Comparative Studies on Biocatalyzed Hydrolysis and Transesterification of Karanj Oil

A Dissertation

Submitted in partial fulfillment of the requirement

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Submitted by:

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CERTIFICATE

This is to certify that the dissertation entitled **Comparative Studies on Biocatalyzed Hydrolysis and Transesterification of Karanj Oil** submitted by Chandanpreet Manshahia in partial fulfillment of the requirement for the award of the Degree of Master of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University.

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DECLARATION

I hereby declare that the work presented in the dissertation entitled **Comparative Studies on Biocatalyzed Hydrolysis and Transesterification of Karanj Oil** in partial fulfillment of the requirement for the award of the degree of Master of Sciences in Biotechnology, is an authentic record of my own work during a period of six months from January 2013 to June 2013, under the guidance of Dr. Tejo Prakash, Professor, Department of Biotechnology and Environmental Sciences and Dr. Ranjana Prakash, Associate professor, school of chemistry and biochemistry, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other University.

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This is to certify that the above statements made by the student are correct and true to the best of our knowledge and belief.

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CONTENTS

Summary

The study presents the observations on the alkyl ester generation by transesterification reaction by vegetable oils using *Aspergillus* and *Rhizopus* as whole-cell biocatalysts. Time dependent modulations in hydrolysis and transesterification reactions have been using whole cell catalyst as well as immobilized culture. Work also represents difference in transesterification reaction on one time addition of alcohol over addition of alcohol at periodic interval. No significant difference was observed in transesterification reaction with one time addition of alcohol and addition of alcohol at periodic interval of 12 h. In addition, the study indicated a distinctive difference in the potentials of strains to hydrolyze and transesterify vegetable oils to alkyl esters.

Introduction

Petrol and diesel one of the most important commodities that consumers rely on daily, these are the non-renewable resources. Since recent past, researchers are in the process of developing gasoline alternatives, electric cars, hydrogen fuel cells and biofuels are some of the alternative energy sources for transportation and industrial applications. Biofuels are the fuels made up of biological ingredients rather than fossil fuels.

Biodiesel is one of the chief biofuels and it is non-toxic, biodegradable, renewable resource of energy, clean burning diesel replacement which is reducing any nation's dependence on imported diesel and reduces global warming by reducing the emission of harmful gases. Biodiesel is usually made by using various chemical reactions such as pyrolysis, microemulsion and transesterification. Biodiesel produced by transesterification is one of the most efficient way in which triglycerides in combination with alcohol in the presence of catalyst produce fatty acid alkyl esters and glycerol. Biodiesel is usually blended with standard diesel fuels which are indicated by the abbreviation BXX i.e.B20 indicates 20% biodiesel blended with 80% of diesel and chemical composition of biodiesel depends upon the feedstock from which it made, B100 refers to pure biodiesel. NBB (National Biodiesel Board, India) defines biodiesel as a fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oil or animal fats, designated B100 and meeting the requirement of ASTM (American society of testing and material) D 6751. Among the alcohols, ethanol is the most commonly used alcohol for biodiesel production because of its less cost.

In transesterification reaction, for every one mole of TAG which undergoes complete conversion 3 moles of biodiesel and 1 mole of glycerol are produced. Transesterification reaction for biodiesel production can be achieved by three approaches acid catalysis, alkali catalysis, and enzymatic catalysis. Wide range of catalysis may be used for production of biodiesel, such as homogenous and heterogeneous acids and bases, sugars, lipases, ion exchange resins, zeolites etc. Chemical transesterification, although being extensively used for commercial production of biodiesel, has various limitations such as difficulty in recovery of glycerol and catalysts, ncecssity for alkaline waste water treatment, interference of free fatty acid with reaction, etc.

Biocatalyzed transesterification in the form of both extracellular and intracellular lipases have certain obvious advantages over chemically catalyzed reaction. Chemical transesterification generally results in intense energy consumption and formation of undesirable byproducts such as soaps in addition to difficulty in recovery of important byproduct such as glycerol is difficult. IN addition unlike chemical transesterification there is no free fatty acids and water which interferes the reaction. Biocatalysts are used to catalyze the transesterification of triglycerides in either aqueous or non-aqueous system. The major limitation with the use of lipase as catalyst is the couse of pure enzyme. As an effective alternative, the use of whole cell biocatalyst has been studied extensively since recent past. Microorganisms have the ability to produce lipase enzyme, which has potential to carry out transesterification. Various fungal species i.e. *Aspergillus species* and *Rhizopus species* have been used to carry out whole cell catalyzed transesterification reaction. In addition, whole cell immobilization is also very efficient way to carry out the same for biodiesel production.

Keeping this in view, the present study aimed at time dependent kinetics of hydrolysis and esterification reaction by using several selected fungal strains.

Literature Review

Fuels, concentrated store of energy, are combustible substances which are used to produce heat and energy, petrol and diesel are one of the most important commodity as they are used everywhere. But these are the limited non-renewable resources that would vanish soon. Continuous depletion of known petroleum reserves and hike in price of petrol and diesel has resulted in need of search for alternative resources. In search of alternatives, researchers have been exploring various alternative fuels. Amongst them, biodiesel has proven to be one of the promising alternatives (Ma et al., 1999).

Biodiesel refers to a vegetable oil or animal fat based diesel fuel, containing long chain of fatty acid alkyl esters (FAAE) which is produced by chemical or enzymatic reactions meeting the requirement of ASTM D 6751(ASTM 2008). It is a clean fuel, less toxic, biodegradable, and non-renewable resource of energy. Biodiesel is a biofuel of biological ingredients rather than fossil fuels. Various agricultural co-products and byproducts such as soybean oil, rapeseed oil, jatropha oil, karanj oil, cotton seed oil and other natural oils; feedstocks such as soapstocks, acid oils, used cooking oils, waste restaurant grease, various animal fats (tallow), non-edible oils obtained from trees and microorganisms such as algae are diverse variety of important substrates available for producing biodiesel (Moser, 2009). Biodiesel is generally used in blends with diesel (Fukuda et al., 2001), biodiesel blends are denoted as "BXX" where "XX" represents the percentage of biodiesel contained in the blend (i.e. B20 is 20% biodiesel). Biodiesel offers obvious advantages over diesel including sustainability, reduction of green house gases emission, regional and agricultural development, security of supply; and potential to reduce the level of pollutants. Because of these advantages, various nations are eager to use biodiesel to decrease their dependence on foreign oil and contribute to their own economy (Reijiders, 2006). Over the past decade, the global biodiesel industry has grown significantly.

Diesel and Biodiesel

Although performing the same function i.e. used as a fuel in diesel vehicles, diesel and biodiesel comes from different sources. Diesel comes as a byproduct of petroleum distillation which is a non-renewable fossil fuel while biodiesel can be obtained from vegetable oils or animal fats by various chemical or enzymatic processes. Regarding the chemistry of diesel and biodiesel, diesel has long hydrocarbon chain while biodiesel has long hydrocarbon chain along with an ester functional group which makes it distinct from diesel; biodiesel fuels produced from vegetable oil have viscosity close to those of diesel but have high cetane number and flash point (Srivastava et al., 2000; Yamane et al., 2001). The direct use of vegetable oil into diesel engines is restricted by various unfavorable physical properties particularly the viscosity of vegetable oils. Because of high viscosity vegetable oil causes poor fuel atomization, incomplete combustion, carbon deposition on the injector etc. that result into serious engine damage (Ma et. al.1999). Because of various problems like carbon deposits in the engine, engine durability and lubricating oil contamination, associated with the use of oils and fats as diesel fuels, they must be derivatized to be compatible with existing engines.

For this various methods like pyrolysis, microemulsions and the most efficient process of choice being transesterification (Fangrui et al., 1999). Pyrolysis is a process which involves chemical change by the application of thermal energy in the presence of air or nitrogen sparge. By pyrolysis of triglycerides, product so obtained is suitable for diesel engines (Schwab et al., 1988). Various vegetable oils shows large difference in composition when they are thermally decomposed, pyrolysed soybean oil, contains 79% carbon and 12% hydrogen. Pyrolysis results into low viscosity and high cetane number as compare to vegetable oils (schwab et al., 1988). Therefore pyrolysed product is suitable for diesel engines. Another approach to produce biodiesel is microemulsions, microemulsions are isotrophic, clear or translucent thermodynamically stable dispersions of oil, water, a surfactant, and a small amphiphillic molecule, called a cosurfactant, the use of microemulsions with solvents like methanol, ethanol etc has been used to solve the problem of high viscosity of vegetable oils (Ziejewski et al., 1984).

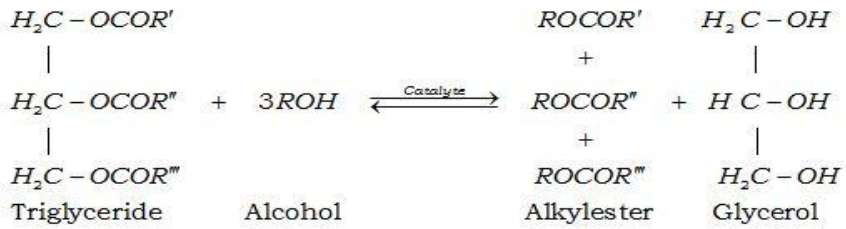
There are various reasons that justify biodiesel's development i.e. it provides a market for excess production of vegetable oils and fats, it decreases the country's dependence on imported petroleum, the exhaust emission of carbon monoxide, unburned hydrocarbons, and particulate

emissions from biodiesel are lower than with regular diesel fuel (Gerpen, 2005). Biodiesel is renewable and does not contribute to global warming due to its closed carbon cycle. A life cycle analysis of biodiesel showed that overall carbon dioxide emissions were reduced by 78% compared with petroleum based diesel fuel (Sheehan et al., 1998). Being non-renewable, overuse of petroleum and diesel has lead to total annihilation of its natural resources, whereas biodiesel is a renewable fuel which can be obtained from vegetable oils or fats by addition of alcohol and form fatty acid alkyl esters (Fukuda et al., 2001). Non edible oils like jatropha (*jatropha curcas*), Karanj (*Pongamia pinnata*) have been converted into biodiesel by transesterification reaction and blended with diesel to use as substitute fuels of diesel engines (Sahoo et. al., 2009).

Transesterification

Transesterification is a two step process involving hydrolysis and esterification, in first step fats get converted into free fatty acids and in second step these free fatty acids combine with alcohol to form fatty acid alkyl esters in the presence of a catalyst. A catalyst is used to increase the efficiency of reaction. Because the reaction is reversible, excess alcohol is used to shift the reaction to product side (Barnwal and Sharma, 2005; Mehar et al., 2006). Amongst the alcohols, methanol and ethanol are most commonly used due to low cost. This process has been widely used to reduce the viscosity of triglycerides, hence enhancing the physical properties of renewable fuels to improve engine performance (Clark, 1984). Glycerol, which is a high viscosity component, is removed and therefore product so obtained has low viscosity like fossil fuels (Parawira, 2009).

Transesterification can be achieved by two approaches, either by chemical catalysis or enzymatic catalysis (Fukuda et al., 2001). Chemical catalysis involves the use of acid and base catalysis while enzymatic transesterification involve use of pure enzyme or as wet biomass. Transesterification is a reversible reaction involveing number of consecutive steps. The first step is the conversion of triglycerides to diglycerides, which is followed by the conversion of diglycerides into monoglycerides and of monoglycerides to glycerol, yielding three molecules of ester (Noureddini et al., 1997). The reaction of transesterification can be shown as-



Stepwise transesterification process can be shown as-

1. Triglycerides (TG) + R'OH $\xleftrightarrow{\text{catalyst}}$ Diglycerides (DG) +R'$COOR_1$
2. Diglycerides (DG) + R'OH $\xleftrightarrow{\text{catalyst}}$ Monoglyceride (MG) +R'$COOR_2$
3. Monoglycerides (MG) +R'OH $\xleftrightarrow{\text{catalyst}}$ Glycerol + R'$COOR_3$

After transesterification of triglycerides, the products include a mixture of esters, glycerol, alcohol, catalyst and tri, di and monoglycerides. Obtaining pure ester is generally not an easy process due to presence of di and monoglycerides as impurities in the esters (Ma et. al., 1998). The co-product glycerol also can be recovered because of its value as an industrial chemical. Glycerol is separated by gravitational settling or centrifugation (Fangrui et al., 1999).

Feed stocks for biodiesel production

Non edible oils sourced from jatropha, karanj, mahua etc., can be used as substrate oil for biodiesel production. Tamalampudi et al. (2008) carried out pioneering studies on utilizing whole-cell catalyzed alcoholysis of the non-edible jatropha oil with *Rhizopus oryzae* cells that had been immobilized into biomass support particles. There are four major biodiesel feed stock categories: algae, oilseeds, animal fats (tallow) and various low-value materials such as used cooking oils, soapstocks and greases (Moser, 2009). Microalgal triglycerides have also attracted attention as potential economical substrate for biodiesel production, its use has advantage of capturing greenhouse gases for their metabolism and growth, and therefore use of microalgal triglycerides is environmental friendly (Meng et al., 2009; Chisti 2008; Chisti 2007). Non edible oils such as *jatropha curcas oil* and *Pongamia pinnata oil* also known as jatropha oil and karanj oil respectively have attracted remarkable interest as a feedstock for biodiesel production in India and other climatically favourable regions of the world (Mohibbeazam et al.,2005 and

kumartiwari et al., 2007). Jatropha tree is a poisonous perennial oil shrub whose seeds contain up to 30 wt % oil that can be found in tropical and subtropical regions such as Africa, Central America, Indian subcontinent and other countries in Asia (Mohibbeazam et al., 2005). Jatropha oil acquires a relatively high percentage of saturated fatty acids (34 wt %) therefore the corresponding alkyl esters exhibit relatively poor low temperature operability (Kumartiwari et al., 2007). Karanj is a medium-sized deciduous tree which grows rapidly in humid and subtropical environments and takes 4 to 7 years for maturation and provide fruit that contains one to two kidney- shaped kernals. The oil content of karanj kernals ranges between 30 and 40 wt % (Mohibbeazam et al., 2005). The low temperature operability of the alkyl esters of karanj oil is superior as compare to jatropha oil because of the presence of high percentage of oleic acid in karanj oil (Srivastava and Verma 2008).

Chemical Transesterification

Chemical transesterification involves the use of either acid or base catalyst. Acids used for transesterification includes sulfuric acid, phosphoric acid, hydrochloric and organic sulfonic acids. Acid-catalyzed transesterification is more suitable for glycerides that have relatively high free fatty acid contents and more water (Freedman et al., 1984). Alkali catalysts for transesterification include NaOH, KOH, carbonates and alkoxides such as sodium methoxide, sodium ethoxide etc.

