Effect of frying on bio-catalyzed hydrolysis and transesterification of used soybean oil

A dissertation submitted
in partial fulfillment for the award for the
Degree of

Master of Science

in

Chemistry

Submitted by

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July 2011

Candidate's Declaration

I hereby declare that the work which is being presented in the dissertation entitled **" Effect of frying on bio-catalyzed hydrolysis and transesterification of used soybean oil"** in partial fulfillment of the requirements for the award of the degree of **Master of Science in Chemistry**, School of Chemistry and Biochemistry, Thapar University, Patiala is an authentic record to my own work carried out during a period of six months from January 2011 to July 2011, under the supervision of **Dr. Ranjana Prakash**, 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.

Place: Patiala

Date: July 13, 2011.

Mandeep Kaur

Mandeep Kaur

Certificate

This is to certify that the project entitled " **Effect of frying on bio-catalyzed hydrolysis and transesterification of used soybean oil**"being submitted by **Mandeep Kaur** in partial fulfillment of the requirement for the award of degree for the Master of Science in Chemistry at the School of Chemistry and Biochemistry, Thapar University, Patiala, is a bonafied 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.

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Summary

Biodiesel consists of long-chain fatty acid esters produced by transesterification reaction of vegetable oils with short chain alcohol using some catalyst or even used cooking. Amongst the various catalysts used for facilitating transesterification reaction, biocatalysts using either enzymes or whole-cells are gaining interest amongst researchers. As an alternative to enzymes which are cost-intensive, whole cell catalysts are getting increasingly popular.

The present study entitled, "Effect of frying on biocatalyzed hydrolysis and transesterification of used soyabean oil" was therefore focused to achieve twin objectives of (a) exploiting the potential of a fungus strain *Aspergillus* sp. (RBD01), as dry biomass, for catalyzing transesterification, and (b) using the used-soybean oil as raw material for generating alkyl esters through transesterification.

The observations obtained in this study could demonstrate the use of dry biomass of a fungus isolate, *Aspergillus* sp. (RBD01) for exploting its potential to transesterify used cooking oil and esterify fatty acids.

Contents

Introduction

The continued rising in global prices of vegetable oil, increasing threats to the environment by exhaust emissions, global warming, and threats of supply instabilities have adversely impacted the developing countries, more so to the petroleum importing countries like China and India. It is important to find a safe alternative fuel to relieve the escalating energy crisis and to protect the environment. Historically, many biomass and agricultural derived materials have been suggested as alternative energy sources and the use of biodiesel as fuel presents a promising potential (Al-Widyan and Al-Shyoukh 2002; Mushrush et al., 2001). Biodiesel consists of long-chain fatty acid esters (Haas et al., 2001; Abreu et al., 2004) produced by transesterification reaction of vegetable oils with short chain alcohols (Noureddini et al., 1998; Encinar et al., 2002) using some catalyst (Zhang., 2003) or even used cooking oils (Issariyakul et.al., 2008). This is due to its ecofriendly nature and to its role as a strategic source of renewable energy in substitution to diesel oil and other petroleum-based fuels(Cardone et al., 2002). In addition, compared to petroleum diesel, biodiesel enhances the engine lubricity (Kurki et. al., 2006) and contributes to sustainability (Kurki et.al., 2006; Khan et al., 2007). The increasing production of used cooking oil from household, restaurants and industrial sources is a growing problem in the world. This residue is regularly poured down the drain, resulting in problems for wastewater treatment plants or integrated into the food chain through animal feeds thus becoming a potential cause of human health problems (Neto et al., 2000). The conversion of the waste cooking oil into fuel also eliminates the environmental impacts caused by the harmful disposal of these waste oils, such as disposal into drains (Utlu., 2007) . The production of biodiesel from such waste cooking oils reduces the cost of biodiesel as compared to the production from virgin oils. The use of waste cooking oils can therefore be preferred over the neat (unprocessed) vegetable oils as

biodiesel feedstock which is more economical and environmentally friendly (Phan et al., 2008).

Biodiesel production can be carried out by transesterification reaction catalyzed either chemically or enzymatically. Chemically catalyzed processes, including alkali and acid, both the process show disadvantages toward the catalysis of waste cooking oils. Only well refined vegetable oil with less than 0.5 wt% free fatty acid (FFA) can be used in the case of alkali catalysis (Watanabe et al., 2003) as the high FFA in the WCO leads to saponification reaction which further affects the extent of transesterification reaction. On the other hand acid catalysis take a longer time and is carried out at very high temperature, with very high concentration of alcohol in the reaction mixture, leading to cost-intensive and hazardous operation.

Biocatalysts, particularly lipases, can be used in place of alkali or acid to catalyze transesterification reaction. Biodiesel production through biocatalysis, offers several potential advantages (Haas et al., 2002) such as mild reaction conditions, no saponification, use of a variety of lower-quality and low-cost feedstocks. In addition, biocatalysis is possible even in the presence of water and requires less alcohol and produces no salts. However, The high cost of lipase and other enzymes due to purification procedures, making the process uneconomical (Gerpen et al., 2004; Nourreddini et al., 2005). An alternative to purified lipase is to utilize the whole cell capable of producing lipase in specific culture conditions as catalyst.

Keeping in view, the importance of the above aspect in the current context, the present study was carried out to examine the influence of frying biocatalyzed hydrolysis and transesterification of soyabean oil.

Literature Review

The idea of using vegetable oils as fuel is not new, it comes from more than 100 years ago when Rudolph Diesel tested this raw material as fuel for his engine (Demirbas, 2003). Later on, due to the petrol crisis in the early 70's there was another attempt of making vegetable oils as a feasible alternative to fossil diesel. But it was not until 80's when this interest became real with the investment in research and development of projects to produce biodiesel.

Dominantly, the production of biodiesel has been through direct use by blending of oil with petroleum diesel or through microemulsions, thermal cracking (pyrolysis) and transesterification. The most common way to produce biodiesel is by transesterification, which refers to a catalyzed chemical reaction involving vegetable oil and an alcohol to yield fatty acid alkyl esters (biodiesel) and glycerol (by-product) (figure1).

Figure 1. general reaction of transesterification

Transesterification is the general term used to describe the important class of organic reactions where an ester is transformed into another through interchange of alkoxy moiety. The term transesterification is generally used as synonym for alcoholysis of carboxylic esters. This is an equilibrium reaction and transformation occurs essentially by mixing of reactants. However, the presence of a catalyst (typically a strong acid or a base) accelerates considerably the adjustment of the equilibrium towards transesterification or alcoholysis.

Transesterification is the displacement of alcohol from an ester by another in a process similar to hydrolysis, except that alcohol is used instead of water (Meher *et al*., 2006; Srivastava and Prasad, 2000). Vegetable oils/animal fats mainly consist of triglyceride molecules where R', R", and R"' represent the hydrocarbon chains (more than 10 carbon atoms) of the fatty acid of the triglyceride (Figure 2.). Triglycerides are highly viscous, and therefore, in order to reduce the viscosity and to make the fuel usable in a diesel engine, pure oil is converted from natural triglyceride into mono-alkyl esters (separated long chain carbon molecules) by transesterification. During transesterification, triglyceride reacts with an alcohol in the presence of a strong acid or base, producing a mixture of fatty acids alkyl esters and glycerol (Figure 2).

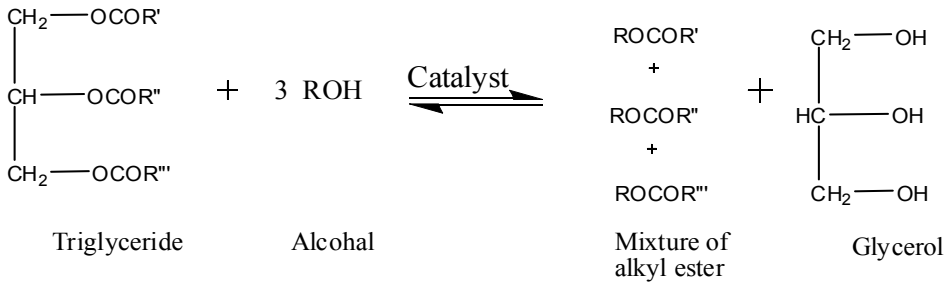

Figure 2 : Transesterification reaction of triglyceride and alcohol to fatty acid alkyl ester(biodiesel) and glycerol.

