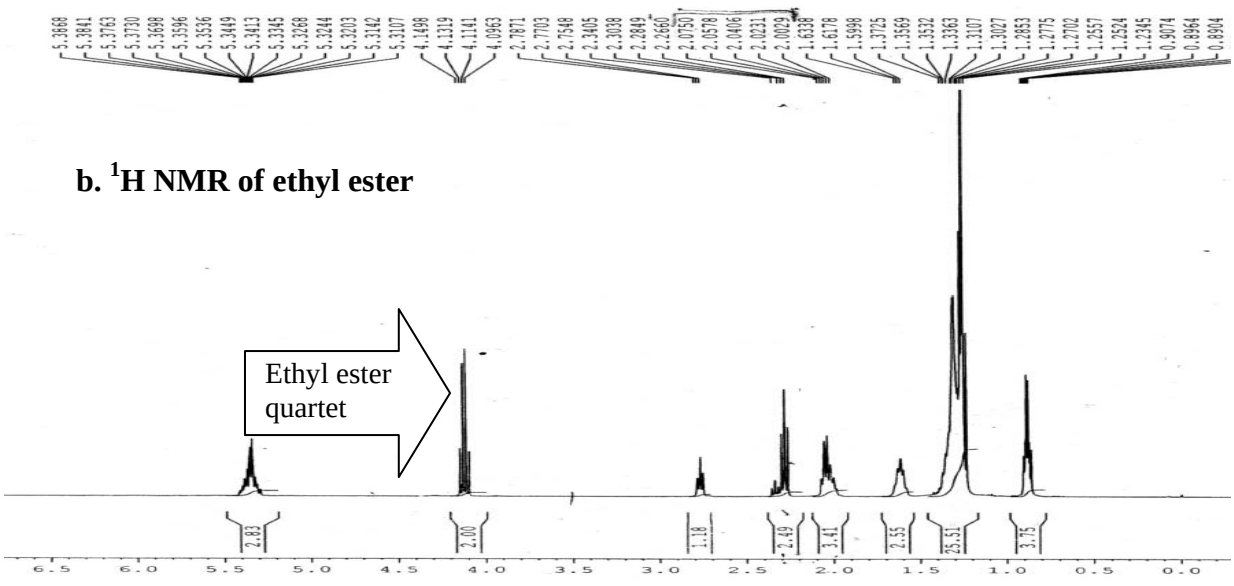


Figure 2: ^{1}H-NMR of (a) pure oil and (b) ethyl ester

Filamentous fungi have arisen as the most robust whole-cell biocatalyst for industrial applications. Nakashima.reported promising processes for the industrial interesterification and methanolysis of plant oils[44]. Fukuda et al[13].reviewed the use of immobilized mycelium of *Rhizopus oryzae* within BSPs for the methanolysis of soybean, jatropha and rapeseed oil which resulted in 70 to 90% of tranesterified products in 24 to 72 h. Interestingly, immobilization within BSPs increased the specific intracellular lipase activity of R. chinensis fourfold to sevenfold compared to that of suspension cells [45]. Limited observation on the use of dried biomass of *Rhizopus chinensis* indicated its application in continuous transesterification of different fats and oils[46]. The observations of the present study confirming formation of ethyl esters using *Aspergillus* sp. are important in the context of achieving enhanced transesterification in lesser time than other reports.