Acid catalyzed transesterification

Biodiesel can be produced by using acid as a catalyst to catalyze transesterification reaction. The amount of acid catalyst varies from 0.5 mol% to 1 mol%, although some had used up to 3.5 % (Zhang et al., 2003). There are few problems associated with the use of acid catalyst, such the corrosiveness of acid which might deteriorate the equipment, low reaction rate and need for longer residence time (Freedman et al., 1986). Another problem associated with acid catalyzed transesterification is formation of water along with ester which inhibits the transesterification (Canakci., 2007). Guan et al. (2009) reported the transesterification of corn oil with methanol using *p*-toulene sulphonic acid in the presence of dimethyl ether and obtained 100% oil conversion using a methanol/oil ratio of 6:1 at 80˚C for 2 h. Freedman et al. (1986) reported the conversion of soyabean oil was completed in 20 h by using 1% sulphuric acid as a

catalyst with an alcohol to oil molar ratio of 30:1. Acid catalyzed transesterification is preferred where free fatty acid content of oil is more than 2% w/w.

Base catalyzed transesterification

Transesterification by the use of base as a catalyst is also used for biodiesel production. The alkaline catalyst used in concentration range of 0.5 -1% by weight is efficient to have 94-99 % conversion of oils into esters. (Barnwal and Sharma, 2005; Srivastava and Prasad, 2000). It proceeds many times faster than acid catalysed transesterification (Formo, 1954) and is also less corrosive to industrial equipments, therefore used for industrial scale production of biodiesel (Agarwal, 2007). Alkali transesterification should be substantially anhydrous because water causes a partial reaction change to saponification, resulting in formation of soap (Wright et al., 1944). Also for alkali catalyzed transesterification, amount of fatty acids in oil should be as low as possible (Ma et al., 1988) and stiochiometry of the alkali transesterification requires three moles of alcohol per mole of triglyceride to yield 3 moles of fatty esters and one mole of glycerol but higher molar ratios result in greater ester conversion in a shorter time. Although chemical transesterification using a homogenous alkali catalysis process gives high conversion levels of triglycerides to their corresponding alkyl esters in short reaction time, this approach involves major limitations i.e. it is energy intensive and generate undesirable byproducts such as soaps and polymeric pigments, recovery of glycerol as a byproduct is difficult, alkali catalyst has to be removed from the product, alkaline waste water require treatment and free fatty acids and water interfere with the reaction. In addition, the content of free fatty acids present in oils should be lower than 0.5% by weight (Ma et al., 1998a) and water concentration should be limited to 0.1% by weight or less (Hass, 2004). The presence of free fatty acids and water contamination results in soap formation i.e., diverts transesterification reaction towards saponification, making downward recovery and purification very difficult and expensive (Fukuda et al. 2001). Although, alkali catalyzed approach is most widely used process at industrial scale of biodiesel production, some of the major limitations are outlined below.

- Moisture and free fatty acids influence transesterification reaction, for chemical transesterification all materials should be substantially anhydrous, in alkali transesterification presence of water causes soap formation which consume the catalyst and divert the reaction toward saponification and reduces the catalytic efficiency (Fangrui et al., 1999).

- Bradshaw and Meuly (1944) and Feuge and Grose (1949) also stressed the importance of oils being dry and free (<0.5) of fatty acids. Freedman et al. (1984) stated that ester yields were significantly reduced if the reactants didn't meet these requirements.
- Molar ratio is an another factor that influence transesterification reaction, the stoichiometric ratio for transesterification requires 3 moles of alcohol and one mole of glyceride to yield 3 moles of fatty acid ester and one mole of glycerol. The molar ratio is associated with the type of catalyst used (Ma et al., 1999). An acid catalysed reaction needed a 30:1 ratio of BuoH to soybean oil, while an alkali catalyzed reaction require only 6:1 ratio to achieve the same ester yield for a given reaction (Freedman et al., 1986).
- Bradshaw and Meuly (1944) stated that the practical range of molar ratio was from 3.3 to 5.25: 1 methanol: vegetable oil. Generally higher molar ratio results in greater conversion in a shorter time.
- Reaction time varies according to the type of oil and fat used; also transesterification occurs at different temperature, depending upon the oil used. Generally transesterification in most of the cases is carried out between 20-35˚C (Fangrui et al., 1999).

As an alternative to overcome the limitations of chemically catalyzed transesterification reaction, researchers, in recent past, have been exploiting the potential nature of biological catalysts namely pure enzymes and whole cells.

Enzymatic Transesterification

Biocatalytic approach to transesterification reaction involves the use of enzyme either in pure form or as a wet biomass overcome the various drawbacks of chemical transesterification (Fukuda et al., 2001). Amongst various enzymes, lipases are proven to efficiently carry out transesterification. Both extracellular and intracellular lipases are able to effectively catalyze the transesterification of triglycerides in either aqueous or non aqueous systems. Lipase catalyzed transesterification is more suitable for the production of biodiesel from feedstocks containing higher free fatty acids and water, because the free fatty acids are directly esterified into biodiesel. Waste or recycled oils and greases are among those having higher free fatty acid content and therefore can be efficiently converted into biodiesel by using enzymatic transesterification (Hsu et al., 2003; Nelson et al., 1996). In enzymatic transesterification, recovery of byproduct i.e.

glycerol is easy, no acid or alkali catalyst residue, free fatty acids present in waste oils and fats can be completely converted to alkyl esters (Fukuda et al., 2001). Nelson et al. (1996) reported the ability of lipase for transesterification of triglycerides with alcohols to produce alkyl esters. Harding et al. (2007) made a comparative study between alkali and enzyme catalysis for biodiesel production and showed that enzymatic biodiesel production route was environmentally more favourable. There are various advantages of enzymatic transesterification over chemical transesterification as reaction can be carried out at room temperature conditions (30-40˚C), no saponification take place instead free fatty acids in raw material get converted into ethyl esters, there is no interference by water in case of enzymatic transesterification, and this method is relatively inexpensive as compare to chemical transesterification (Crabble et al., 2001; Watanabe et al., 2000). Although enzymatic transesterification has various advantages, the approach also has few drawbacks such as lower lipase activity than chemical catalysis and therefore requiring more reaction time, and deactivation of enzyme by lower alcohols (Parawira, 2009). Among the enzymes lipase is efficient to carry out enzymatic transesterification, this enzyme is produced intracellularly and extracellulary in several microorganisms, such as *Candida antartica, Rhizopus oryzae, Aspergillus species, Pseudomonas species* etc. (Du et al., 2004; Hama et al.,2004; Noureddini et al.,2005; Oda et al.,2005; Shieh et al., 2003).). Lipases are enzymes that catalyze number of reactions such as transesterification, hydrolysis and ester synthesis and therefore have biotechnological importance, filamentous fungi have tremendous potential to produce various types of lipases that are generally used as purified enzyme but extraction of purified lipase is complex process and also involves high cost which makes pure form of lipase costlier.

Whole-cell catalyzed transesterification

One of the major limitations in enzymatic catalysis is the cost of the lipase enzyme, as the cost of pure form of lipase is very high. This problem can be overcome by the use of biomass as a whole cell catalyst (Fukuda et al., 2008). Whole cell biocatalyst can effectively be used for biodiesel production as it is easy to prepare and has relatively higher enzyme recovery potential. Sun et al. (2009) reported that *Rhizopus oryzae* IFO4697 can be used as whole cell catalyst for biodiesel production. Whole cell biocatalysis involves the use of microorganisms such as bacteria, yeast and filamentous fungi that serves as whole cell catalyst based on their ability of

immobilization and the display of functional protein of interest on their cell surface. Amongt all of these whole cell biocatalyst systems, filamentous fungi have arisen as most efficient whole cell biocatalyst for industrial applications. Various fungal and yeast have been used so far for the production of biodiesel i.e. *Rhizopus oryzae, Rhizopus chinensis, S.cerevisiae* etc. (Fukuda et al., 2008). Success of transesterification reaction using whole cell catalyst depends upon various factors i.e. selection of lipase preparation, substrates for biodiesel production, selection of organic solvents used, temperature, water content in transesterification reaction, lipase microenvironment etc. (Antczak, et al., 2009). Stability of whole cell biocatalyst is important for its further application on large scale for biodiesel production. Sun et al. (2009) reported that water content was important for achieving high catalytic activity and good stability of the biocatalyst, the optimum water content found to be 5-15%. Also both particle size and desiccation methods showed no obvious effect on the stability of biocatalyst. For the proper activity of various lipases to carry out transesterification, optimum temperature required ranges between 30˚C-55˚C (Haas et al, 2002). Both intracellular and extracellular lipase can be used as whole cell catalyst (Fukuda et al, 2001). Comparing whole cell catalysis with enzymatic catalysis using pure form of lipase, former provides various advantages over latter with regard to inexpensive method of catalyst preparation and operational stability (Fukuda et al, 2008).

To enhance the efficacy of whole cells as catalysts, immobilization has been extensively carried out for various biocatalyzed processes. Filamentous fungi that were batch cultivated could be spontaneously immobilized on biomass support particles (BSPs) made out of polyurethane foam (Fukuda et al., 2008). These immobilized biomass support particles allowed for a separation of whole cell catalysis from reaction mixture and facilitate their repeated use in bioconversion process i.e. once the culture immobilized then it can be used again to carry out the reaction and after immobilization the efficiency of culture increases as enzyme remain entrapped on biomass support particles (Fukuda et al., 2008). Chain length of alcohol influence immobilization process, immobilized lipase was easily deactivated by lower linear alcohol. Degree of deactivation was found to be inversely proportional to the number of carbon atoms in the linear chain of alcohols (Chen and Wu 2003). Dried *Rhizopus chinensis* cells that were immobilized on biomass support particles could be successfully used to carry out transesterification of different oils and fats (Kyotani et al., 1991). Specific intracellular lipase activity of *Rhizopus chinensis* immobilized on biomass support particles increased by fourfold to

sevenfold compare to that of suspension cells (Nakashima et al., 1990). For biodiesel production, lipases with different immobilization carriers can be used i.e. fiber cloth, porous kaolinitr, hydrotalcite, macroporous resins, silica gel etc. (Iso et al. 2001; Du et al. 2005; Orcaire et al. 2006; Yagiz et al. 2007). Among the various techniques for immobilization, a technique using biomass support particles (Atkinson et al., 1979) has various advantages over other methods in terms of industrial application i.e. no chemical additives are required, no need for preproduction of cells, the particles are reusable, particles are durable against mechanical shear, bioreactor scale up is easy, economical. Comparing the lipase activity of immobilized culture and suspension, it was observed on *Rhizopus oryzae* cells that for suspension intracellular activity decreases with increasing cultivation time while extracellular activity remained relatively high throughout. Immobilized systems generally show higher intracellular lipase activity and lower extracellular activity with the former being approximately half that of suspension cells (Hama et al., 2006). Also in *Rhizopus chinensis* cells, it was observed that cell aggregation followed by immobilization within biomass support particles is a trigger for intracellular lipase production (Nakashima et al., 1990), therefore for an economical process wet biomass of culture are immobilized on biomass support particles which are used as whole cell biocatalyst (Hama et al., 2006). It is also reported that *Candida Antarctica* lipase (Novozym 435) immobilized on acrylic resin was the most effective lipase among any of the lipases tested for methanolysis (Shimada et al., 1996).

Lacunae

Enzymatic and whole cell catalysis, time consuming processes in compare to acid and alkali catalysis. These processes require around 48 to 72 hours for significant transesterification. To best of our knowledge there are no reports on time dependent kinetics of whole cell catalysis i.e. how reaction proceeds in 48-72 hours. It is also reported that transesterification is reversible reaction. There are very limited reports in case of whole cell catalyst to prove the reversibility of reaction during transesterification.

Objective

The present study aimed at time dependent kinetics of hydrolysis and esterification reaction by using selected fungal strains.

Materials and Methods

Materials

Karanj oil was procured from Medors Biotech Ltd. and cottonseed oil was obtained from retail market. Culture media *viz*., mycological peptone, Bushnall Hass broth (BHB) and potato dextrose broth (PDB) was purchased from HiMedia, India. Other chemicals such as ethanol, hexane, ethyl acetate, silica gel (G) for TLC, diammonium hydrogen ortho-phosphate ($(NH_4)_2HPO_4$), potassium hydroxide (KOH), ethyl alcohol, diethyl ether and phenolphthalein were purchased from SD Fine-Chem limited, India. All the reagents used were analytical grade.

Cultivation of test organisms

Three test organisms *viz*., *Aspergillus sp.*, (RBD-01), AKS-08 (γ irradiated strain of RBD-01) and *Rhizopus sp.* were used in the present study. RBD-01 was obtained from bio-contaminated butter and was reported by our group to exhibit significant oil tolerance and transesterification potential (Aulakh and Prakash, 2010, Prakash and Aulakh, 2011). AKS-08 was generated through irradiation of RBD-01 using 300 Gy γ irradiation. *Rhizopus sp.*, known to have transesterification potential, was used as a test species for comparative studies.

The spores of RBD-01, AKS-08 and *Rhizopus sp.* was inoculated aseptically in 500 ml Erlenmeyer flask containing 200 ml of sterile PDB separately and incubated at 30°C, 120 rpm for 72 h. The active culture obtained from PDB was further used for experimentation. The minimal media BHB containing $MgSO_4$ (0.2 g/l), $CaCl_2$ (0.02 g/l), KH_2PO_4 (1.0 g/l), K_2HPO_4 (1.0 g/l) and $FeCl_3$ (0.05 g/l) was supplemented with mycological peptone (0.5% w/v), $(NH_4)_2HPO_4$ (0.5% w/v) and virgin cottonseed oil (for *Rhizopus sp.*) or karanj oil (for RBD-01 and AKS-08) (30% v/v), was used as growth medium. Mycological peptone and $(NH_4)_2HPO_4$ were used to supplement nitrogen and cottonseed or karanj oil was used as main carbon source for fungal growth.

Hydrolysis of oils to fatty acids

In case of RBD01, active culture was inoculated in growth medium supplemented with karanj oil (70 % oil and 30% media) and incubated at 30˚C at 120 rpm for 120 h for carrying out hydrolytic reaction. Through this period of incubation, samples were collected at periodic intervals of 12 h and subjected to thin layer chromatography (TLC) with silica gel G as stationary phase and hexane:ethyl acetate (9:1) as a mobile phase. The chromatogram was developed in the iodine chamber (Samukawa *et al*., 2000). The progress of the reaction was checked regularly by thin layer chromatography and further analysed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) (400 MHz; JEOL JNM-ECS 400). Deuterated chloroform ($CDCl_3$) was used as solvent and tetra methyl silane (TMS) as internal standard. ^{1}H NMR spectra were recorded with pulse duration of 2.18 sec with a relaxation delay of 4 sec and 16 scans. Following derivation (Satyarthi *et al.*, 2009) was used to determine the FFA yield through hydrolysis.