The overall process is a sequence of three consecutive and reversible reactions, in which di-glyceride and mono-glycerides are formed as intermediates. The stoichiometric reaction requires 1 mole of a triglyceride and 3 mole of the alcohol. However, an excess of the alcohol is used to increase the yields of the alkyl esters and to allow its phase separation from the glycerol formed. Several aspects, including the type of catalyst (alkaline or acid), alcohol/vegetable oil molar ratio, temperature, purity of the reactants (mainly water content) and free fatty acid content have an influence on the course of the transesterification.

Transesterification significantly reduces the viscosity of vegetable oil, without affecting the heating value of original fuel, resulting in better fuel atomization, combustion and emission characteristics if ester of vegetable oils are used in engines. When triglyceride is converted stepwise to di-glyceride, monoglyceride and finally to glycerol one mole of fatty ester liberated at each step. Vegetable oils and fats contain small amount of water and free fatty acid. Alcohols such as methanol, ethanol, butanol can be used in transesterification and monoesters thus formed are named as methyl ester, ethyl ester or butyl ester respectively.

Transesterification reaction can be catalyzed chemically either by an acid or an alkali (Freedman *et al*., 1986, Noureddini *et al*., 1997). For acid catalysis, diverse varieties of acids have been used for transesterification which include sulfuric, phosphoric, hydrochloric and organic sulfonic acids (Ma *et al*., 1999; Freedman *et al*., 1984). Although, acid catalysis facilitated high yield of esters but the reaction is relatively slow. In addition, use of excess of alcohol in the process to obtain better conversion of triglycerides, results in making recovery of glycerol more difficult. Serious environmental and corrosion related problems also make their use non-practical for biodiesel production at the industrial scale (Canakci *et al*., 1999; Li *et al*., 2008).

With reference to alkali catalysis, alkalies commonly used for transesterification include 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 results in saponification (Wright *et al*., 1944). The soap reduces the catalytic efficiency, and increases the viscosity through the formation of gels, resulting in difficult separation of glycerol (Fukuda *et al*., 2001). In addition, alkali catalyzed reaction necessitate low free fatty acid content in oil (≤0.5%) (Ma *et al*., 1998). Ester yields were observed to reduce significantly if the reactants did not meet these requirements (Freedman *et al*., 1984).

A variety of heterogeneous catalysts have also 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) and 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).

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 were carried out on the lipase-catalyzed transesterification of triglycerides. 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.

Transesterification of the triglycerides viz., sunflower oil, fish oil, and grease with ethanol *i.e.* ethanolysis, has been studied by different research groups. In each case, higher yields beyond 80% could be achieved using the lipases from M. *miehei ,Candida antarctica* and *Pseudomonas cepacia* respectively.

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*, *Rhizopus chinensis,* recombinant *Saccaromyces cerevisiae* and most recently *Aspergillus niger* as whole-cell biocatalysts*,* have been studied and reviewed by different research groups (Fukuda *et al*. 2008, Hama *et al* 2008,). These studies however have reported transesterification to a maximum extent of 90% (yield of FAME). In addition, there are **no reports** on the use of cell-suspension in these studies. **This lacuna necessitates** a focused exploration on use of whole cell biocatalyst with lipase producing organisms for transesterification reactions.

Raw material used for biodiesel production

Investigations of alternative renewable energy resources continue, with many studies focused on biodiesel fuels. One of the alternative renewable energy sources for diesel engines is vegetable oil. A variety of biolipids can be used to produce biodiesel. These are (a) virgin vegetable oil feedstock; rapeseed and soybean oils are most commonly used, though other crops such as mustard, palm oil, sunflower, hemp and even algae show promise; (b) waste

vegetable oil; (c) animal fats including tallow, lard and yellow grease and (d) non-edible oils such as Jatropha, neem oil, castor oil, tall oil, etc. There are more than 350 oil-bearing crops identified, among which only soybean, palm, sunflower, safflower, cottonseed, rapeseed and peanut oils are considered as potential alternative fuels for diesel engines (Goering *et al*., 1982; Pryor *et al*., 1982). Worldwide consumption of soybean oil is the highest in 2003 (27.9 million metric tons). Vegetable oils have become more attractive recently because of their environmental benefits and the fact that these are made from renewable resources Vegetable oils are a renewable and potentially inexhaustible source of energy with energy content close to diesel fuel. However, extensive use of virgin edible vegetable oils may cause other significant problems such as availability of edible oils for human consumption.

A major barrier in the commercialization of biodiesel production from vegetable oil is its high manufacturing cost, which is due to the higher 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.* 2003). The use of waste cooking oil as biodiesel feedstock reduces the cost of biodiesel production (Canacki 2007) since the feedstock costs constitutes approximately 70-95% of the overall cost of biodiesel production (Kulkarni *et al.* 2006).

The properties of the biodiesel from waste cooking oil would be largely dependent on the physicochemical properties of these feedstocks. Depending on the degree of heating, various physical and chemical changes occur in food constituents. Frying is one of the most

popular methods of food preparation in modern times, with the reason being the excellent taste of the fried food. The use of oil (lipids) is an integral part of frying. During frying, oil is heated in air and in the presence of light at temperatures of 160-200^{0}C for relatively longer duration. For economical reasons, the same oil/fat is used many times or continuously (Cvengros *et al.* 2004). Generally, in public/commercial restaurants, frying is conducted in the same oil for several hours; however, in household frying, the oil in use undergoes only limited number of fryings (Cvengros *et al.* 2004). Obviously, the conditions used for frying cause major physical and chemical changes in the oil, which differ significantly with virgin oil in its composition. Some common physical changes observed in vegetable oil after frying are (i) an increase in the viscosity, (ii) an increase in the specific heat, (iii) a change in the surface tension, (iv) a change in colour, and (v) an increase in the tendency of fat to foam (Cvengros *et al.* 2004).

The steam produced during the preparation of food causes the hydrolysis of triglycerides, resulting in the formation of free fatty acids, glycerol, monoglycerides and diglycerides (Mittelbach *et al.* 1999). As a combined result of all these chemical reactions, many unknown/unidentified compounds form. In addition, the polar content of the oil increases upon repetitive heating (Guesta *et al.* 1993). The quality of the edible oil after frying is generally assessed based on its polar content. The polar content of fresh unused oil is usually between 0.4-6.4 mg/100 g and most European countries have set maximum polar content level of 25% in edible oil as upper limit for re-use (Bastida *et al.* 2001). A study conducted on sunflower oil and olive oil; and a mixture of the two oils showed that after 20 fryings, the polar content of sunflower oil is increased by 640% and that of olive oil by 480%. Further, the frequent addition of fresh oil throughout frying minimized thermoxidative and hydrolytic changes in the frying oil, even after 20 repeated fryings (Bastida *et al.* 2001).

Waste cooking oil is an economical choice for biodiesel production, because of its availability and low cost. This oil has many undesirable compounds such as polymers, free fatty acid (FFA), and many other chemicals that are formed during frying, which are of major concern during the transesterification reaction. Pretreatment of the waste cooking oil to remove these undesirable chemicals is not practical. Depending on the water and FFA content of the waste cooking oil, a suitable transesterification method can be selected. If the FFA and water contents are <1 wt % and <0.5 wt %, respectively, then an alkaline catalyst is more suitable for the ester production. If the FFA content of oil is high (>1 wt %), then an acid catalyst is a good choice. However, because of the requirement of high catalyst concentration and high molar ratio, and because of corrosion problems, these catalysts are also not recommended for the transesterification of waste cooking oil. A two-phase method (an acid-catalyzed step, followed by an alkaline-catalyzed step) is not feasible, because it requires many steps, which makes the biodiesel process costl-intensive. Alternatively, bio-catalyzed transesterification is a very good option to all chemical-catalyzed reactions; however, cost effective production and purification of lipase is an important hurdle in its commercialization, which however, can be overcomed with the whole cell catalysed transesterification.

As on date, no reports are available on the effect of frying on the extent of hydrolysis and transesterification of soyabean oil using whole cell catalysis Keeping this in view, the present study was designed in two phases; in first phase soybean oil was fried for different time intervals and in second phase biocatalysed hydrolysis and transesterification was carried out by using whole cell system under shake flask condition as well as by using dried biomass.