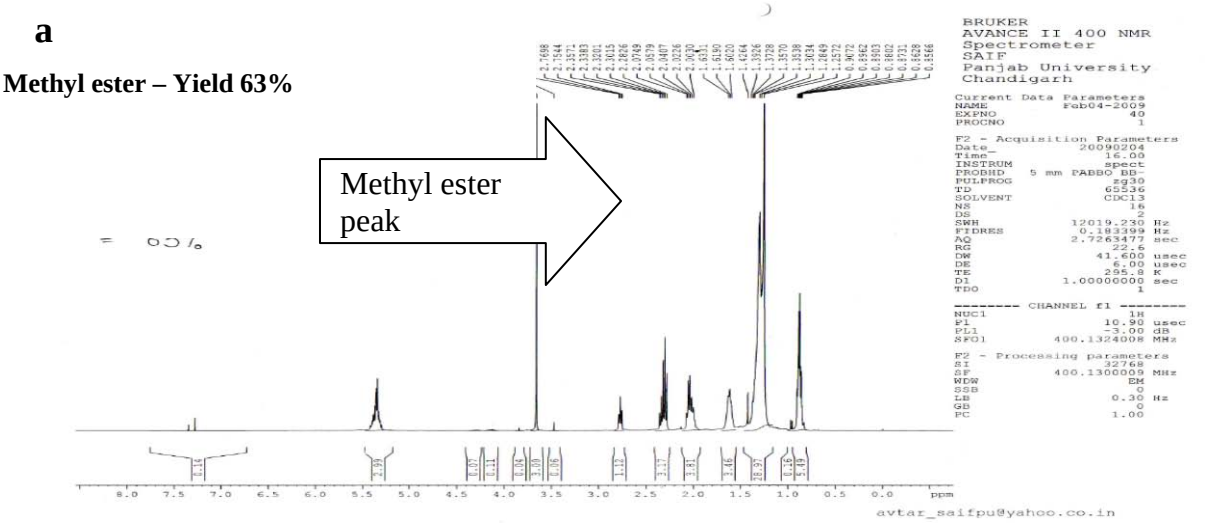
On confirmation of the synthesis of alkyl ester by the dry biomass of *Aspergillus* sp. further work was focused on optimizing the conditions that would enhance the extent of transesterification. The parameters that were considered included (i) presence or absence of water; (ii) type of alcohol (ethanol/methanol); (iii) amount of biomass (4g / 6g); (iv) incubation time (2h / 12h); (v) periodicity of alcohol addition; and (vi) reaction time.

5.2 *Optimization of reaction conditions*

5.2.1 Effect of the type of alcohol

The effect of chemically different alcohols was examined on alcoholysis process using ethanol and methanol as reactants as per the process conditions outlined in Table 1. Amongst these two alcohols as reactants, the presence of ethanol was observed to yield 90%

of ethyl ester whereas methanol as reactant resulted in 63% of methyl ester. Figure 3 (a & b) represent the ^{1}H-NMR spectra of alkyl esters as obtained with ethanol and methanol as reactants respectively. Ethanol was observably a better reactant than methanol in the yield of alkyl esters.