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

Wherein α-CH_2 is α-acyl methylenic hydrogens in oil and FFA at 2.20-2.40 ppm.

A similar protocol was followed for AKS-08 using karanj oil and medium in ratio of 70:30 where as in case of *Rhizopus sp.* cottonseed oil was used for supplementation. The cultures were incubated in similar conditions mentioned above. For quantification of fatty acids generated through hydrolysis, trimetric method was followed using protocol outlined by AOCS Ca5a-40 (AOCS, 1989) in the case AKS-08 and ^{1}H NMR in case of *Rhizopus* sp.

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

Transesterification

Subsequent to the hydrolytic reaction carried out for 120 h, ethanol was added at 1:4 molar ratio of oil to alcohol through (a) single step addition and (b) in two steps at interval of 12h. The varying additions were carried out to understand the effect of time interval of alcohol addition on extent of transesterification. Followed by alcohol addition, samples were collected at an interval of 1.5h upto 31h. Samples were washed thrice with hexane to remove excess of oil from ester. Hexane was evaporated out on water bath. Progress of the reaction was monitored at regular intervals by TLC followed by quantification by ^{1}H NMR. The ester was quantified using ^{1}H NMR (400 MHz; Jeol JNM-ECS 400). $CDCl_3$ (deuterated chloroform) was used as solvent and tetra methyl silane as internal standard. ^{1}H NMR spectra were recorded with pulse duration of 2.18 sec with a relaxation delay of 4 sec and 16 scans.

Ethyl ester quantification by ^{1}H NMR spectroscopy is complex, due to a superimposition of the glyceryl methylenic hydrogens in oil and the $-OCH_2$ from ethyl ester where partial conversion was obtained (Ghesti *et al.*,2007). However, where the peak due to glyceryl methylenic hydrogens in oil at 4.25-4.35 ppm completely disappeared, the process of oil conversion was considered to be nearly complete. The product formed from the transesterification reaction was quantified by using the equation proposed by Silva (2005).

$$\%C_{EE} = 100 \left[\left(I_{TAG + EE} - I_{TAG}\right) / I_{\alpha CH2} \right]$$

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 hydrogen superimposed at 4.10-4.20 ppm; and

(iii) $I\alpha_{CH2}$ - integration of α-acyl methylenic hydrogens in oil and ethyl esters at 2.20-2.40 ppm.

Hydrolysis and transesterification with immobilized whole cell as catalyst

The test strains (RBD01, AKS-08 and *Rhizopus* sp.) were inoculated aseptically in 250ml Erlenmeyer flask containing 100 ml of sterile PDB and incubated at 30°C, 120 rpm for 3 days. To immobilize the active culture, polyurethane foam was used. Culture strains (RBD01, AKS-08, *Rhizopus sp.*) were inoculated in sterile media and oil (70:30) having pieces of polyurethane foam (PUF) and incubated at 30˚C at 120 rpm for 120h separately. After 120h of incubation, the culture(s), immobilized on pieces of polyurethane foam, was washed with hexane and distilled water, dried with blotting paper and kept overnight at 40˚C. The immobilized culture was then inoculated in the minimal media using composition mentioned earlier (supplemented with 70% oil) and incubated at 30˚C at120 rpm for 3 days. For RBD01 and AKS-08, karanj oil was used for supplementation where as in case of *Rhizopus* sp. cottonseed oil was used. Samples were collected each at interval of 12 h i.e. up to 72h to determine the extent of hydrolysis by TLC. Alcohol was added into 1:4 molar ratio after 72h incubation. Samples of alcoholysis were collected each at regular intervals of 1.5h up to 48 h. The product so formed was separated and determined using ^{1}H NMR. Quantification of alkyl esters were carried out using the derivation mentioned earlier.

The present study was aimed at estimating the time dependent modulations in hydrolysis and esterification of vegetable oils through whole-cell biocatalysis by different fungal strains organisms *viz*., *Aspergillus sp.*, (RBD-01), AKS-08 (γ irradiated strain of RBD-01) and *Rhizopus sp.* The *Aspergillus sp*. (RBD-01 and (AKS-08) were tried both in cell suspension and immobilized form.

Hydrolysis and transesterification reaction catalyzed by RBD-01

Hydrolytic reaction carried out with RBD-01 (*Aspergillus species*) showed gradual increase in FFA from 29% in 12 h to 89.3% in 72 h during hydrolysis (**Figure 1)**.

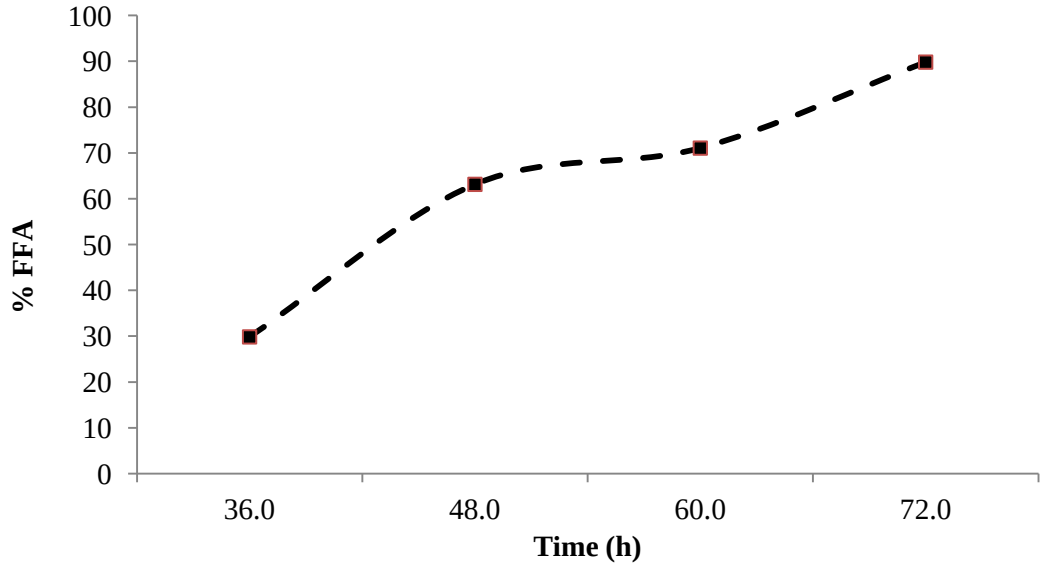


Figure 1. Hydrolysis of oil to fatty acids by RBD-01

One time addition of alcohol (1:4 molar ratio) resulted into the formation of alkyl esters with increase in ester content except at few points which is expected due to reversibility of reaction (**Figure 2)**. FFA decreased from 89.3% to 40% within 1.5 h of alcohol addition and ester produced was around 33.3 %. The yield of esters further increased to 49.5% in 7.5 h. Evidence of the reversibility of the reaction was evident at 10.5h where in there was marginal decrease in ester content i.e. 48.1% in 10.5 hand corresponding increase in FFA to 47%. The ester formation regained by 25 h with increase upto 63.2% and FFA content decreasing upto 22%. The reaction

which was continued upto 31 h resulted in ester yield of 55.3% with corresponding decrease in FFA to 19%.

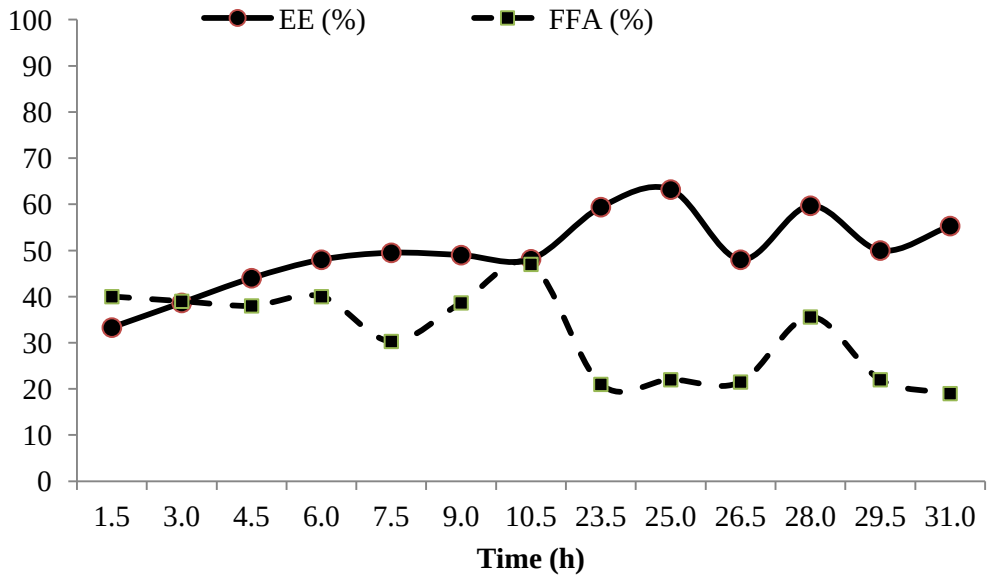


Figure 2. Modulations in hydrolysis vis-à-vis esterification on addition of alcohol with RBD-01 as catalyst

Hydrolysis and transesterification reaction catalyzed by AKS-08

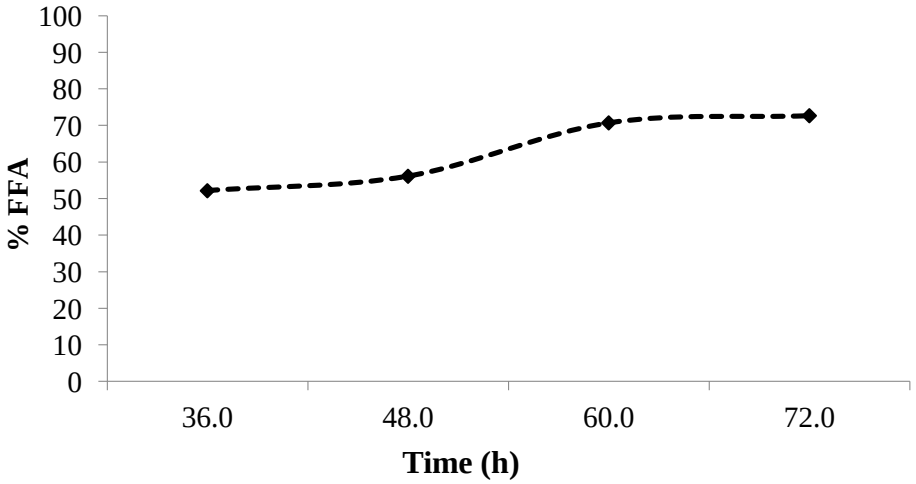


Figure 3. Hydrolysis of oil to fatty acids by AKS-08 as whole cell catalyst

Transesterification reaction carried out with AKS-08 showed gradual increase in FFA content from 37.8% in 12 h to 72.7% in 72 h during hydrolysis (**Figure 3)**. Addition of alcohol resulted

in the formation of alkyl esters with increase in ester content (**Figure 4**). The FFA content decreased from 72.7% to 51% within 1.5 h of alcohol addition and ester produced was around 27% which increased gradually upto 71% in 25h, there is slight decrease in ester content i.e. 65% in 28 h, it shows reversibility. Further, the ester formation increased upto 74% within 46h. The reaction, continued upto 31 h resulted in an ester yield 72% with decrease in FFA to 26%.

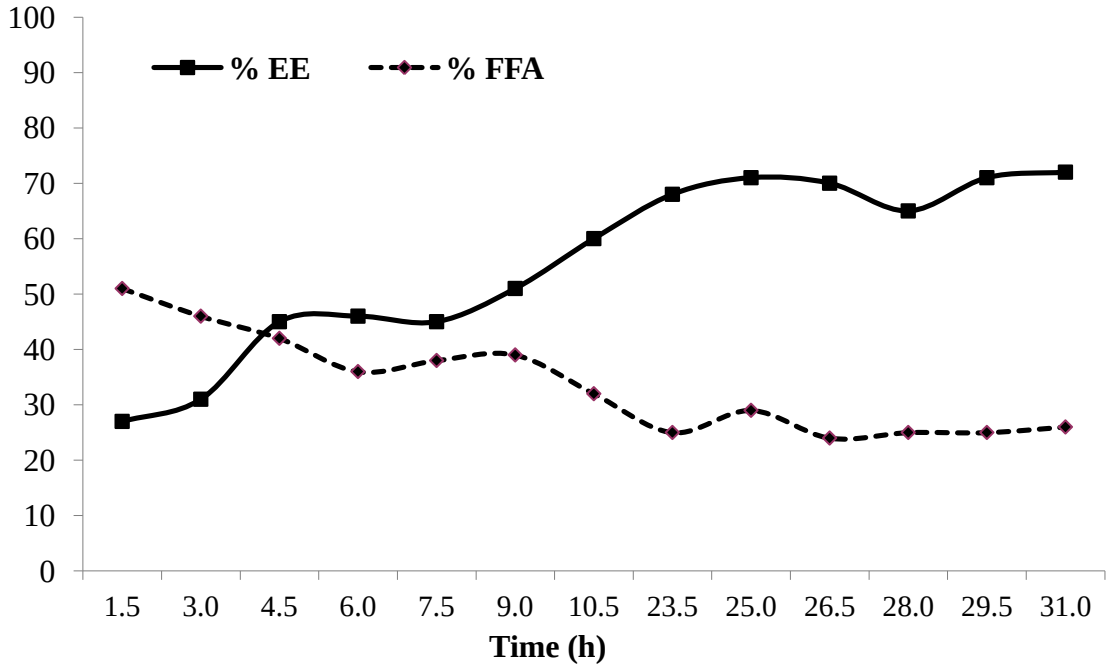


Figure 4. Modulations in hydrolysis vis-à-vis esterification on addition of alcohol with AKS-08 as catalyst

Comparative study on whole cell catalyzed hydrolysis and esterification of oil

RBD-01 and AKS-08, both being *Aspergillus* sp.; the comparison on the hydrolytic potential of both species RBD-01 and AKS-08 showed no statitistically significant result in terms of the FFA generation wherein it was 90% in case of RBD-01 and 73% in case of AKS-08 (**Figures 1** and **3)**. With reference to the esterification, the yield of ethyl ester was significantly lower in RBD-01 (55.3%) when compared to AKS-08 (72%) in 31 h of esterification reaction. **Figure 5** depicts the variations in the yield of ethyl ester between AKS-08 and RBD-01. FFA during alcoholysis in case of RBD-01 declines from 40% to 19% in 31 h and in case of AKS-08 from 51% to 26% within the same time period. **Figure 6** shows comparison between RBD-01 and AKS-08 for remaining FFA during alcoholysis.