Materials and Methods

Soybean oil, used in the present study, was purchase from commercial qutlets, Bi-ammonium hydrogen ortho-phosphate [$(NH_4)_2HPO_4$] and mycological peptone, culture media viz., potato dextrose agar (PDA) and potato dextrose broth (PDB) were procured from HiMedia, India. Other chemicals such as ethanol, hexane, ethyl acetate, glacial acetic acid, silica gel grade G, KOH, Neutral ethyl alcohol and phenolphthalein indicator were sourced from SD Fine Chem., India

Growth of *Aspergillus* in medium. The fungal strain, *Aspergillus* sp (RBD01), isolated from contaminated fat material through plating on PDA at 28ºC for 96 h following standard protocols (Krieg et al. 1984), was used in the present study. Freshly sporulating culture was inoculated under sterile conditions into 500 mL Erlenmeyer flask containing 200 mL of PDB at 28 ºC and kept at 120 rpm for 72h. This active culture was further used for experimentation. The mineral medium used in the experiments comprised magnesium sulphate (0.20 g/l), calcium chloride (0.02 g/l), mono-potassium phosphate (1.0 g/l), di-potassium phosphate (1.00 g/l) and ferric chloride (0.05 g/l). The medium was supplemented with mycological peptone and bi-ammonium hydrogen ortho-phosphate (0.5%w/v) as nitrogen source. Waste frying oil was used as supplement to carbon source.

Generation of used soybean oil. Two different samples of (OS1 and OS2) virgin soybean oil (5 L) was fried for a duration of 9h, for frying oil is heated in open pans at 160-200^{0}C for relatively long period of time (Mangesh et al. 2006), 500 ml of sample was drawn at an interval of 1h. Approximately 7- 9 fryings of food materials were carried out at each hour and 58 fryings were carried out in 9 h frying time. No fresh oil was supplemented during this period.

Free Fatty Acid (FFA) Determination. The FFA of virgin oil and collected fried oil samples were determined using the standard method outlined by American Oil Chemists Society (Rukunudin et al., 1998). 10g of used oil was dissolved in 1:1 ethanol and diethylether. The mixture was titrated, under constant stirring, against 1.0 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.

$$\%\ \text{FFA as oleic acid} = \frac{\text{Alkali volume (ml)} \times \text{Alkali normality} \times 28.2}{\text{Sample weight (g)}}$$

Further studies on the biocatalysis of tranesterification reaction with fresh whole cells and dry biomass was carried out with OS1, keeping in view the similar trend in the FFA content of both OS samples.

Whole cell catalysed hydrolysis and transesterification/esterification reaction under shake flask condition. 140ml of frying oil samples were collected at periodic intervals were cooled at room temperature and supplemented with 60ml MS medium (70:30,v/v; Oil sample : MS media) in 500ml Erlenmeyer flask. The sample was inoculated with freshly grown biomass of *Aspergillus* (RBD01) . The reaction mixture was incubated at 28 ºC for 72 hours on shaker set at 120 rpm. After 72 hours, a 10g sample was withdrawn and the FFA content was estimated. Following incubation, 1:3 molar ratio of oil to ethanol was added at periodic interval of 12 hours a little excess ethanol was added to avoid reversible reaction. After additional 24 h incubation, the product was allowed to settle and upper layer was separated through separating funnel.

In the similar way whole cell catalyzed esterification was carried out under shake flask condition by varying the percentage of oleic acid (30% to 90%) in the growth medium,

ethanol was added step wise in 1:1 molar ratio, rest of the process is same as explained above.

Whole cell catalyzed transesterification by using dried biomass. For generating dry biomass the freshly grown biomass was introduced in oil: minimal media ratio of 30:70 and inoculated for 120h. Biomass produced was separated and dried by using simple filter paper. Dried biomass was then immersed in liquid nitrogen and crushed to powder. This powdered biomass was used for further in the experiments.

General procedure for Transesterification reaction. Powdered biomass was added to waste cooking oil and the suspension stirred at 35°C in a round bottom flask on hot plate magnetic stirrer. 5ml ethanol was added at an interval of 12hr in parts and stirring continued to facilitate complete conversion. Intermittently, the progress of reaction was checked by thin layer chromatography.

Identification and Quantification of product. The product (ethyl ester) obtained was analyzed using thin layer chromatography with silica gel G as stationery phase and hexane: ethyl ester: acetic acid (90:10:1) as a mobile phase. The chromatogram was developed in the iodine chamber (Samukawa et al. 2000). The product was further quantified ^{1}H NMR (Bruker-Advance II-400 with 5mm BBO probes) with $CDCl_3$ as solvent and tetra methyl silane as internal standard. The spectra were recorded with pulse duration of 2.72 sec with a relaxation delay of 1 sec and 16 scans. The product was quantified using equation proposed by Ghesti et al. (2007).

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

Wherein

(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

(iii) The numbers 4 and 6 in equation are related to four glyceryl methylenic hydrogens present in TAG molecule and to six hydrogens formed in three ethyl ester products.

Quantification of Ethyl Ester produced from oleic acid. For the quantification of ethyl ester produced from oleic acid the residual free fatty acid was calculated after transesterification of oleic acid. Free fatty acid content was quantified by using formula given by Satyarthi et al. (2009).

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

Quantification of the FFA content by ^{1}H NMR is based on the fact that α-CH_2 peaks of fatty acids appear at δ values higher than those of the methyl (biodiesel) or glyceryl (vegetable oil) 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 α-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 (vegetable oil or biodiesel) shows a quartet like spectral pattern in the α-CH_2 region of the 1H 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 (Figure 2b). The area (AFFA) 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 area

corresponding to the α-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 α-CH_2 of both FFA and ester can be determined by integrating the spectral region 2.20-2.41 ppm.

Results and Discussion

The present study was focused to examine the effect of frying on the whole cell catalyzed hydrolysis, transesterification and fatty acid esterification with Aspergillus sp. (RBD01). The product, ethyl ester, was identified through TLC with chemically synthesized ethyl ester as standard which was further quantified using ^{1}H-NMR.

Thin layer chromatograph (Figure 1) indicates two corresponding spots of bio-catalysed as well as chemically catalysed ethyl ester. Spots corresponding to free fatty acids, di-glycerides and mono-glycerides were observed in the whole cell catalysed biodiesel, with no indication of the presence of oil.

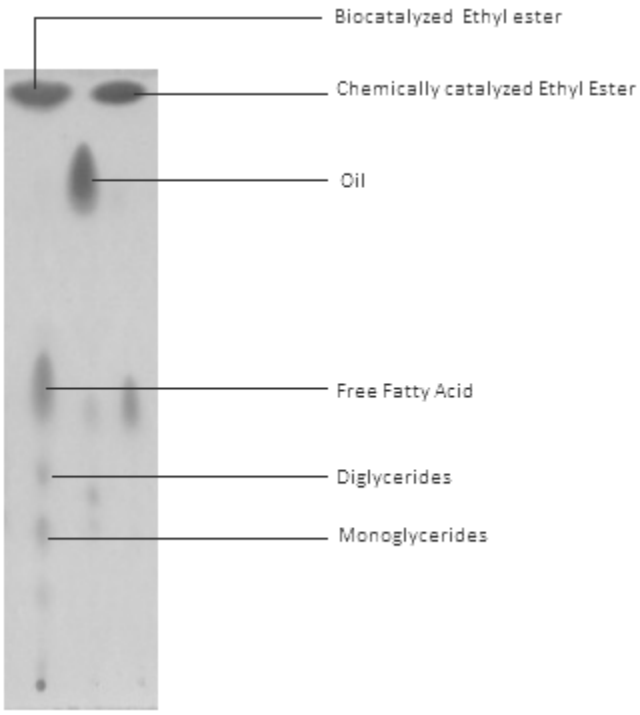

Fig: 1. Thin Layer Chromatograph of bio-catalysed ethyl ester, chemically catalysed ethyl ester and oil

^{1}H NMR spectra of ethyl ester (>98) and oil (Figure 2) showed that the peak due to glyceryl methylenic hydrogens at 4.25-4.35 ppm was completely replaced by peak of -OCH_2 of ethoxy hydrogens at 4.10-4.20 ppm representing the complete conversion of oil to ethyl ester.