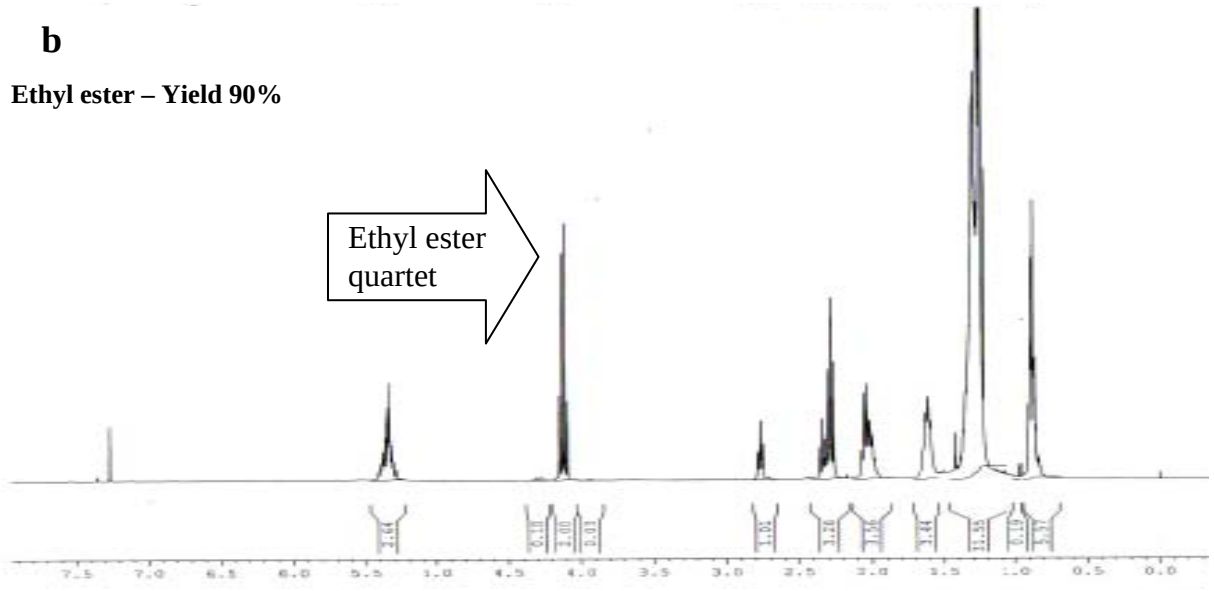


Figure 3: ^{1}H-NMR spectra of alkyl esters synthesized with (a) methanol and (b) ethanol as reactants

As on date, most of the reports are based on methanol as reactant and whole cell as a biocatalyst. However, observations by other researchers on pure enzyme as catalyst and various alcohols as reactants, indicate that although linear and branched alcohols with short

alkyl chains show high reaction rate and conversion, methanol does not favour the same probably due to low miscibility with oil resulting in lower conversion. [47]

5.2.2 Effect of the presence of water in reaction medium

Presence of water is generally considered as a pre-requisite for effective biocatalyzed reactions with most of the findings by other researchers indicating use of aqueous buffer to facilitate transesterification reactions [40]. This aspect was studied following experimental conditions similar to the above (Section 5.1) by setting the experiment devoid of water in the medium. The products thus obtained were analyzed using ^{1}H-NMR (Figure 4 a & b). The peaks in (4 a & b) at 4.25-4.35 ppm show the presence of residual oil fractions indicating incomplete transesterification in non-aqueous conditions.