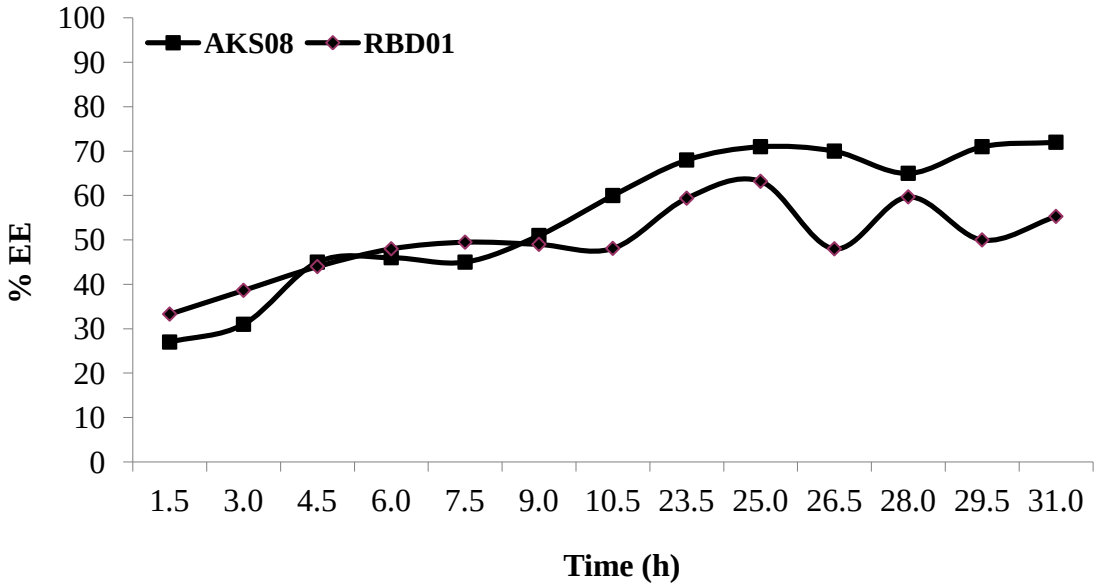


Figure 5. Comparison in ester yield with RBD-01 and AKS-08 as whole cell catalysts

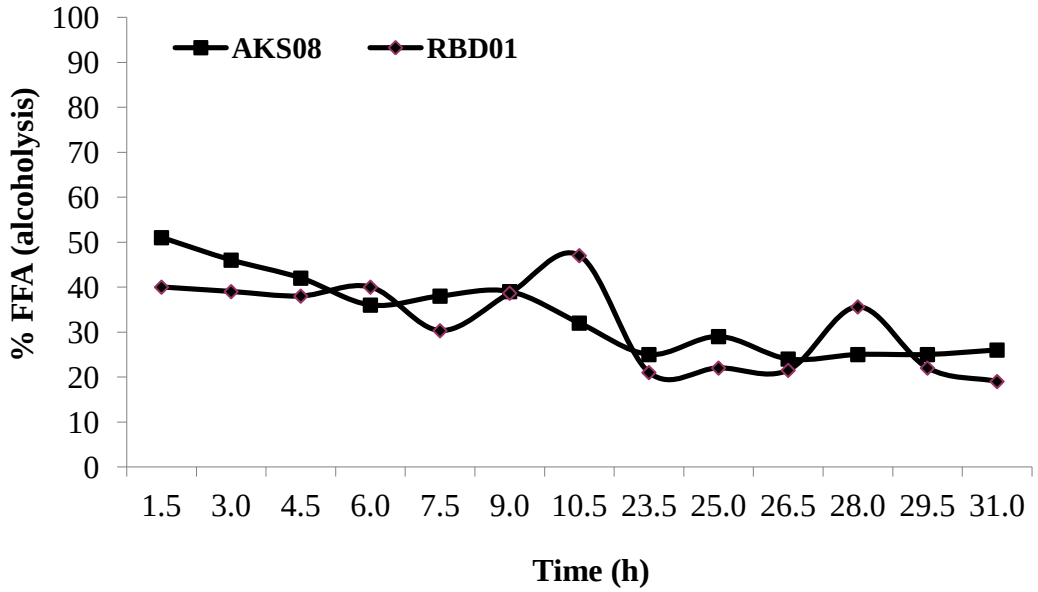


Figure 6. Comparison in FFA content after addition of alcohol with RBD-01 and AKS-08 as whole cell catalysts

Effect of variations in time interval of alcohol addition (one time vs periodic intervals)

In the transesterification reaction carried out using RBD-01 strain, it was observed that one time addition of alcohol (30ml) resulted in ester yield of upto 55.3% in 31 h while ethyl ester yield was 56.2% in 31 h when adding alcohol in two parts (15ml/12h) at an periodic interval of 12 h indicating no significant effect of single supplementation of higher concentration alcohol (**Figure 7**).

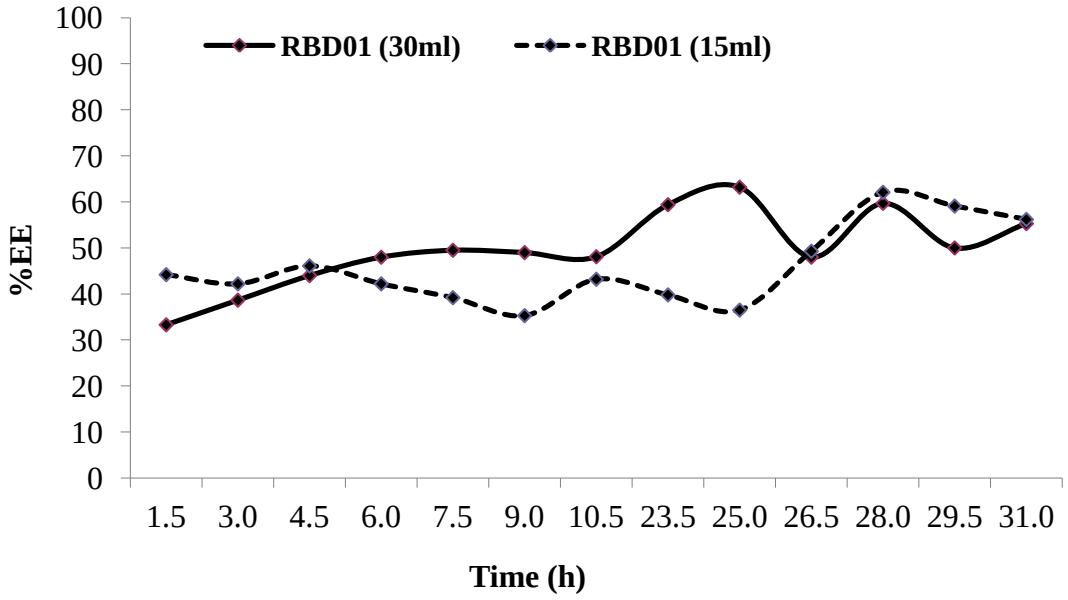


Figure 8. Variations in ethyl ester yield with one time addition of alcohol vis-à-vis addition at periodic intervals of 12 h

Effect of immobilization on hydrolysis and transesterification of oils with RBD-01 and AKS-08 as whole-cell catalysts

Both (RBD-01 and AKS-08) were immobilized on polyurethane foam pieces to examine the effect of immobilization on hydrolysis and transesterification reactions. **Figure 9** depicts the comparative observations on hydrolysis using immobilization of RBD-01 and AKS-08. It was observed that FFA produced during hydrolysis in 12 h was 34% in case of AKS-08 while in case of RBD-01 it was 30%. The FFA, further increased to 91% in 72 h in case of AKS-08 while it

increased only to 49% in 72 h in case of RBD-01 indicating that AKS-08 gave good result of hydrolysis over RBD-01.

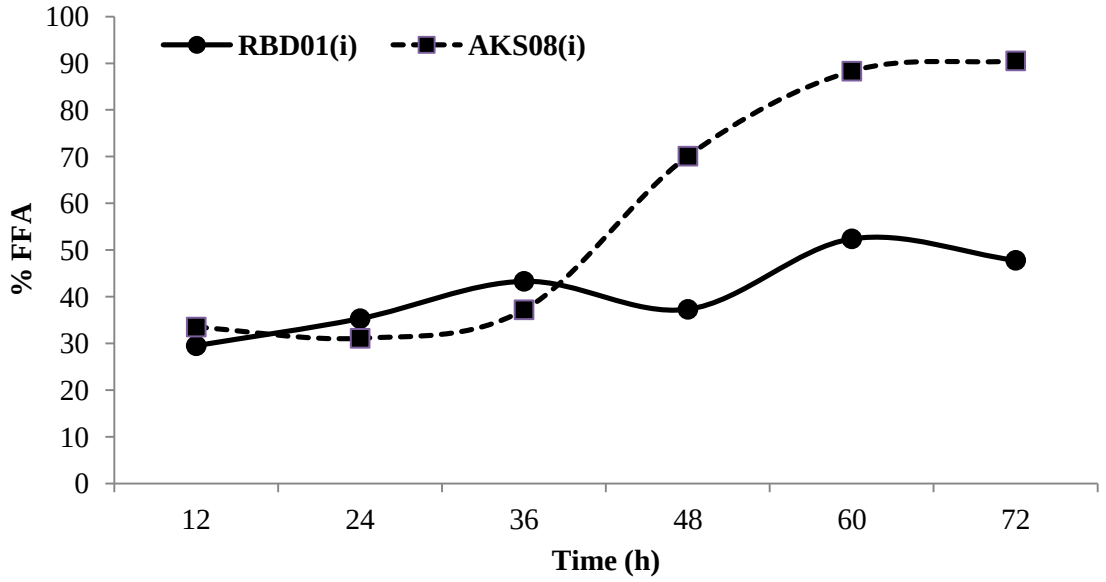


Figure 9. Variations in generations of FFA during hydrolytic activity of RBD-01 and AKS-08

During esterification, in case of RBD-01 (**Figure 10**), 44% FFA was present at 1.5 h of alcohol addition which further decreases upto 27% within 26.5 h followed by increase upto 48% within 28 h of alcoholysis, caused due to reversibility of the reaction. The FFA content further decreased to 25% within 31 h of alcohol addition. Correspondingly, ester produced during 1.5 h was 19% which further increased to 20% in 10.5 h and later on increased to 34% within 31 h of alcohol addition. There was no specific or definite trend observed during the transesterification reaction in ester yield or FFA content along across 31 h, in the case RBD-01.

In transesterification reaction with AKS-08 (**Figure 11**), 53% FFA were present produced within 1.5 h of alcohol addition which further decreased to 38% in 23.5 h followed by increase upto 43% in 26.5 h. The FFA further decreased to 26% in 31 h of alcohol addition. Ester produced during 1.5h was 10% which further increased to 17% in 10.5 h and later on 38% in 31 hd of alcohol addition. In the case if AKS-08, the increase in ester yield corresponding to the decrease in FFA content was clearly evident.

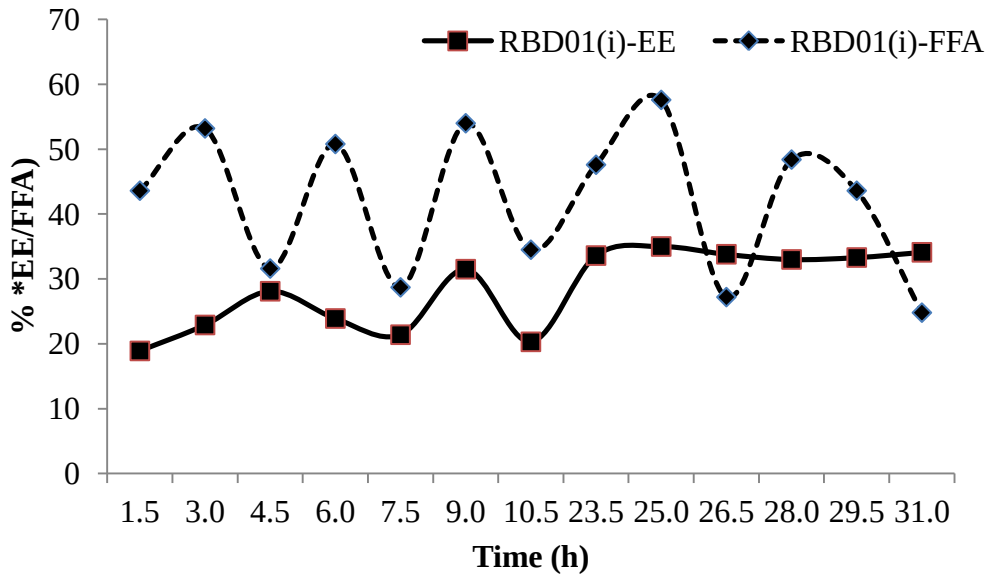


Figure 10. Modulations in hydrolysis vis-à-vis esterification on addition of alcohol with immobilized RBD-01 as catalyst

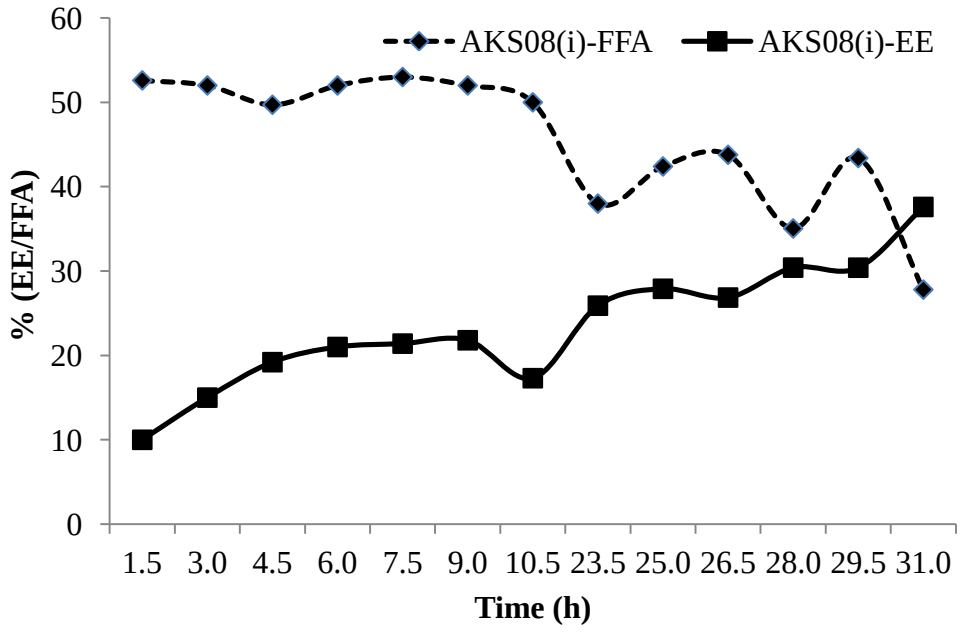


Figure 11. Modulations in hydrolysis vis-à-vis esterification on addition of alcohol with immobilized AKS-08 as catalyst

The hydrolysis reaction by *Rhizopus*, using cotton-seed oil as carbon source, resulted in FFA yield of 27% during 12 h of hydrolysis which further increases to75% FFA in 72 h of hydrolysis. However, But corresponding changes in FFA and ester yield during transesterification were insignificant in the case of cell suspension as well as in immobilized form. The observations clearly indicate that the potential of the fungal strains to hydrolyze oil as well as transesterify follow independent mechanisms and necessarily are not exhibited by the same strain.