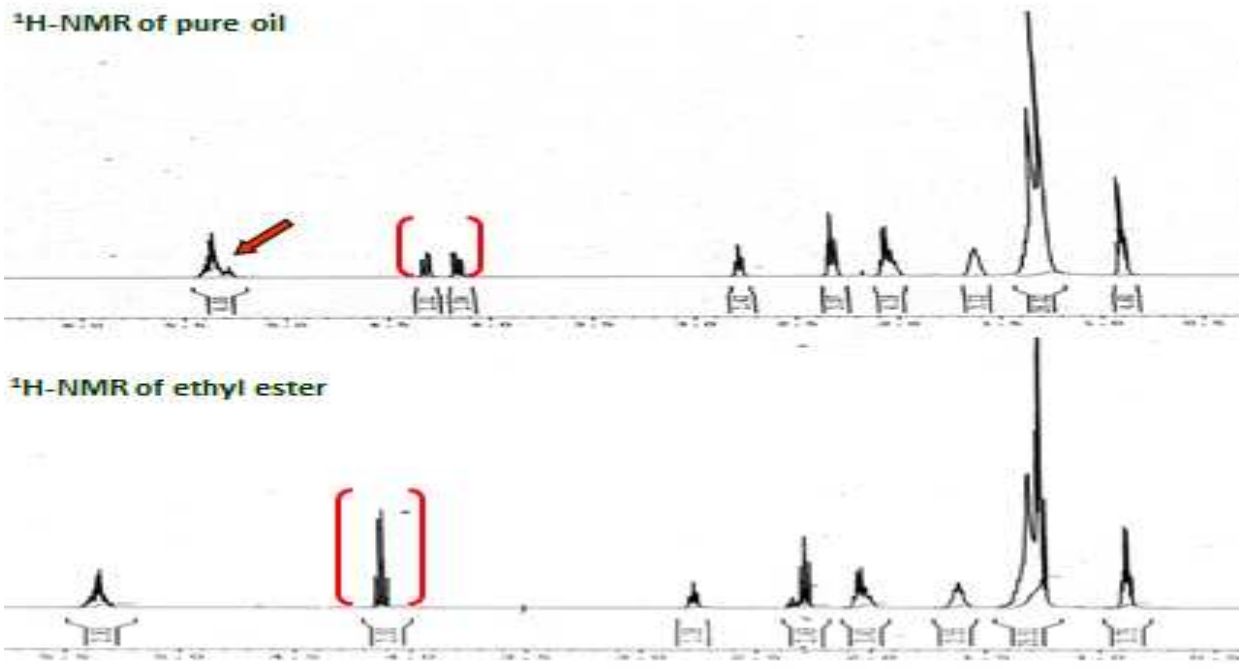

Fig: 2. ^{1}H NMR of pure oil and bio-catalyzed ethyl ester

Effect of frying on free fatty acid (FFA) generation

The first phase of the study was to understand the influence of frying time on the FFA generation. Two different samples (OS1 and OS2) soybean oil were collected after frying of virgin soybean oil as given in the methodology. The observations on FFA generation in OS1 showed an increasing trend in FFA content from 0.1% to 7.6% with increase in frying time from 0 h to 9 h (Figure 3.) Further, the FFA levels remained constant until 2nd frying (0.29% FFA) followed by gradual increase till 5th frying (0.56% FFA) and steep increase till 9th frying (7.63%). There was a minor deviation in the case of OS2 wherein the FFA was constantly increased from 1st frying to 5th frying (0.1% to 0.7%) (Figure 3) followed by steep

increase from 5th frying to 9th frying (0.7% to 6%). This showed that with increase in the time of constant frying of oil there was an increase in the percentage of FFA due to hydrolysis of oil in the presence of moisture released during frying.

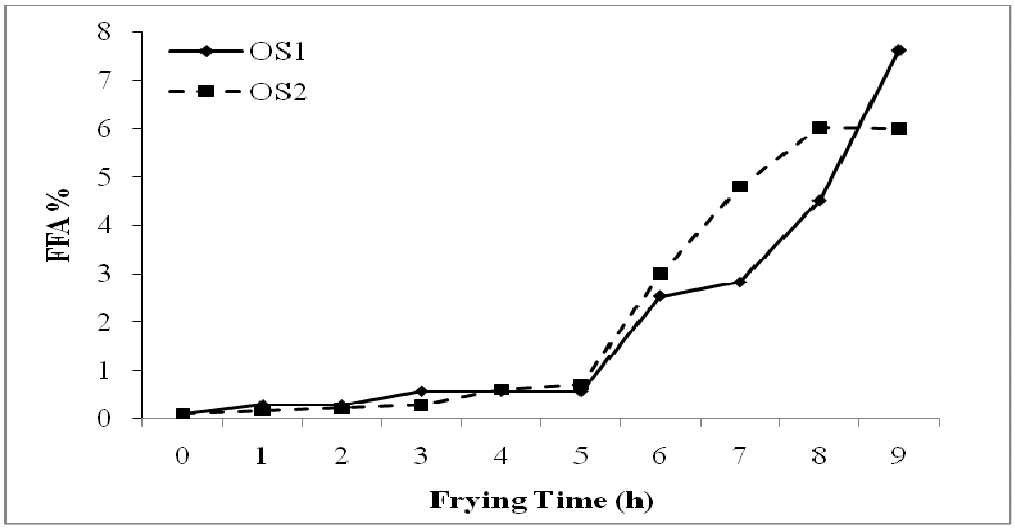

Fig: 3. Trend in free fatty acid generation of OS1 and OS2 during frying

Effect of frying on whole cell catalyzed hydrolysis

The samples collected after frying at different time intervals were further subjected to hydrolysis using whole cells of *Aspergillus sp*. (RBD-01). The results indicated that with increase in frying time, the extent of bio-catalyzed hydrolysis decreased from 80 % (0 h) to 35.81% (9 h) for OS1 (Figure 4). Where as in the case of second sample (OS2), with increasing frying time, the extent of whole cell catalyzed hydrolysis decreases from 0 h to 9 frying (86% to 47.3%) (Figure 4). When the samples (OS1 and OS2) are correlated, it was found that whole cell hydrolysis of OS2 resulted in higher FFA yield as compared to OS1.

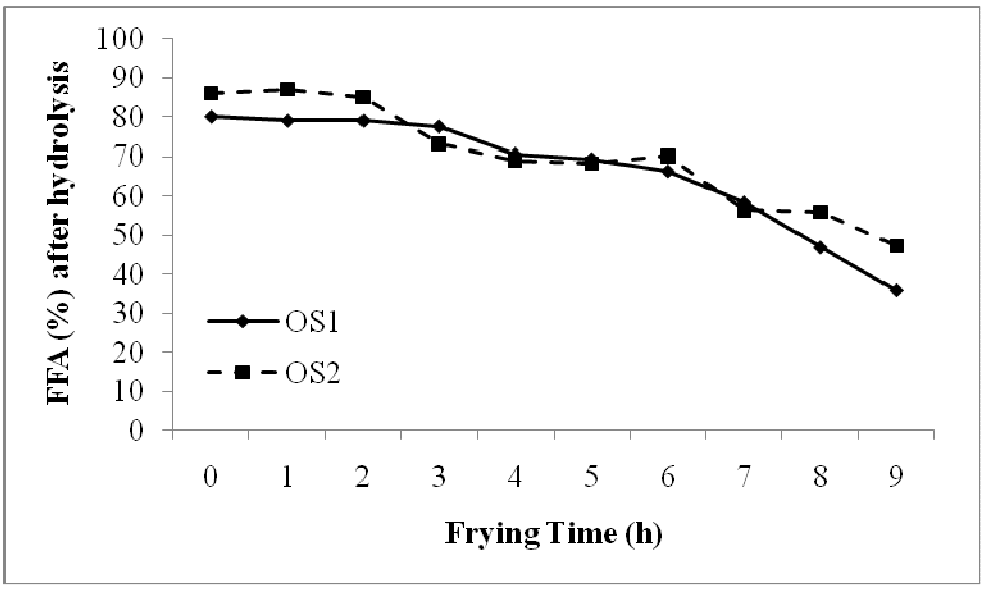

Fig: 4. Effect of frying on whole cell catalyzed hydrolysis of OS1 and OS2

A comparative and average profile of the FFA levels (%) in both oil samples, before and after biocatalyzed hydrolysis, is presented in Table 1 (in the following page). The trends in the profile indicate that there is a reverse trend in the FFA content before biocatalyzed hydrolysis when compared to after the catalysis, which is presumably due to thermolytic, oxidative and hydrolytic reactions occurring during the frying process resulting in the formation of many potentially inhibitory unknown/unidentified compounds (Mittelbach et al., 2000) which would affect the biocatalytic mechanisms (Prakash et al., 2010).