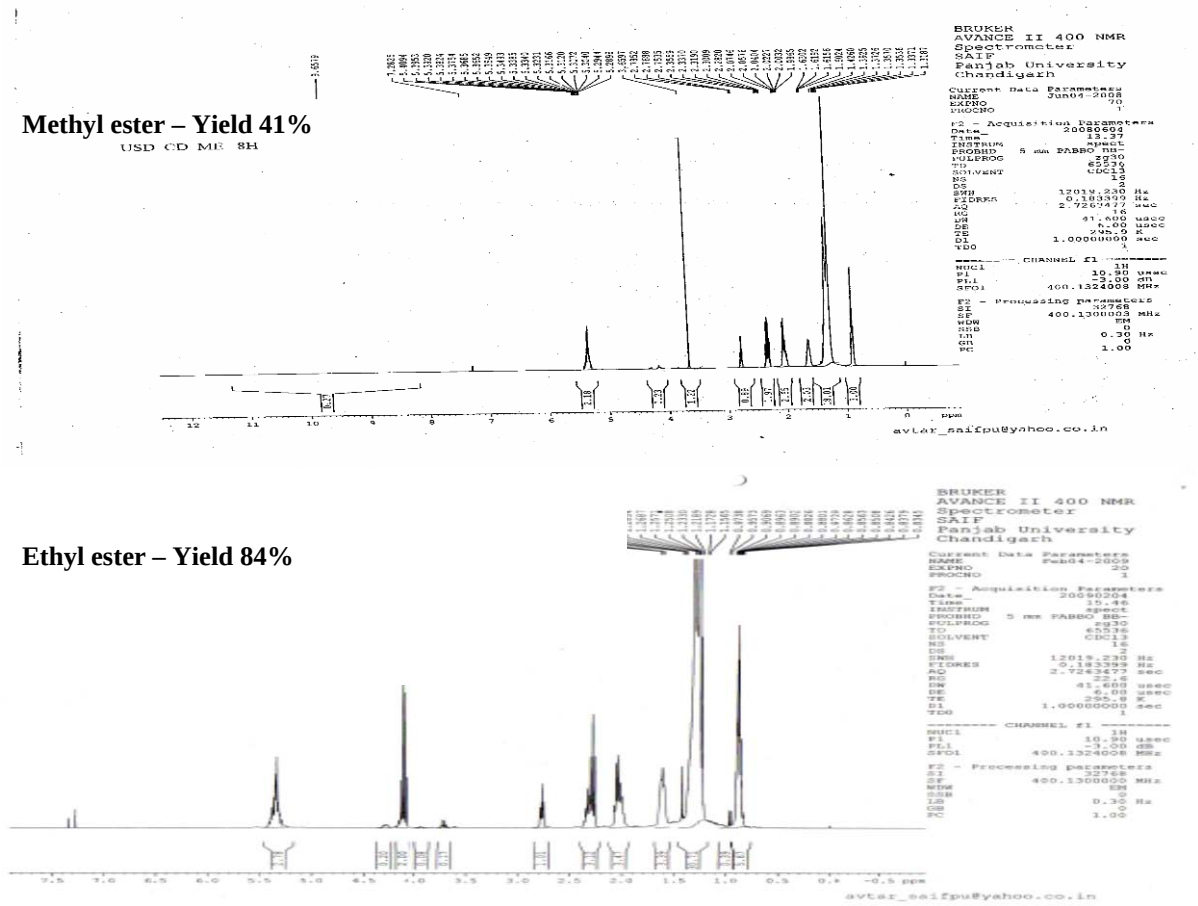


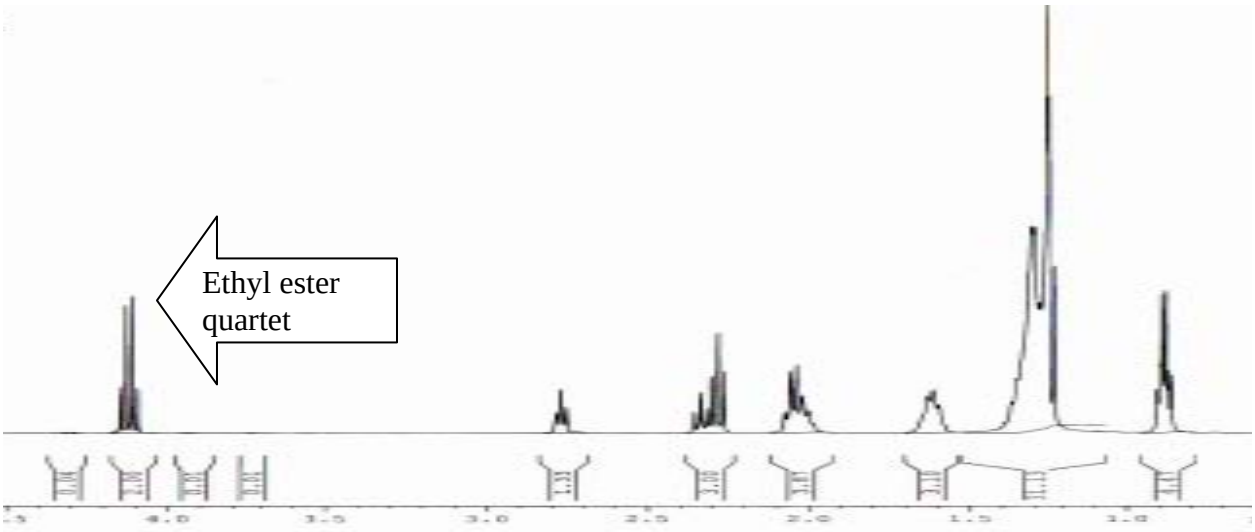
Figure 4: ^{1}H-NMR spectra of alkyl esters synthesized with (a) methanol and (b) ethanol synthesized in the non-aqueous medium

The results indicated that presence of water enhances the yield of alkyl ester with either methanol (63%) or ethanol (90%) as reactants when compared to reaction in absence of water i.e., 41% and 84% respectively. Similar observations were obtained by Ban et al. with *R.oryzae* as catalyst and methanol as reactant, gradual decrease in water content (as buffer)

from 15 wt.% (water/substrate) to 3 wt. % and absence of water resulted in gradual decrease in yield of methyl ester 91%>54%>33%[40]. An important observation to be noted is that, in the present study the yield of methyl ester was higher (41%) in the absence of water with *Aspergillus* sp. as catalyst when compared to *R. oryzae* (33%) reported by Ban et al.[40].