Various microorganisms such as bacteria, yeast and fungi can be used as a whole cell biocatalyst for biodiesel production. Filamentous fungi are one of the most prominent one for biodiesel production. (Ban et al. 2001; Fujita et al. 2002 ; Narita et al. 2006). In the present study, hydrolysis and transesterification reactions were carried out using three fungal strains-RBD-01, AKS-08, *Rhizopus* sp. Alkyl ester produced was 20.4% in 31 h of alcohol addition by using *Rhizopus* sp. when cotton-seed oil used as supplemented carbon source. Wei et al. (2007) reported 72% ethyl ester generation by using *Rhizopus oryzae* in non-aqueous medium containing t-butanol. Among ethanol and butanol, butanol is less toxic to culture and hence gives better conversion but ethanol is more economical therefore commonly used to carry out transesterification reaction. It was observed that transesterification reaction shows reversibility, as noted by other researchers. Scwab et al. (1987) and Freedman et al. (1986) also reported that transesterification consists of number of consecutive and reversible reactions. The triglyceride is converted stepwise to diglyceride, monoglyceride and finally glycerol and a mole of ester is liberated at each step, although the reactions are reversible, equilibrium lies towards the production of FFA and glycerol. Nelson et al. (1996) reported the ability of lipase for transesterification of triglycerides with short chain alcohols to produce alkyl esters. Quin et al. (2008) reported the use of whole cells of *Rhizopus chinensis* for biodiesel production and obtain yield of 86% methyl ester.

Non-edible oil like karanj oil can be used for the production of alkyl esters as demonstrated in the present study in case of RBD-01 as well as AKS-08 as catalysts. Karmee and Chadha (2005) reported biodiesel/alkyl ester production from karanj oil by transesterification of crude oil with methanol and KOH as catalyst. A maximum conversion of 92% was reported using 1:10 molar ratio of oil to methanol at 60°C. Tiwari et al. (2007) reported the production of biodiesel

from jatropha oil by using sulphuric acid as a catalyst. The use of whole cell catalyst is preferred over acid and alkali catalyst. Prakash and Aulakh (2011) reported the use of non-edible oil karanj, jatropha and used cottonseed to produce alkyl ester. They reported near complete conversion with 70% oil media after 48 h of reaction and with stepwise addition of ethanol at an interval of 12 h. It is also reported that one time addition of alcohol decreased the yield of alkyl ester, to the extent of 44.2%, 52.6% and 78.3% from karanj, jatropha and used cottonseed oil.

There have been very limited studies on transesterification of oils with immobilized biomass using ethanol as acyl acceptor. All the studies examined till date, dominantly involved use of methanol for such reactions. Immobilized culture strains also have potential to carry out transesterification reaction. Immobilization facilitates the repeated use of potential microbial cultures along with extended viability and limited loss in activity. Shimada et al. (1999) reported biodiesel production by immobilization of *Candida antartica* on acrylin resin. Chen and Lin (2010) reported transesterification of soybean oil using *R. oryzae* bed immobilized on non-woven fabric as matrix They predicted the yield value of 72.6% under the optimum conditions and reaction time of 23 h. Li et al. (2007) reported *R. oryzae* IFO4697 whole cell immobilized within BSPs in tert-butanol as solvent resulted in 72% yield of butyl ester from soybean oil under optimal conditions. Tert-butanol as a solvent have a beneficial effect that it could eliminate the negative effects of excess methanol and by-product glycerol which were mainly responsible for the poor activity and stability of the lipase in alkyl ester (biodiesel) production (Li et al. 2006; 2007). Ban et al. (2001) reported methanolysis of soybean oil by using *Rhizopus oryzae* IFO4697 (with a 1,3-positional specificity lipase) immobilized within biomass support particles (BSPs).

Thus, in conclusion, the salient findings of the study are as follows:

- **The biocatalyzed transesterification reaction is reversible in nature over period of time;**
- **One time addition of alcohol for facilitating transesterification reaction inhibits biocatalytic activity especially in immobilized state; and**
- **The potential of the fungal strains to hydrolyze oil as well as transesterify follow independent mechanisms.**

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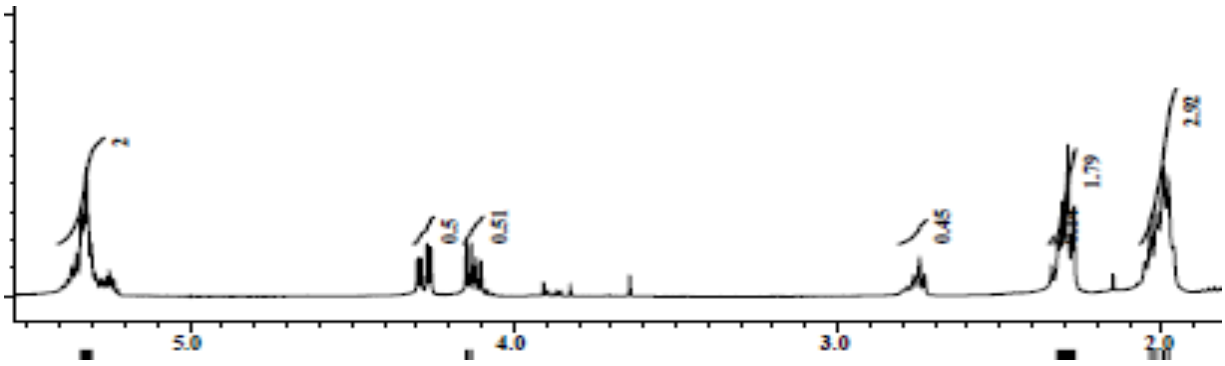
NMR representing transesterification reaction using RBD-01.



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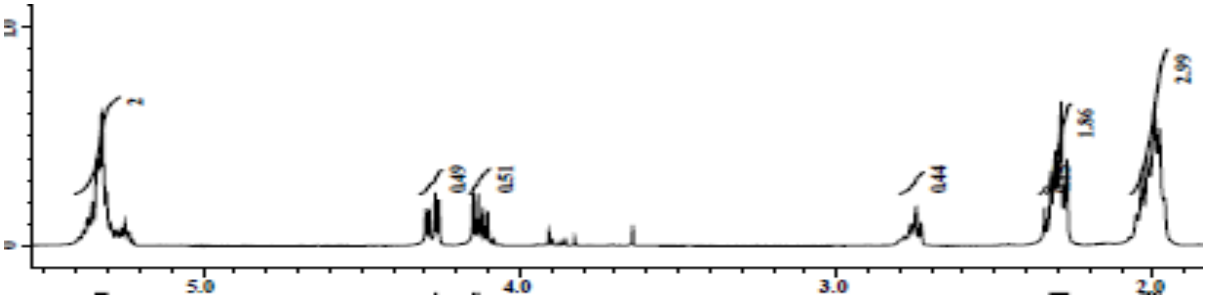

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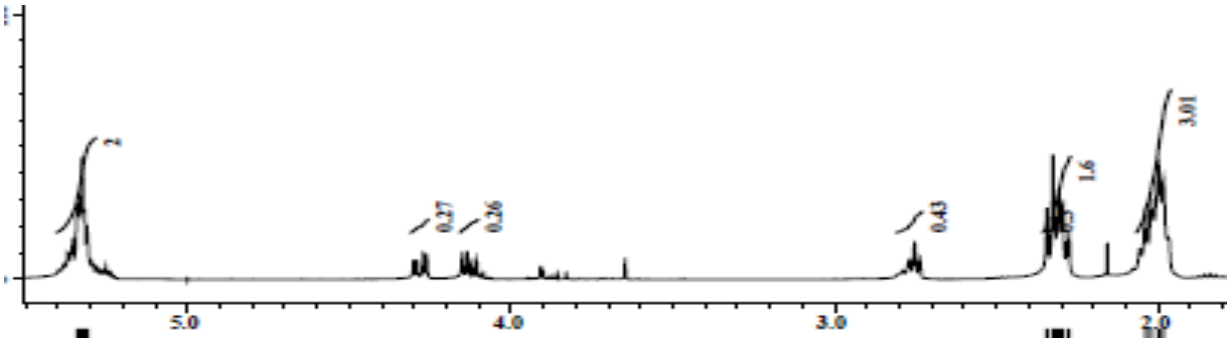

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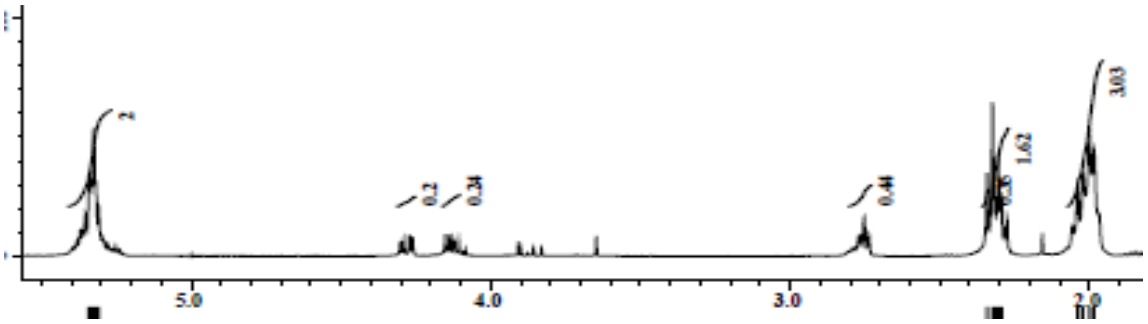

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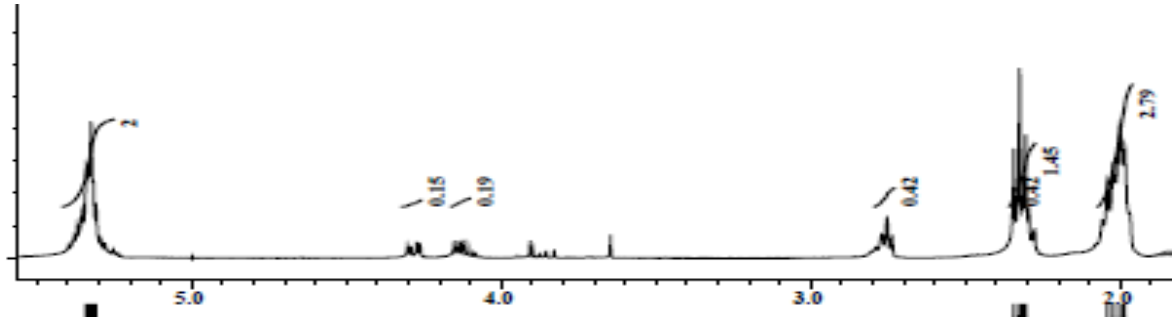

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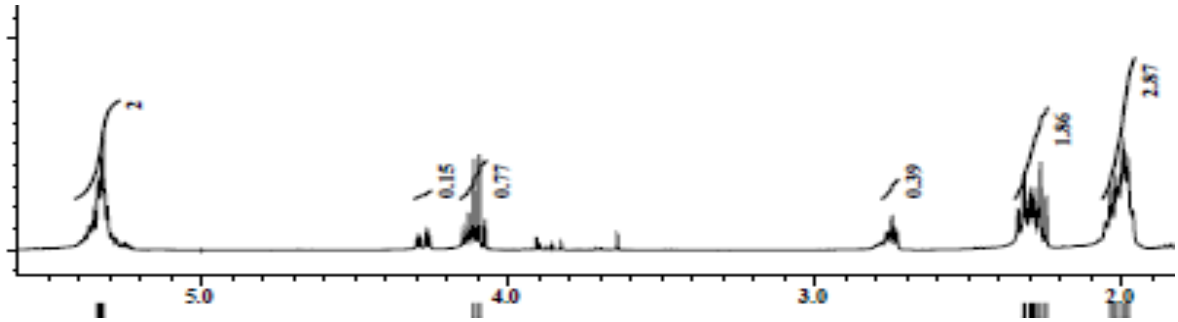

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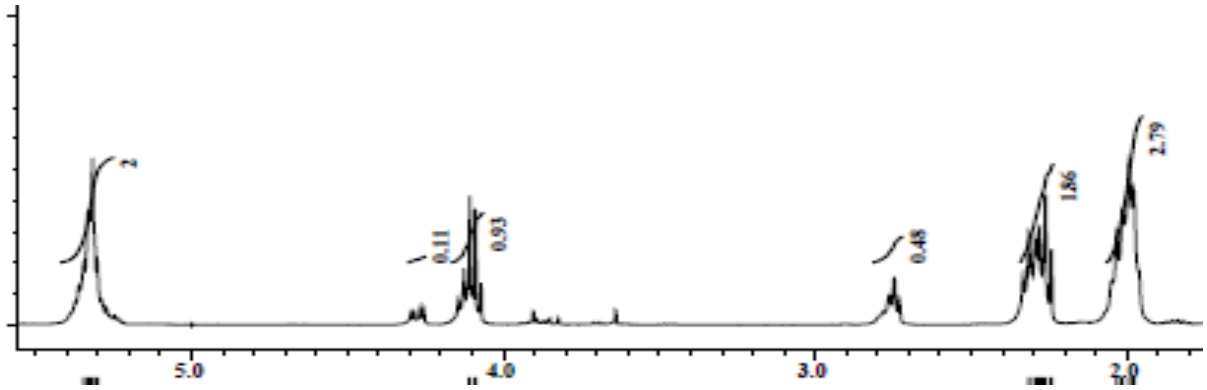