Effect of frying on transesterification reaction

Keeping in view, similar trends in FFA content of OS1 and OS2, further studies on whole cell catalyzed transesterfication was carried out with OS1 sample. Two different approaches were used to catalyzed transesterification reaction – (1) Transesterification reaction under shake flask condition; and (2) Transesterification reaction using dried biomass

Table 1. FFA levels in frying oil samples before and after biocatalyzed hydrolysis

Frying Time	Sample code	FFA% after frying	Mean (SD)	FFA% after whole cell catalysed hydrolysis	Mean (SD)
0h	OS1	0.1	0.10	80	83.00 (4.24)
	OS2	0.1		86	
1h	OS1	0.28	0.22 (0.08)	78.96	82.98 (5.69)
	OS2	0.16		87	
2h	OS1	0.28	0.25 (0.04)	78.96	81.98 (4.27)
	OS2	0.22		85	
3h	OS1	0.56	0.42 (0.20)	77.55	75.38 (3.08)
	OS2	0.28		73.2	
4h	OS1	0.56	0.58 (0.03)	70.5	69.75 (1.06)
	OS2	0.60		69	
5h	OS1	0.56	0.64 (0.11)	69.37	68.84 (0.76)
	OS2	0.71		68.3	
6h	OS1	2.54	2.77 (0.33)	66.27	68.19 (2.71)
	OS2	3.00		70.1	
7h	OS1	2.82	3.81 (1.40)	58.37	57.34 (1.46)
	OS2	4.80		56.3	
8h	OS1	4.51	5.27 (1.07)	46.81	51.31 (6.36)
	OS2	6.02		55.8	
9h	OS1	7.61	6.81 (1.14)	35.81	41.56 (8.12)
	OS2	6.00		47.3	

Transesterification reaction under shake flask condition

With the increase in the frying time, the extent of transesterification showed a decreasing trend after 4th frying (98%) to 58% in 5th frying and 24% in 9th frying samples. This is correlated with the whole cell hydrolysis wherein the FFA levels decreased from 1st frying to 9th frying. The generation of ethyl ester as depicted by H^1 NMR (Figure 5) is shown in terms of percentage in Figure 6.

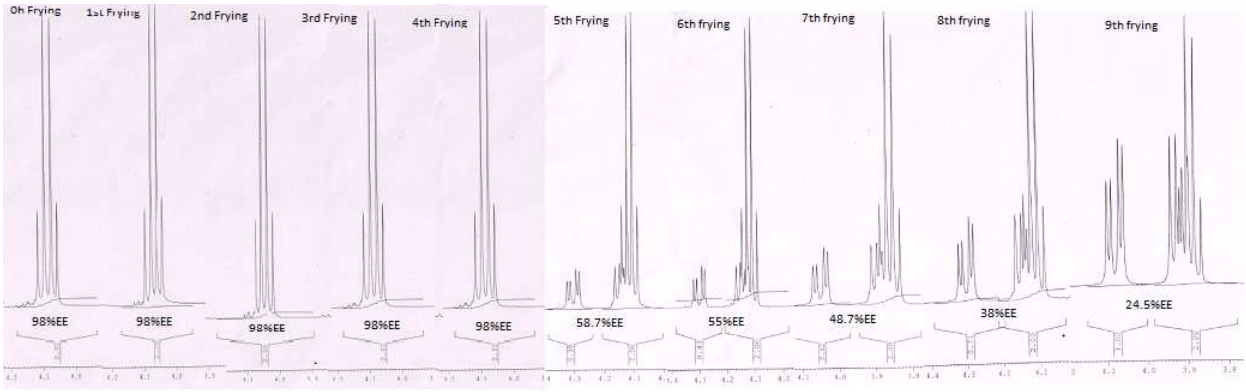

Fig: 5. ^{1}H NMR of frying oil sample showing incomplete conversion

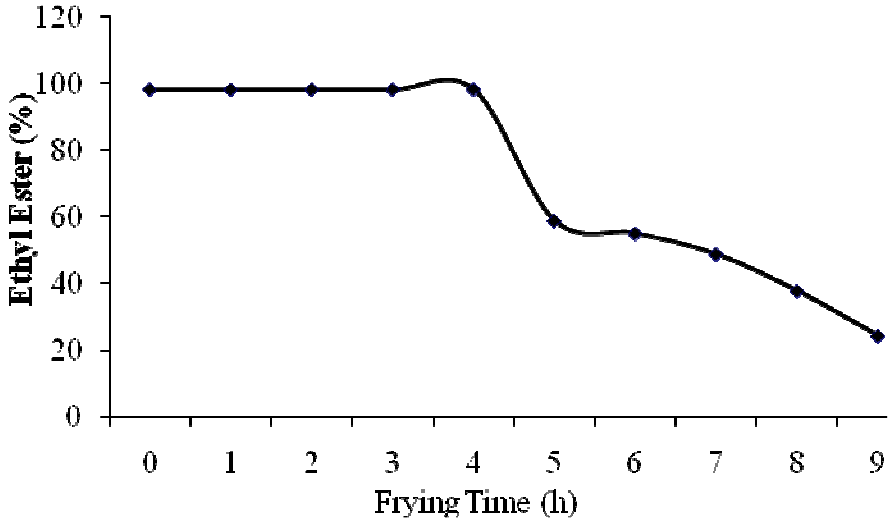

Fig: 6. Ethyl ester (%) produced through whole cell catalyzed transesterification

In addition to use of fresh and active biomass for catalyzing transesterification reaction, studies were also carried out to examine the use of dried biomass to catalyze such reactions.

Transesterification reaction by using dried biomass

Transesterification reaction with dried biomass indicated relatively different trend when compared to the fresh growing biomass. From the H^{1} NMR spectra (Figure 7), it is evident that the yield of ethyl esters (EE) significantly decrease from 76% to 24% as the frying time extended to 9 h (Figure 8).

This showed that the whole cell catalyzed transesterification under shake flask condition with growing biomass resulted in better yield of ethyl esters than the dried biomass. In addition, complete conversion of oil to ethyl ester was obtained with growing biomass in 48 h where as the reaction was incomplete with dried biomass with maximum yield of 76% in 52 h.

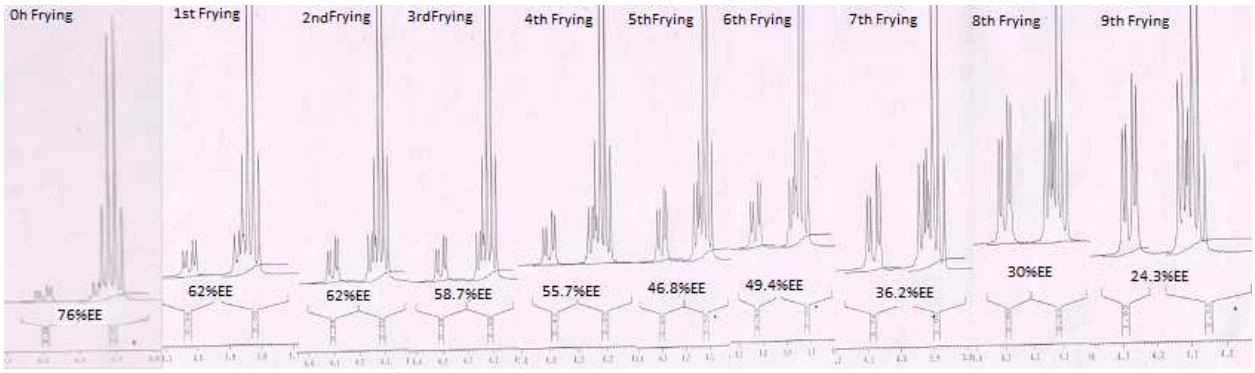

Fig: 7. ^{1}H NMR of transesterified products showing incomplete conversion

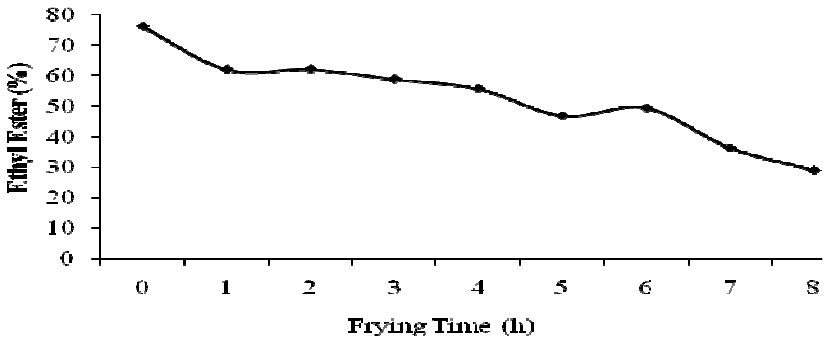

Fig: 8. Ethyl ester produced during transesterification using dried biomas

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). 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 *Rhizopus oryzae* (ATCC10260) was grown and incubated at room temperature without immobilization. Results showed that whole-cell biocatalyst was able to

produce biodiesel 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-hr transesterification reaction using no excess methanol. However, in contrast to these observations (Jin et al 2009), the present study demonstrates near total transesterification in lesser reaction time of 48 h with used frying oil containing higher FFA content as substrate. In addition, reduced yield of alkyl esters as observed by Jin et al.(2009) is possibly due to the use of methanol as reactant, which is contrast to the present study where in a better yield of alkyl ester was obtained with ethanol as a reactant. This may be attributed to (a) better tolerance and compatibility of biocatalyst to ethanol over methanol (Fukuda et al. 2009); (b) better enzymatic compatibility to long chain alcohols (Hsu et al. 2004) ; and (c) faster esterification of FFA by ethanol when compared to methanol (Jin et al. 2008).