5.2.3 Effect of amount of biomass

The effect of the amount of biomass on the transesterification process was considered with a view to obtain complete transesterification. Study was set with 4g and 6g of biomass with other reaction conditions outlined in table 4.2. The observations indicated that the mentioned reaction conditions and 4 to 6 g biomass could facilitate complete transesterification (>98%) of oil to methyl esters (Figure 5). The reaction conditions along with the biomass to oil ratio of 1:2.5 were thus considered to be optimum for obtaining complete transesterification. Further studies on the effect of the periodicity of alcohol addition and the time-dependent and other kinetics were carried out with the biomass to oil



ratio of 1:2.5.

Figure 5: NMR spectra showing >98% transesterification (obtained with 4g and 6g biomass)

5.2.4 Effect of periodicity of alcohol addition on transesterification

Since high alcohol concentration causes irreversible denaturation of the lipolytic enzymes, studies carried out by other researchers indicated that step-wise addition of alcohol can facilitate transesterification without inhibition or deactivation of the enzyme [31]. The study on the periodicity of ethanol was carried by one time addition (1.5ml) and periodic addition of alcohol (0.5 ml/h) during the reaction time. The reaction conditions in this experiment are outlined in table 4.3.

The results showed that one time addition of 1.5 ml ethanol ceased the reaction completely, where as step-wise addition of the same volume of ethanol at the rate of 0.5 ml per hour resulted in 72.5% alkyl esters. These observations confirm that one time addition of ethanol significantly affects the catalytic potential of biological systems either as enzymes or whole cells.

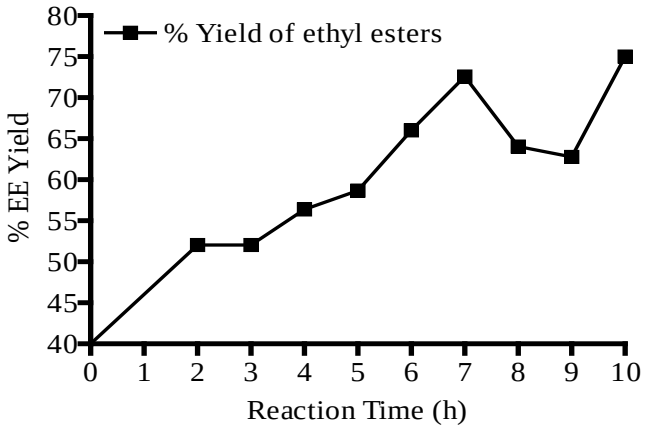
5.3 *Time dependent kinetics*

5.3.1 Role of reaction time

The observations on the effect of whole cell catalysis on the extent of transesterification were taken with reference to time taken for transesterification of waste cooking oil to ethyl esters. The study with reference to the reaction time, after step-wise addition of ethanol, indicated that the ethyl ester yield increased (72.5%) over time upto 7 h followed by decrease (62.7%) upto 9 h and further increase (75%) beyond 10 h (Figure 9). Reversibility of the reaction was indicated by variations in glyceridic and ethyl ester peaks of ^{1}H-NMR.

The tranesterification process to produce alkyl esters from triacyl glycerides follows a stepwise mechanism of consecutive reversible reactions: (1) conversion of triacyl glycerides to diacylglycerides and fatty acid methyl esters (FAMEs); (2) conversion of diacylglycerides to monacylgycerides and FAMEs; and (3) conversion of monoacylglycerides into glycerol and FAMEs [48]. Such reversibility was hypothesized by Kaieda *et al*. who reported successive reaction mechanism in enzyme catalyzed tranesterfication using *R.oryzae* lipase[41]. However, no such report has been noted with whole cell catalyzed tranesterification to the best of our knowledge.