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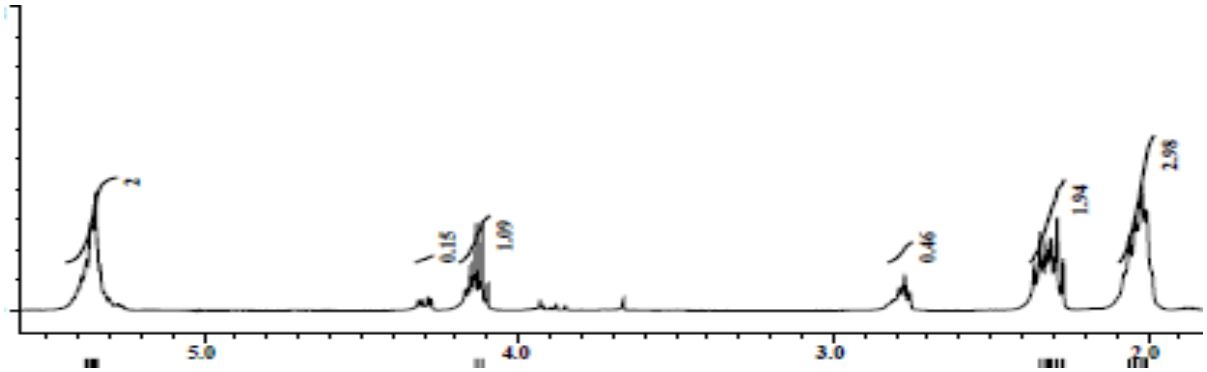

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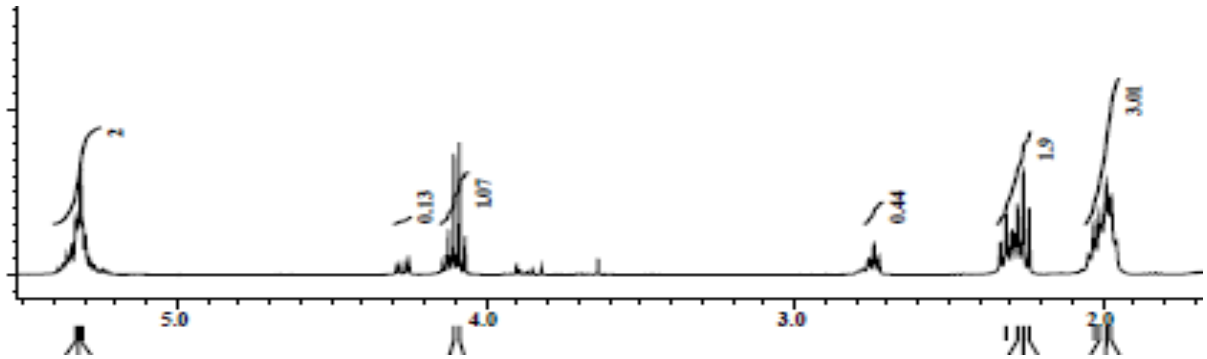

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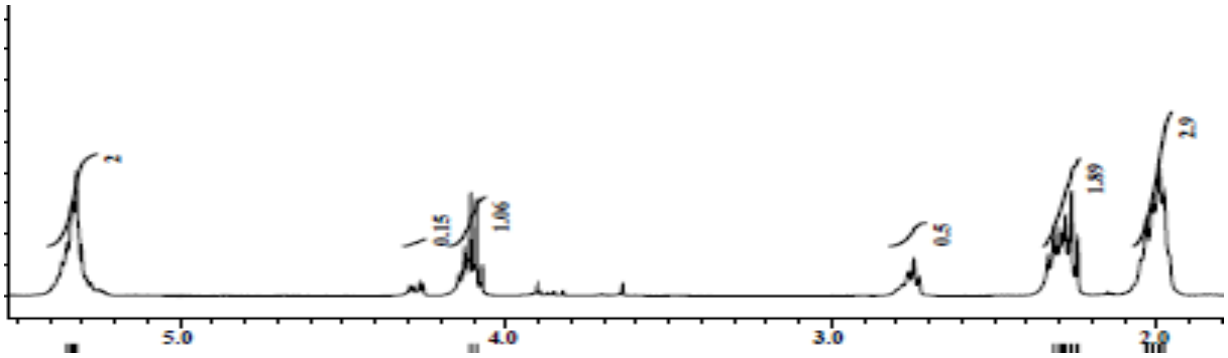

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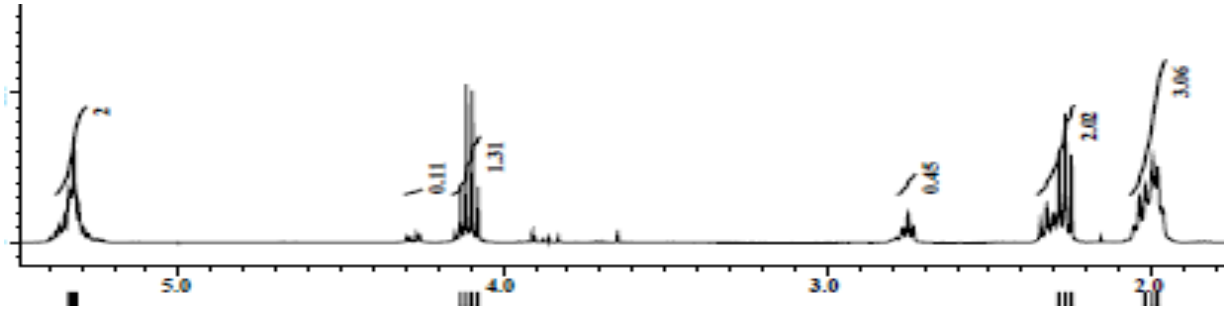

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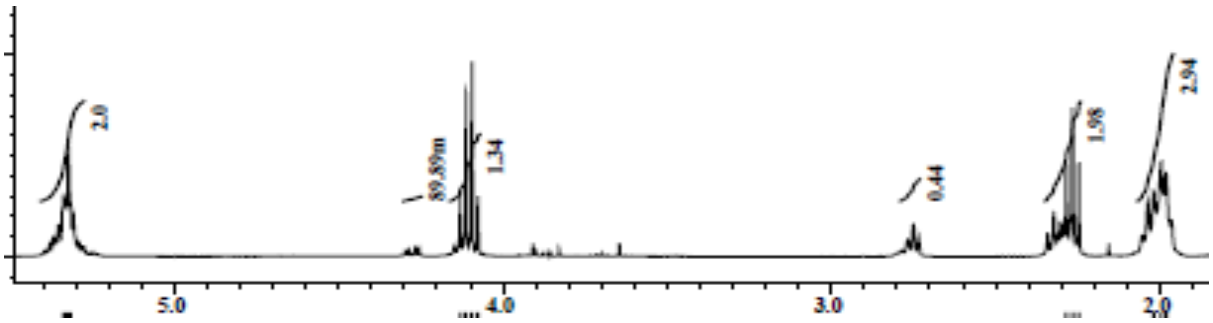

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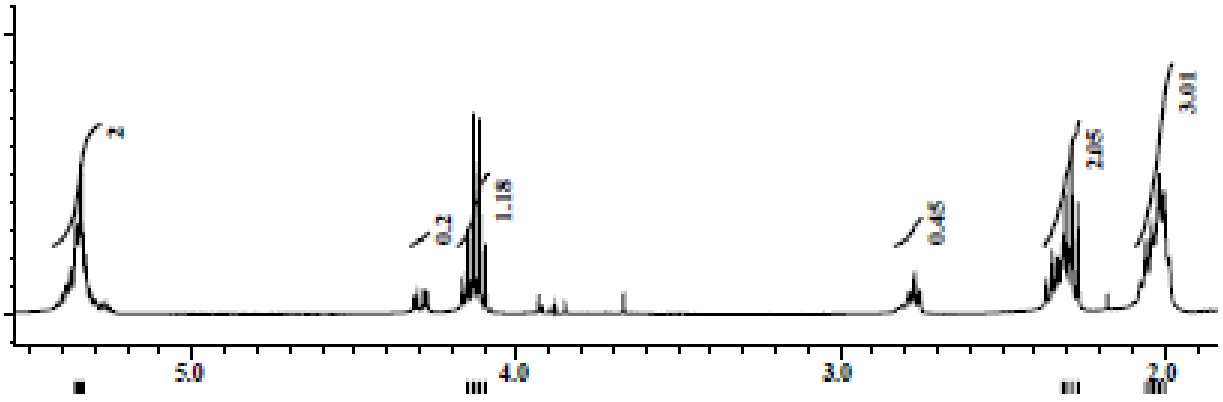

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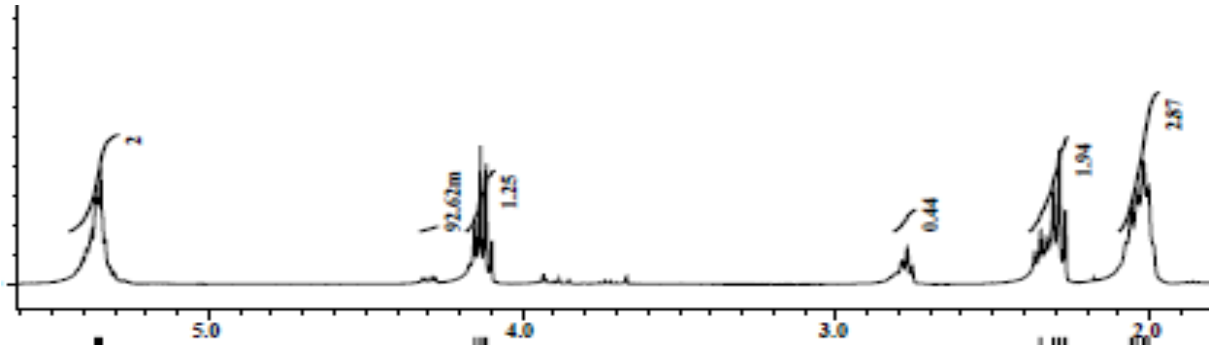

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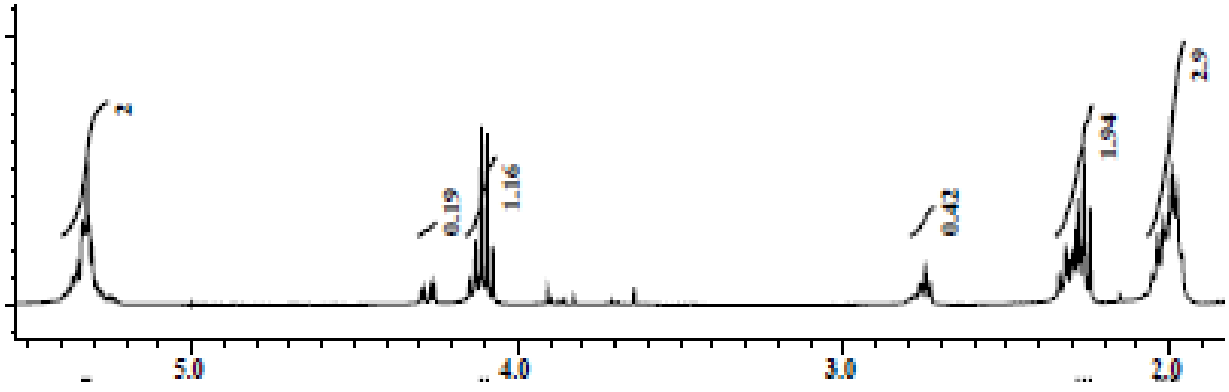

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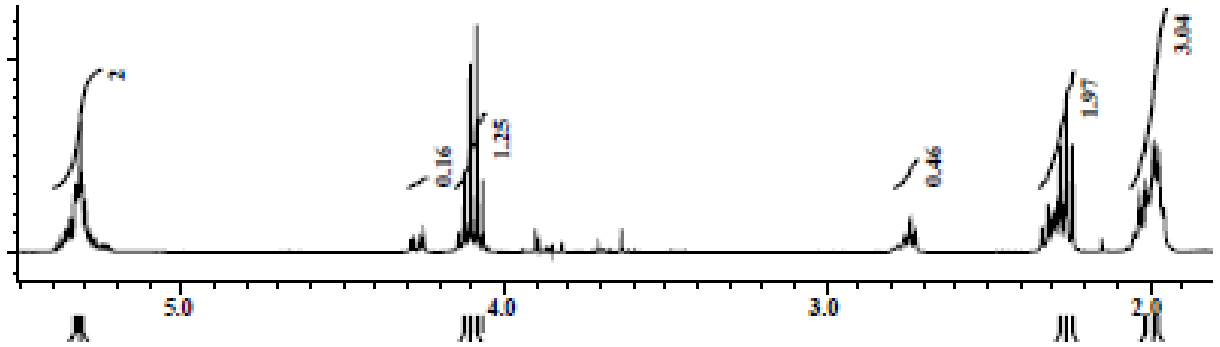

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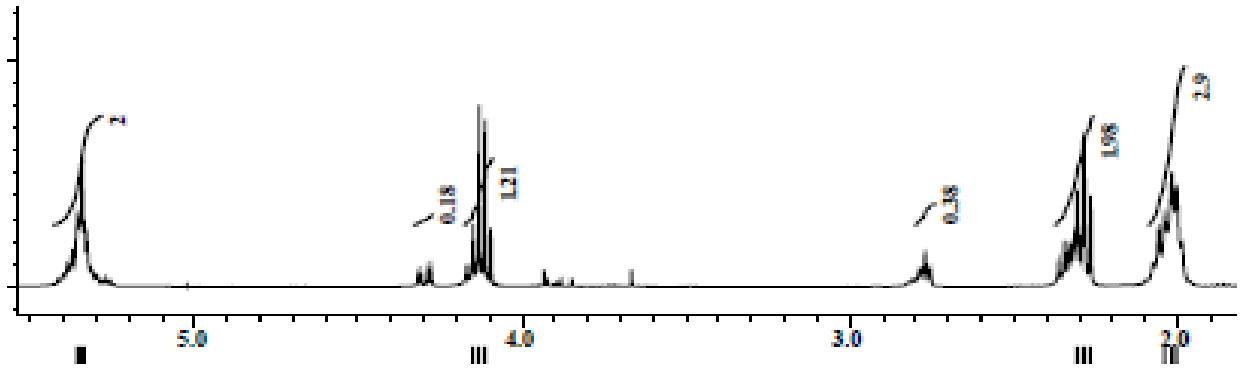