Nuclear magnetic resonance spectrometry (NMR), which is reported to be a suitable method to characterize and quantify alkyl esters, was used in the study (Neto et al. 2004, Geldard et al. 2005, Reddy et al. 2006, Liu et al. 2008, Wahlen et al. 2008, Monteiro et al. 2009). The derivation for quantification of ethyl esters (%) using ^{1}H NMR, as done in the present study, was derived by Ghesti et al. (2007) modified from the derivation reported by Silva (2005). NMR spectra showed that glyceridic peak due to glycerol methylenic hydrogen of oils were completely replaced by ester quartet in case of complete (>98%) transesterification.

Recent studies carried out by this research group with non-edible oils viz., Jatropha (9.8% FFA) and Karanj (4.5% FFA) in 70:30 oil:medium ratio respectively resulted in >98% yield in ethyl esters Thus, the results indicate that factors other than FFA and moisture presumably influence the extent of biocatalyzed tranesterification of used frying oil (Prakash and Aulakh 2011). The results on transesterification of different frying oil (cottonseed) samples was published elsewhere, showed that increased frying time of the oil decreased the

extent of the transesterification reaction and hence alkyl ester production. Nearly complete (>98%) transesterification to ethyl ester was observed with used oil containing a free fatty acid (FFA) content of 3.7%, whereas beyond an FFA content of 4.0% the yield was reduced. Biocatalyzed hydrolysis (in the absence of the ethyl alcohol acceptor) of used frying oil resulted in decreasing yield of FFA from 84.0% to 27.6% with increasing frying time. With fried oil capable of a hydrolysis yield of 82-41% FFA, transesterification reactions were nearly complete. With the lower hydrolysis yields of 38-27% FFA, the transesterified ethyl ester yield decreased to 61-51%. These observations indicate that factors other than the presence of FFA and moisture influence the biocatalytic transesterification of used cooking oils (Prakash *et al*. 2010). Drawing a comparison between the earlier studies with used cottonseed oil with that of the observations obtained in the present study with soyabean oil, it has been observed that the FFA generation during frying is faster in cottonseed oil (1.4% in 1 h) than soybean oil (> 1% in 1h). This factor seems to affect the biocatalyzed hydrolysis also wherein the FFA generation after catalysis was 41% in 2 h in case of cottonseed oil and 80% in soybean oil. The comparison clearly indicates that soybean oil is a better substrate in terms of FFA generation as well as biocatalyzed transesterification.

In addition to the studies carried on biocatalyzed transesterification of used frying soybean oil, to examine if there is interference of FFA in the transesterification process, separate studies were carried out on biocatalyzed esterification of oleic acid using freshly growing biomass under shake flask condition.

Whole cell catalyzed esterification of oleic acid under shake flask condition

Esterification of oleic acid under shake flask condition showed (Figure 9) the maximum ester yield of 82% at 30%, 40% and 70% of acid in the medium.

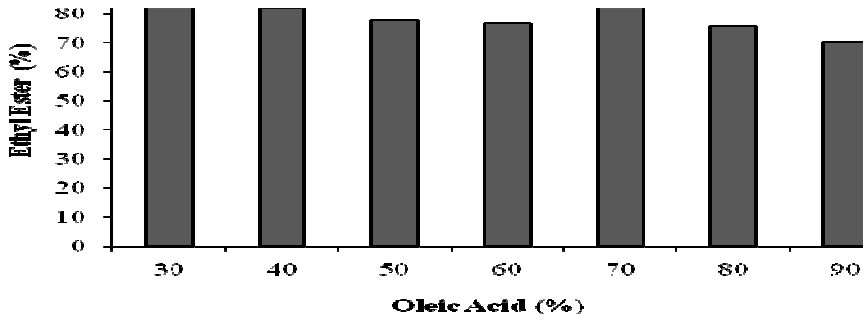

Fig: 9. Esterification of oleic acid

The biocatalyzed esterification of oleic acid resulted in 82% ethyl oleate with 30% oleic acid supplementation followed by decrease to 77% with 60% oleic acid. This further decreased to 70% with supplementation of 90% oleic acid. There was an increase in ethyl oleate yield 82% at 70% oleic acid supplementation, which can possibly be an experimental artifact, but an aspect that needs to be further examined. This observation is one of the few reports on the use of significantly high concentration of oleic acid (upto 90% in the growth medium) as a carbon source as well as lipase inducers wherein *Aspergillus sp*. (RBD01) was observed to grow and esterify the fatty acid.

Limited reports available on the whole cell catalyzed esterification reaction, in general, include use of *R. oryzae* IFO4697 as a catalyst immobilized within biomass support particles for esterification of tert-butanol to biodiesel (Lin et al., 2009); and esterification of long-chain alcohol and oleic acid for producing wax esters using *R. niveous* as a whole cell catalyst (Chen et al., 1997).

In conclusion, this study demonstrates the use of a fungus isolate, *Aspergillus* sp. (RBD01) for exploiting its potential to transesterify used soybean oil and esterify fatty acids.

References

1. Abreu, F. R.; Alves, M. B.; Macedo, C. C. S.; Zara, L. F.; Suarez, P. A. Z. New multi-phase catalytic systems based on tin compounds active for vegetable oil transesterificaton reaction. *J. Mol. Catal. A: Chem.* 1–2, 263-267, **2005.**

2. Al-Widyan, M. I.; Al-Shyoukh, A. O. Experimental evaluation of the transesterification of waste palm oil into biodiesel. *Biores. Technol.* 85, 253–256. **2002.**

3. Antolin, G.; Tinaut, F. V.; Briceno, Y.; Castano, V.; Perez, C.; Ramirez, A. I. Optimisation of biodiesel production by sunflower oil transesterification. *Biores. Technol.* 83, 111-114, **2002.**

4. Ban, K.; Hama, S.; Nishizuka, K.; Kaieda, M.; Matsumoto, T.; Kondo, A.; Noda, H.; Fukuda. H. Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. *J. Mol. Catal. B:Enzym*. 17, 157-165, **2002.**

5. Ban, K.; Kaieda, M.; Matsumoto, T.; Kondo, A.; and Fukuda. H. Whole-cell biocatalyst for biodiesel fuel production utilizing *Rhizopus oryzae* cells immobilized within biomass support particles. *Biochem. Eng. J.* 8, 39-43, **2001.**

6. Bastida, S.; Sanchez-Muniz, F. J. Thermal oxidation of olive oil, sunflower oil and a mix of both oils during forty, discontinous domestic fryings of different foods. *Food Sci. Technol. Int*. 7, 15-21, **2001.**

7. Canakci, M. The potential of restaurant waste lipids as biodiesel feedstocks. *Biores. Technol.* 98, 183–190, **2007.**

8. Canakci, M.; Gerpen, J. V. Biodiesel production via acid catalysis. *Trans. Am. Soc. Agric. Eng*.42, 1203-1210, **1999.**

9. Cardone, M.; Prati, M.V.; Rocco, V.; Senatore, A. Experimental analysis of performances and emissions of a diesel engines fuelled with biodiesel and diesel oil blends. In: Proceedings of MIS–MAC V, Roma, (in Italian), pp. 211–225, **1998.**

10. Carraretto, C.; Macor, A.; Mirandola, A.; Stoppato, A.; Tonon, S. Biodiesel as alternative fuel: experimental analysis and energetic evaluations. *Ener. fuels*. 29, 2195–2211, **2004.**

11. Chiu, C. W.; Schumacher, L. G.; Suppes, G. J. Impact of cold flow improvers on soybean biodiesel blend. *Biomass Bioener*. 27, 485, **2004**

12. Cvengers, J.; Cvengrosova, Z. Used frying oils and fats and their utilization in the production of methyl esters of higher fatty acids. *Biomass Bioener*. 27, 173-181, **2004**.