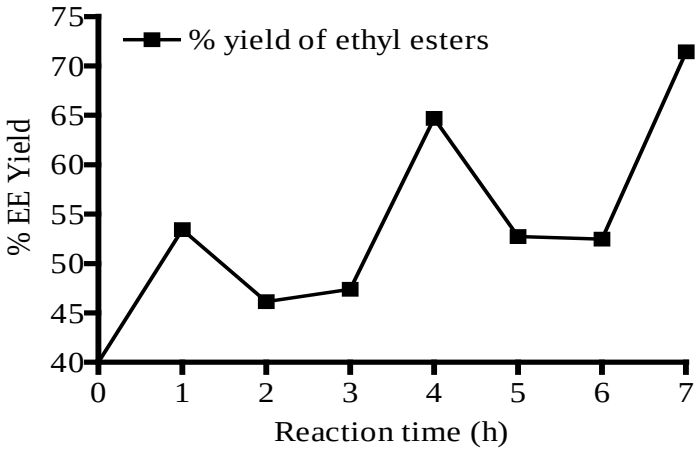
Figure 9: Percent of ethyl esters (EE) yield indicating increase in yield upto 7 h followed by reversibility of reaction from 7-9 and 9-10 h



5.3.2 Role of incubation time

The effect of the incubation time was studied with an aim to reduce the total reaction time by altering the time required for incubation of biomass with subtrate viz., waste cooking oil for generation of fatty acids. The incubation time was varied by shaking the biomass and the substrate for 2 h and 12 h at 33^{o}C and 120 rpm. Alcohol was added as outlined in section 4.2.2.

Figure 10: Percent of ethyl esters (EE) yield with incubation time of 2h

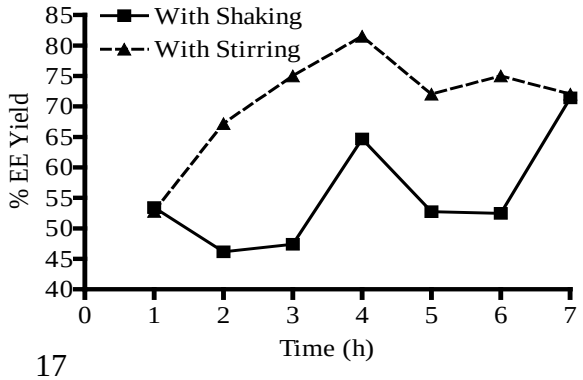


Reduction of incubation time to 2 h also indicated reversibility of transesterification reaction (Figure 10). The significant component of this observation is the comparison of the reaction rates at both the times of incubation viz., 2 h and 12 h [ref. section 5.3.1 & Figure 9]. The observations indicated that the product yield was approximately 71-72% after the total reaction time of 21 h in the case of 12 h incubation time; and 9 h in the case of 2 h incubation time. The reduction in total reaction time so as to obtain optimum transesterification of 71-72% is of significance as it facilitates desired yield of ethyl esters within shorter reaction time.

5.4 *Agitation dependent kinetics*

The study carried out to examine the role of agitation on the transesterification indicated its significant effect on the tranesterification process (Figure 11).

Figure 11: Ethyl esters (EE) yield in reaction carried out at shaking/stirring conditions



The extent of transesterification was 81% with stirring and 64% in case of shaking. The process of stirring over shaking, with other reaction conditions remaining similar, showed that the complete mixing of reactants facilitated by stirring provided better reaction conditions for synthesis of alkyl esters compared to shaking. The observations also confirmed reversibility in transesterification process in either of the conditions indicating that this phenomenon is independent of the mixing of reactions and the extent of transesterification.

6.0 *Conclusions*

The present study carried out to examine the catalytic potential of the dry biomass of *Aspergillus* sp. resulted in following salient findings:

1. **The important finding is the use of waste cooking oil as effective substrate for synthesis of alkyl esters**
2. **Another important observations is the use/function of the dry biomass of *Aspergillus* sp. as potential biocatalyst for transesterification reactions**
3. **In addition, the transesterification could be achieved with optimum biomass to oil ratio of 1:2.5**
4. **Yet another finding is the demonstration of obtaining optimum yield of alkyl esters by carrying out transesterification reaction in non-aqueous conditions**
5. **Another observation of significance is the effect of alcohol which indicated periodic addition of alcohol facilitating transesterification reaction over one-time addition**
6. **The transesterification reaction was observed to be reversible with initial increase followed by decrease and then increase in the yield of ethyl esters over time**
7. **The optimum yield of ethyl esters (>70%) could be achieved with 2 h incubation**
8. **The mixing of reactants (through stirring) plays major role in enhancing the transesterification process and yield of ethyl esters**

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