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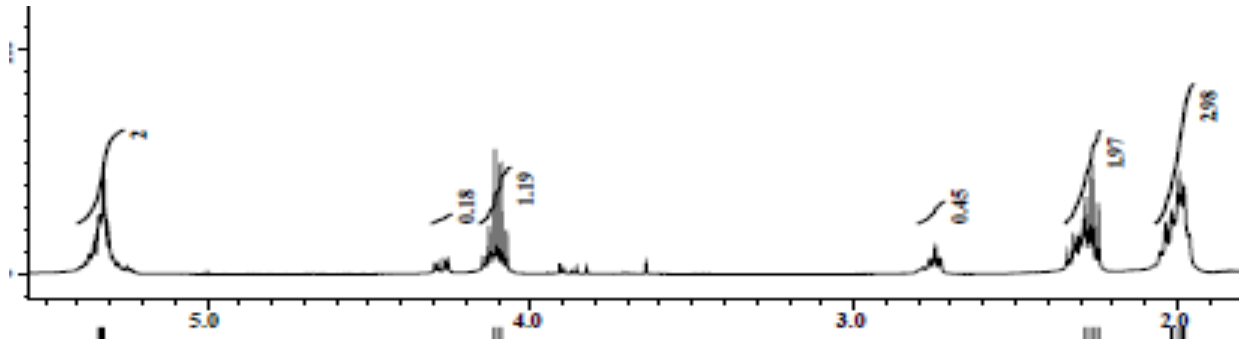

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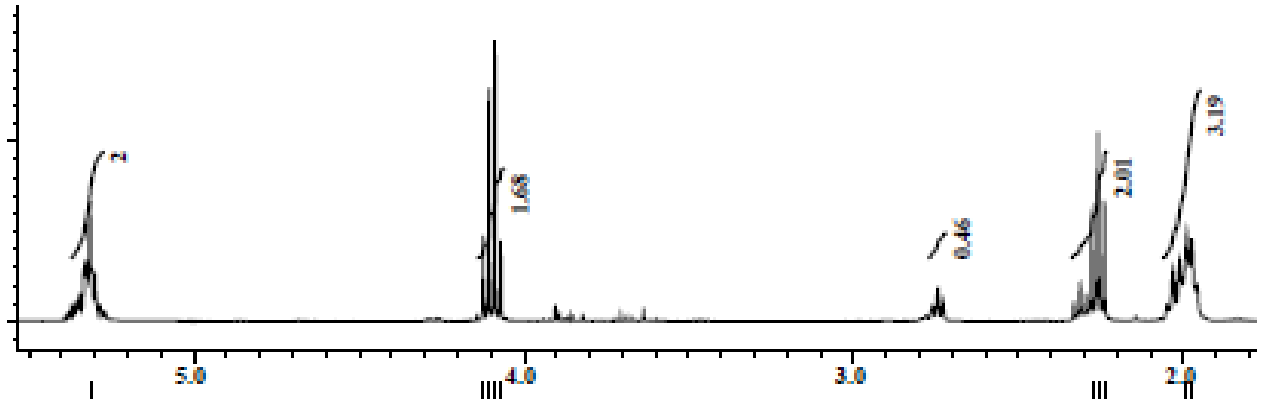

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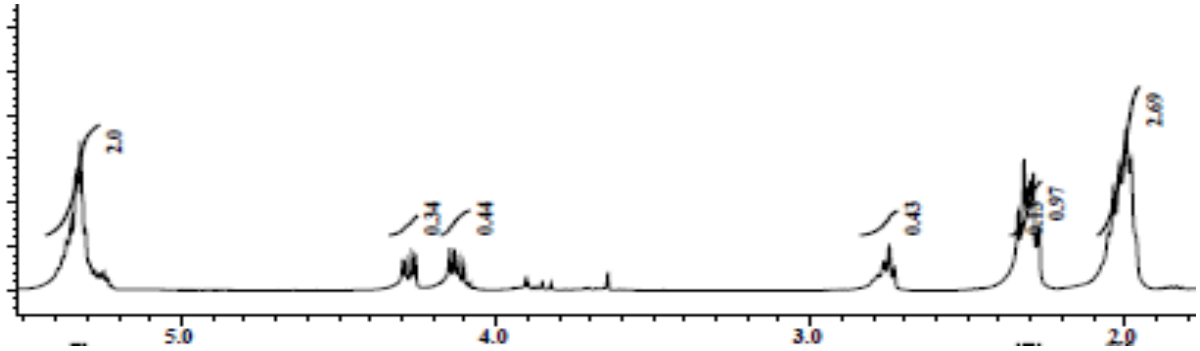
NMR representing transesterification using AKS-08



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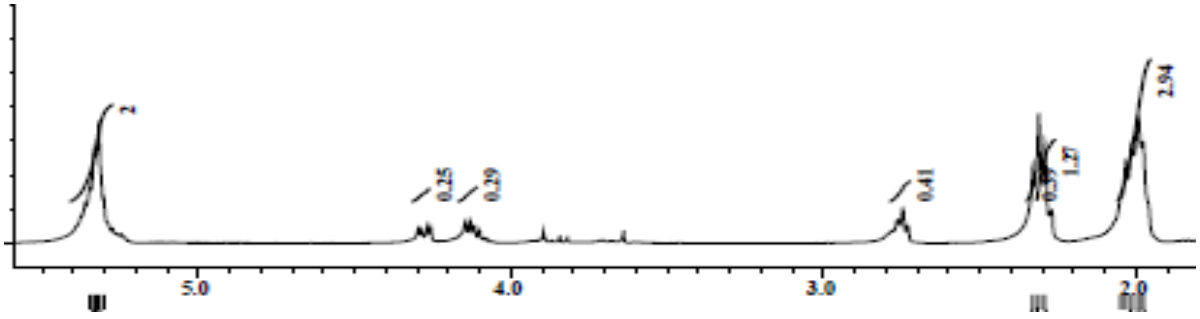

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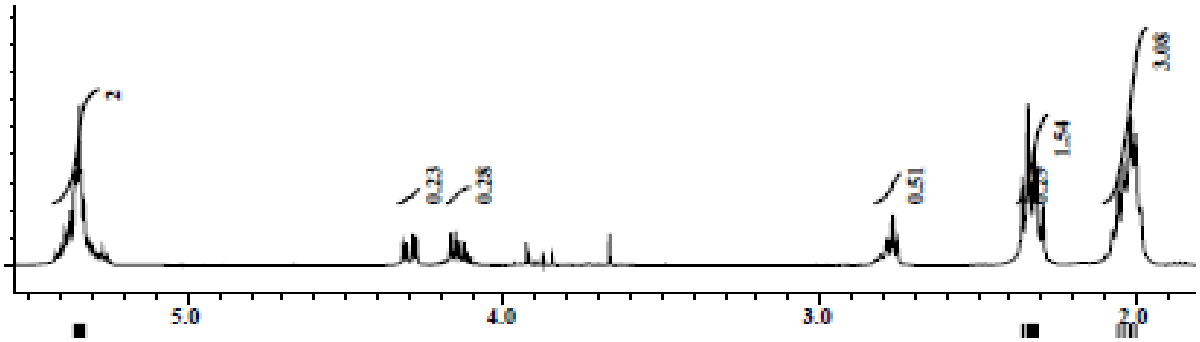

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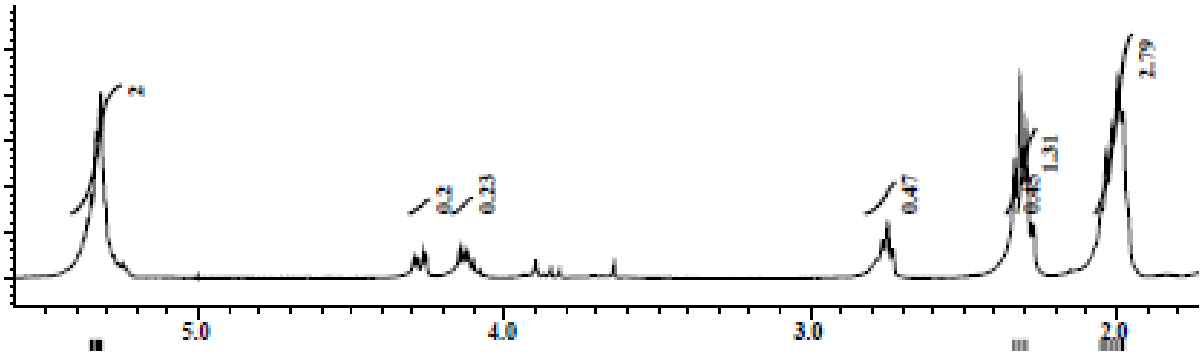

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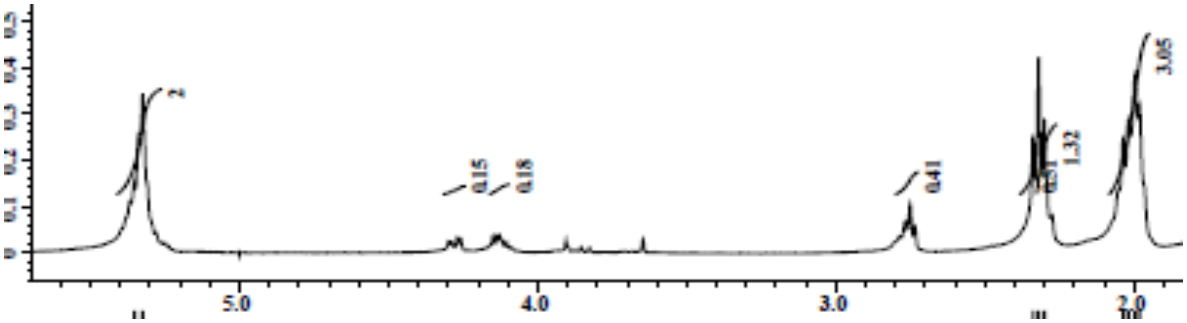

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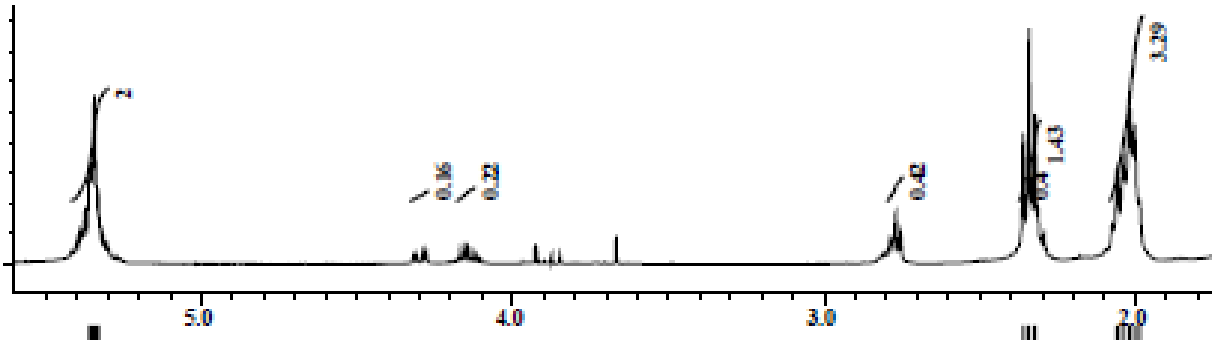

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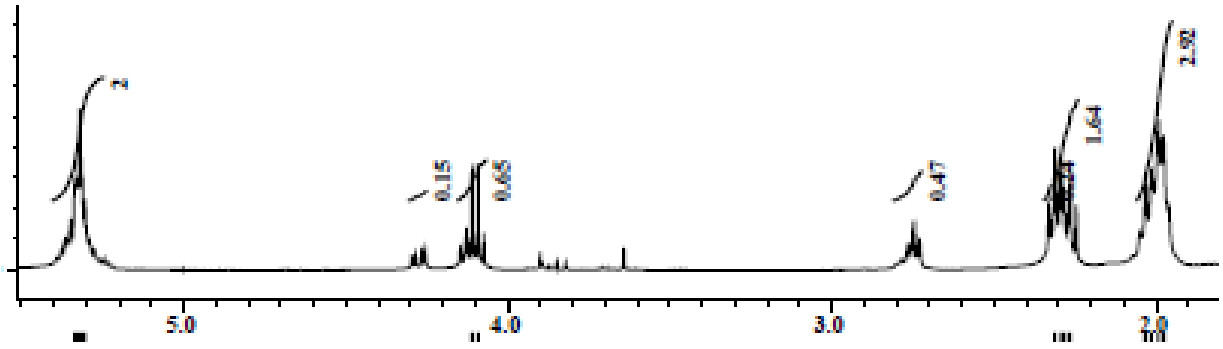

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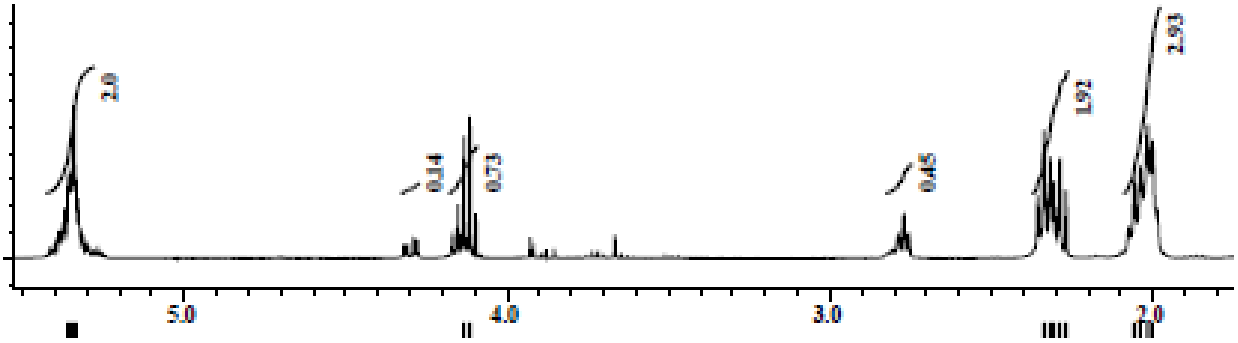

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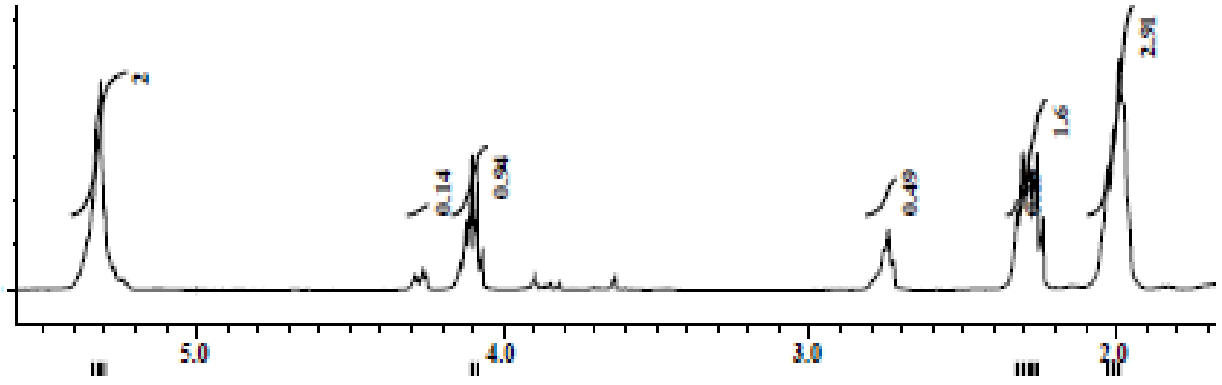