13. Dasari, M. A.; Goff, M. J.; Suppes, G. J. Non-Catalytic alcoholysis kinetics of soybean oil. *J. Am. Oil. Chem. Soc*. 80, 189, **2003.**

14. Demirbas, A. Biodiesel production via non-catalytic SCF method and biodiesel fuel characteristics. *Ener. Convers. Manag.*. 47, 2271–2282, **2006.**

15. Demirbas, A. Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: A survey, *Energy Convers. Manag.* 4, 2093–2109, **2003.**

16. Devanesan. M. G.; Viruthagiri, N. Transesterification of Jatropha oil using immobilized *Pseudomonas fluorescens. Afr. J. Biotech.* 6, 2497-2501, **2007.**

17. Dorado, M. P.; Ballester, E.; Arnal, S.; Gomeq, J.; Gimezn. Testing of waste olive oil methyl ester as a fuel in diesel engine. *Ener. Fuels.* 17.1560-1565, **2003.**

18. Dorado, M. P.; Ballesteros, E.; Lopez, F. J.; Mittelbach, M. Optimization of alkali-catalyzed transesterification of brassica oil for biodiesel production. *Ener. Fuels.* 18, 77-83, **2004.**

19. Encinar, J. M.; Gonza lez, J. F.; Rodri guez, J. J.; Tejedor. A. Biodiesel fuels from vegetable oils transesterification of *Cynara cardunculus L.* oils with ethanol. *Ener. Fuels.* 16, 443. **2002.**

20. Freedman, B.; Butterfield, R. O.; Pryde, E. H. Transesterification kinetics of soyabean oil. *J. Am. Oil Chem. Soc.* 62, 1375-1380, **1985**.

21. Freedman, B.; Kwolek, W. F.; Pryde, E. H. Quantification in the analysis of transesterified soyabean oil by capillary gas chromatography. *J. Am. Oil Chem. Soc.* 63, 1370-1375, **1986.**

22. Freedman, B.; Pryde, E. H.; Mounts, T. L**.** Variables affecting the yields of fatty esters from transesterified vegetable oils. *J. Am. Oil Chem. Soc*. 61, 1638-1643, **1984**.

23. Fukuda, H.; Kondo, A.; Noda, H. Biodiesel fuel production by transesterification of oils. *J. Biosci. Bioeng*. 92, 405-416, **2001**.

24. Gerpen, J. V.; Shanks B.; Pruszko, R.; Clements, D.; Knothe, G. Biodiesel production technology. *National Renewable Energy Laboratory Report,* NREL/SR-510-36244, **2004**.

25. Ghesti, G. F.; DeMacedo, J. L.; Resck, I. S.; Dias, J. A.; Dias, S. C. L. F T Raman spectroscopy quantification of biodiesel in progressive soyabean oil transesterification reaction and its correlation with ^{1}H –NMR Spectroscopy methods. *Ener. Fuels.* 21, 2475-2480, **2007**

26. Goering, E.; Schwab, W.; Daugherty, J.; Pryde, H.; Heakin, J. Fuel properties of eleven vegetable oils. *Trans. ASAE.* 25, 1472–1483, **1982.**

27. Haas, M. J.; Bloomer, S.; Scott, K. Sample high-efficiency synthesis of fatty acid methyl esters from soapstock. *J. Am. Oil Chem. Soc*. 77, 373–379, **2000.**

28. Haas, M. J.; Piazz, G. J.; Fogliz, T. A. Enzymatic approaches to the production of biodiesel fuels. *Lipid Biotechnol*. 587-598, **2002.**

29. Hama, S. Lipase localization in *Rhizopus oryzae* cells immobilized within biomass support particles for use as whole-cell biocatalysts in biodiesel-fuel production. *J. Biosci. Bioeng*. 101, 328– 333, **2006.**

30. Iso, M.; Chen, B.; Eguchi,M.; Kudo,T.; Shreshta, S. Production of biodiesel fuel from triglycerides and alcohol using immobilized lipase. *J.Mol.Catal.B.Enzym.*16, 53-58, **2001.**

31. Issariyakul, T.; Kulkarni, M. G.; Dalai, A. K.; Bakhshi, N. N. Production of biodiesel form waste fryer grease using mixed methanol/ethanol system. *Fuel Proc. Technol.* 88, 429-436, **2007**.

32. Jin, G.; Bierma, T. J.; Hamaker, C. G.; Mucha, R.; Schola, V.; Stewart, J.; Wade, C. Use of whole cell biocatalyst to produce biodiesel in water containing system. *J. Environ. Sci Hlth, Part A*. 43, 21-28, **2009.**

33. Jin, G.; Bierma, T. J.; Hamaker, C. G.; Rhykerd, R.; Loftus, L. A. Production of biodiesel using whole-cell biocatalysts in separate hydrolysis and methanolysis reactions. *J. Environ. Sci. Hlth, Part A*. 43, 589-595, **2008.**

34. Jitputti, J.; Kitiyanan, B.; Rangsunvigit, P.; Bunyakiat, K.; Attanatho, L.; Jenvanitpanjakul, P. Transesterification of crude palm kernel oil and crude coconut oil by different solid catalysts. *Chem. Eng. J.* 1, 61-66, **2006.**

35. Kaieda, M.; Biodiesel fuel production from plant oil catalyzed by *Rhizopus oryzae* lipase in a water-containing system without an organic solvent. *J. Biosci. Bioeng*. 88, 627–631, **1999**.

36. Khan, M. I.; Chhetri, A. B.; Islam, M. R. Analyzing sustainability of community based energy technologies. *Ener. Sour. 2*, 403-419, **2007.**

37. Knothe, G.; Sharp, C. A.; Ryan, T. W.; Exhaust emissions of biodiesel, petrodiesel, neat methyl esters, and alkanes in a new technology engine. *Ener. Fuels.* 20, 403–408, **2006**.

38. Kulkarni, M. G.; Dalai, A. K. Waste cooking oils - An economical source for biodiesel: A Review. *Ind. Eng. Chem. Res.* 45, 2901-2913, **2006**.

39. Kurki, A.; Hill, A.; Morris, M. Biodiesel: The sustainability dimensions. *ATTRA Publication*#IP. 281**,** 1-12, **2006.**

40. Lago, R.; Spiz, R.; Jablonka, F.; Pereira, D. A.; Hartman, L. Extraction and transesterification of vegetables oil with ethanol. *Oleagineux* .40, 147, **1985.**

41. Laforgia, D.; Ardito, V. Biodiesel fuelled IDI Engines: performances, emissions and heat release investigation. *Biores. Technol*. 51, 53–59**, 1994.**

42. Lang, X.; Dalai, A. K.; Bakhshi, N. N.; Reaney, M. J.; Hertz, P. B. Preparation and characterization of biodiesels from various bio-oils. *Biores. Technol.* 80, 53-62, **2002**.

43. Li, E.; Rudolph, V. Transesterification of vegetable oil to biodiesel over MgO-functionalized mesoporous catalysts. *Ener. Fuels.*1, 145-149, **2008**.

44. Ma, F.; Hanna, M. A. Biodiesel production: a review. *Biores. Technol.* 70, 1–15, **1999.**

45. Matsumoto, T.; Takahashi, S.; Kaieda, M.; Ueda, M.; Tanaka, A.; Fukuda, H.; Kondo, A. Yeast whole-cell biocatalyst constructed by intracellular overproduction of *Rhizopus oryzae* lipase is applicable to biodiesel fuel production. *Appl. Microbiol. Biotechnol*. 57, 515-520, **2001.**

46. Meher, L. C.; Dharmagadda, S. S. V.; Naik, S. N. Optimization of alkali catalyzed transesterification of *Pongamia pinnata* oil for production of biodiesel. *Biores. Techol*. 97, 1392-1397, **2006.**

47. Meher, L. C.; Sagar, D. V.; Naik S. N. Technical aspects of biodiesel production by trasesrerification-a review, *Renew Sust. Energ. Rev.*10, 248-268, **2006.**

48. Mittelbach, M.; Tritthart P. Diesel fuel derived from vegetable oils: III, Emission test using methyl esters of used frying oil. *J. Am. Oil Chem. Soc.* 7, 1185–1187, **1988**.

49. Mudge, S. M.; Pereira, G. Stimulating the biodegradation of crude oil with biodiesel preliminary results. *Spill Sci. Technol. Bull.* 5, 353–355, **1999.**

50. Mulkins Phillips, G.J.; Stewart, J.E.; Effect of environmental parameters on bacterial degradation of bunker C oil, crude oils, and hydrocarbons. *Appl. Microbiol.* 8, 915–922. **1974.**

51. Muniyappa, P. R.; Brammer, S. C.; Noureddini, H. Improved conversion of plant oils and animal fats into biodiesel and co-product. *Biores. Technol*. 56, 19–24, **1996.**

52. Mushrush G.W, Mose D. G, Wray C. L, Sullivan K. T. Biofuels as a means of improving the quality of petroleum middle distillate fuels. *Ener. Sources.* 23, 649-655, **2001.**

53. Nakashima, T.; Cell aggregation as a trigger for enhancement of intracellular lipase production by a *Rhizopus* species. *J. Ferment. Bioeng*. 70, 83–89, **1990**.

54. Nelson, L. A.; Foglia, A.; Marmer, W. N. Lipase-catalysed production of Biodiesel. *J. Am. Oil Chem. Soc*. 73, 1101-1195, **1996.**

55. Neto, C.; Rossi, P. R.; Zagonel, L.; Ramos, G. 2000. Producao de biocombustível alternativo ao oleo diesel atraves da transesterificacao de oleo de soja usado em frituras. *Quim. Nova*. 23, 531–537, **2000.**

56. Noureddini, H.; Gao, X.; Philkana, S. Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. *Biores. Technol.* 96, 769-777, **2005.**

57. Noureddini, H.; Zhu, D**.** Kinetics of transesterification of soybean oil. *J. Am. Oil Chem. Soc*.74, 1457-1463, **1997**.

58. Oda, M.; Kaieda, M.; Hama, S.; Yamaji, H.; Kondo, A.; Izumoto, E.; Fukuda, H.; Facilitatory effect of immobilized lipase-producing *Rhizopus oryzae* cells on acyl migration in biodiesel fuel production. *Biochem. Eng. J.* 23, 45-51, **2005.**

59. Perez, G. Analysis of enzymatical alcoholysis reaction with vegitable oils. Doctrol Thesis, Planta Piloto de Ingenieria quimica, UNS-CONICET argentina, **2003**.

60. Phan, A. N.; Phan, T. M. Biodiesel production from waste cooking oils. *Fuel*. 87, 3490–3496, **2008.**

61. Prakash, R.; Aulakh, S.S.; Kalra, R. Effect of frying time on free fatty acid generation and esterification rate in Aspergillus sp. catalysed transesterification of cottonseed oil. *Biocatal. Biotrans.*. 28, 403-407, **2010.**

62. Pryor, R. W.; Hanna, M. A.; Schinstock, J. L.; Bashford, L. L. Soybean oil fuel in a small diesel engine. *Trans. ASAE.* 26, 333–338, **1982.**

63. Qin, H.E. Biodiesel production catalyzed by whole-cell lipase from *Rhizopus chinensis*. *Chin. J. Catal*. 29, 41–46, **2008.**

64. Reddy, C. R. V; Oshel, R.; Verkade, J. G. Room temperature conversion of soyabean oil and poultry fat to biodiesel catalysed by non crystalline calcium oxides. *J. Am. Oil Chem.Soc*. 75, 563-568, **1998.**

65. Roger, A. K.; Jaiduk, J.O. A rapid engine test to measure injector fouling in diesel engines using vegetable oil fuels. *J. Am. Oil Chem. Soc*. 62, 1563-1566, **1985.**

66. Rukunudin, I. H.; White, P. J.; Bern, C. J.; Bailey, T. B. A modified method for determining free fatty acids from small soybean oil sample sizes. *J. Am. Oil Chem. Soc*. 75, 563–568, **1998**.

67. Schumacher, L. G.; Borgelt, S. C.; Fosseen, D.; Goetz, W.; Hires, W.G.; Heavy-duty engine exhaust emission test using methyl ester soybean oil/diesel fuel blends. *Biores. Technol.* 57, 31–36, **1996.**

68. Schuchardt, U.; Sercheli, R.; Vargas, R. M. Transesterification of vegetable oils: A review. *J. Braz. Chem. Soc*. 9, 199-210, **1998.**

69. Schuchardt, U.; Vargas, R.M.; Gelbard, G. Transesterification of soybean oil catalyzed by alkyl guanidines heterogenized on different substituted polystyrenes. *J. Mol. Catal. A: Chem.* 37-44, **1996**.

70. Serdari. A.; Lois, E.; Stornas, S. Impact of ester of mono and di carboxylic acid on diesel fuel quality. *Ind. Eng. Res*. 38, 3543-3548, **1999.**

71. Shay, E. G.; Diesel fuel from vegetable oils: status and opportunities. *Biomass Bioener*. 4, 227–242, **1993.**

72. Sheehan, J.; Cambreco, V.; Duffield, J.; Garboski, M.; Shapouri, H.; An overview of biodiesel and petroleum diesel life cycles*.* *US-DOE Report*. 1–35, **1998**.

73. Shiraga, S.; Kawakami, M.; Ishiguro, M.; Ueda, M. Enhanced reactivity of *Rhizopus oryzae* lipase displayed on yeast cell surfaces in organic solvents: potential as a whole-cell biocatalyst in organic solvent. *Appl. Environ. Microbiol*. 71, 4335-4338, **2005**

74. Speidel, H. K.; Lightner, R. L.; Ahmed, I. Biodegradability of new engineered fuels compared to conventional petroleum fuels and alternative fuels in current use. *Appl. Biochem. Biotechnol*. 84–86, 879–897, **2000.**

75. Suppes, G. J.; Bockwinkel, K.; Lucas, S.; Botts, J . B.; Mason, M. H.; Heppert, J. A. Calcium carbonate catalyzed alcoholysis of fats and oils. *J. Am. Chem. Soc*. 2, 139-146, **2001**.

76. Suppes, G. J.; Dasari, M. A.; Doskocil, E. J.; Mankidy, P. J.; Goff, M. J. Transesterification of Soybean Oil with Zeolite and Metal Catalysts, *Appl. Catal. A: Gen.* 257, 213-223, **2004.**

77. Tan, R. R.; Culaba, A. B.; Purvis, M. R. I. Carbon balance implications of coconut biodiesel utilization in the Philippine automotive transport sector. *Biomass Bioener.* 26, 579–585, **2004.**

78. Teng, Y.; and Xu, Y.; Culture condition improvement for whole cell lipase production in submerged fermentation by *Rhizopus chinensis* using statistical method. *Biores. Technol*. 99, 3900–3907, **2008.**

79. Utlu, Z.; Evaluation of Biodiesel Fuel Obtained from Waste Cooking Oil. *Ener. Sour. Part A*, 29, 1295–1304, **2007.**

80. Varese, R.; Varese, M. Methyl ester biodiesel: opportunity or necessity. *Int. News Fats, Oils Relat. Mater*. 7, 816-824, **1996.**

81. Walker, D.; Petrakis, L.; Colwell, R. R. Comparison of biodegradability of crude and fuel oils. *Can. J. Microbiol.* 22, 598–602, **1976.**

82. Wang, Y.; Nie, J.; Zhao.; Ma, S.; Kuang, L.; Tang, H.; Tang, S. Production of Biodiesel from Waste Cooking Oil via a Two-Step Catalyzed Process and Molecular Distillation. *Ener. Fuels.* 24, 2104–2108, **2010.**

83. Watanabe, Y.; Shimada, Y.; Sugihara, A.; Tominaga, Y. Enzymatic conversion of waste edible oil to biodiesel fuel in a fixed bed bioreactor. *J. Am. Oil Soc. Chem*. 78, 703–707, **2001.**

84. Wei, L.; Optimization of whole-cell catalyze methanolysis of soybean oil for biodiesel production using response surface methodology. *J. Mol. Catal:B Enzym*. 45, 122–127, **2007.**

85. Wright, H. J.; Segur,J. B.;Clark ,H. V.;Coburn, S. K.; Langdon, E. E.; Dupuis, R.N .A report on ester interchange. *Oil Soap*. 21, 145-148, **1944.**

86. Zaher, F. A. Vegetable oil as alternative fuel for diesel engines: a review. *Grasas Aceites* (Seville). 41, 82-91, **1990.**

87. Zhang, X.; Peterson, C.; Reece, D.; Haws, R.; Moller, G. Biodegradability of biodiesel in the aquatic environment. *Trans. ASAE.* 41, 1423–1430, **1998.**

88. Zhang, Y.; Dube, M.A.; McLean, D.D.; Kates, M. Biodiesel production from waste cooking oil: In Process design and technological assessment. *Biores. Technol*. 89, 1–6, **2003.**

89. Zhang, Y.; Dub, M. A.; McLean, D. D.; Kates, M. Biodiesel production from waste cooking oil: 2. Economic assessment and sensitivity analysis. *Biores. Technol.* 90, 229–240, **2003.**