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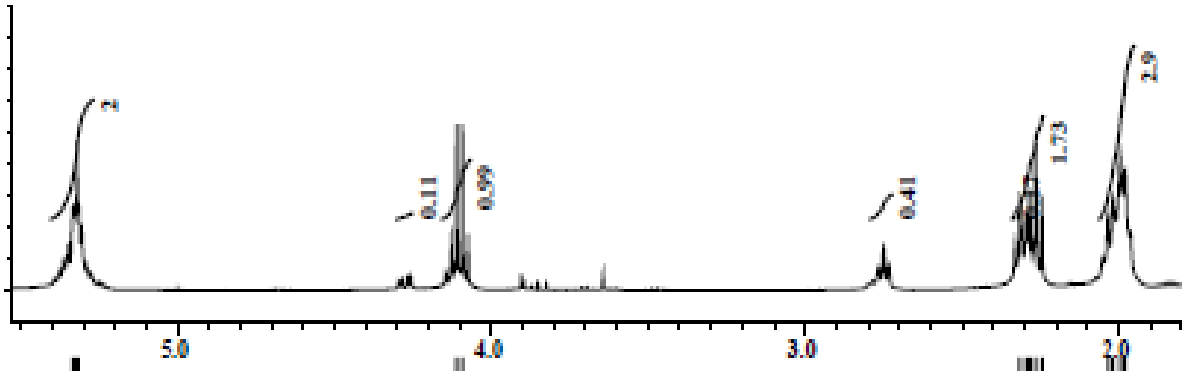

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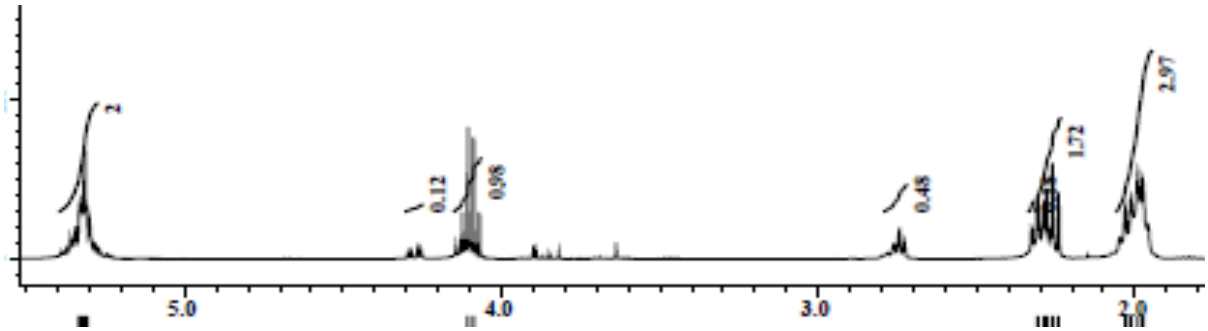

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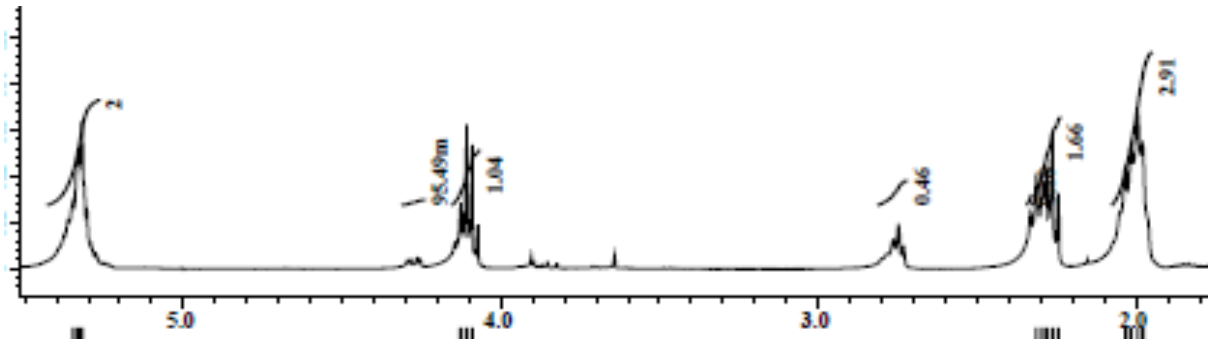

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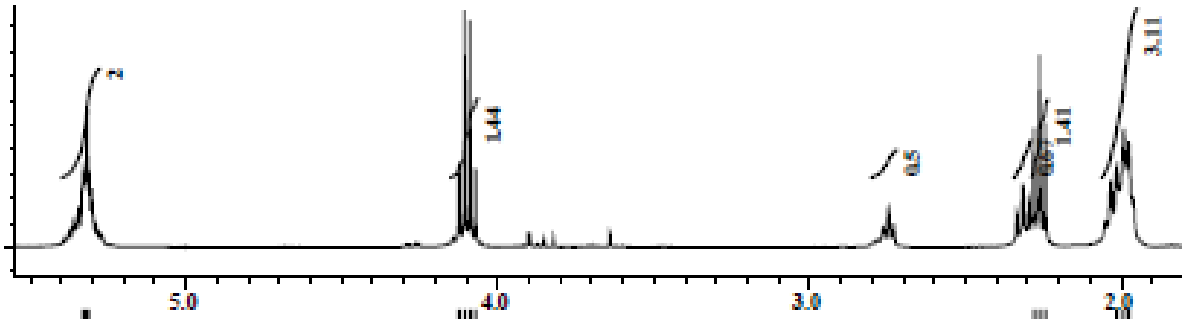

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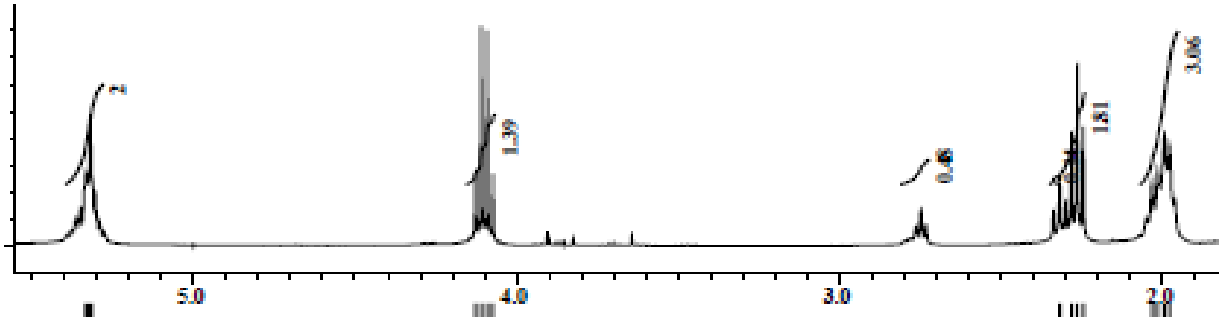

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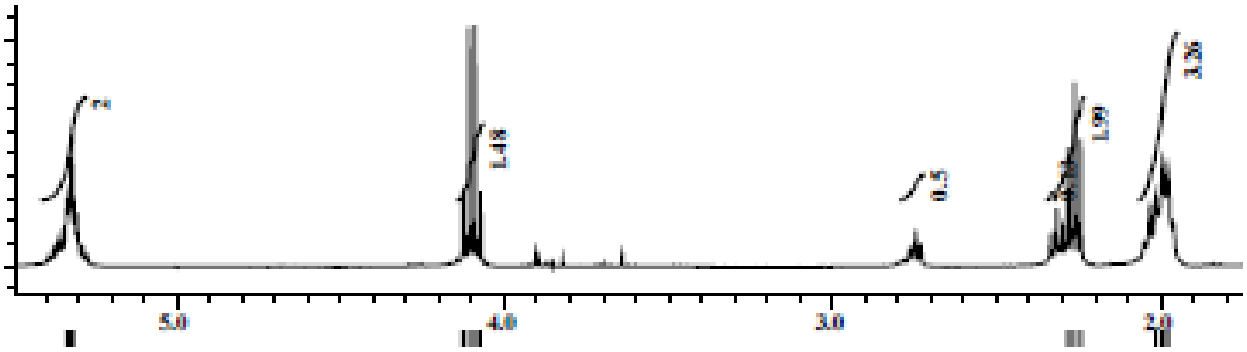

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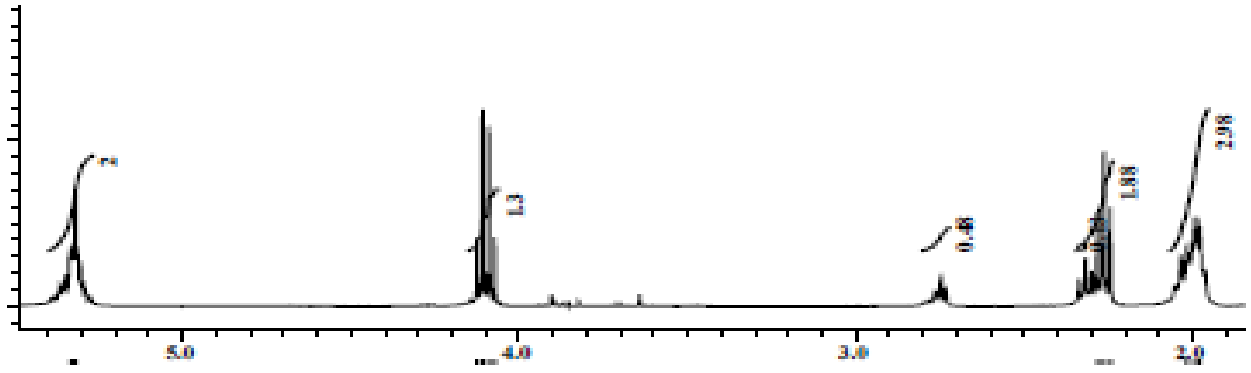

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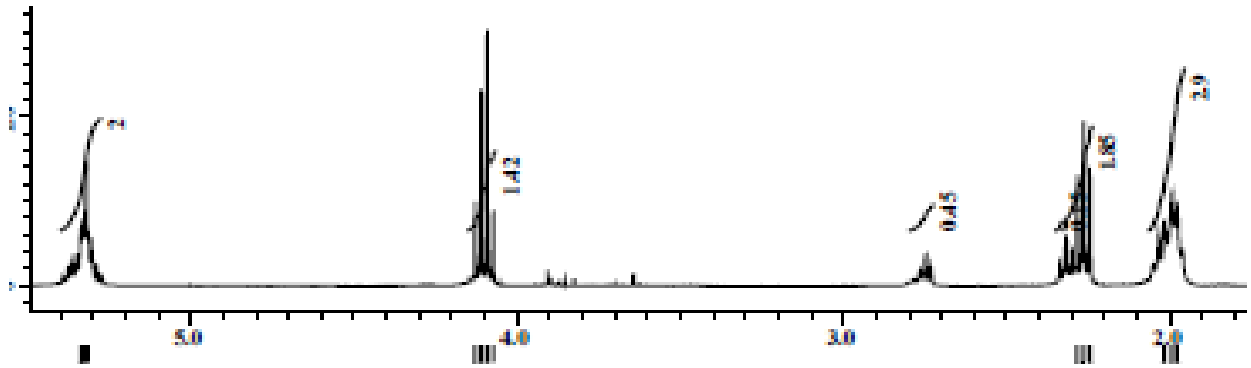

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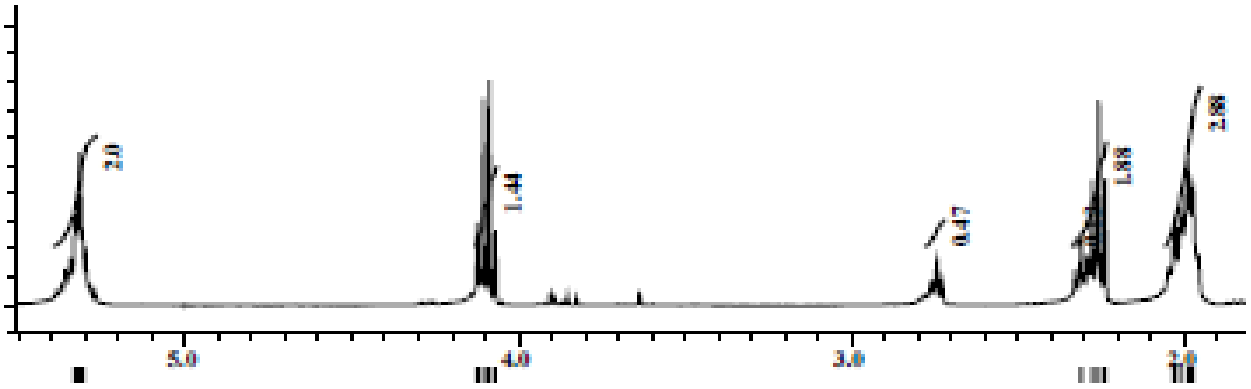

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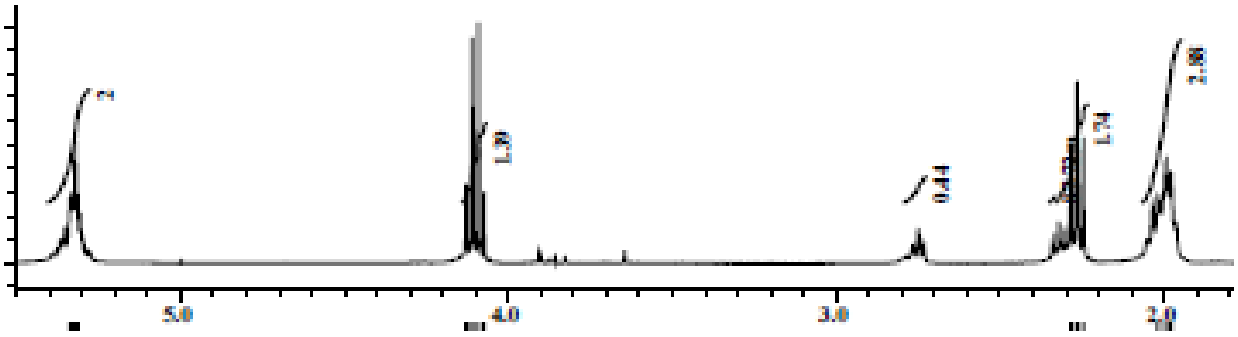

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