Transesterification of Rice Bran Oil Using Biocatalyst

Thesis Submitted

In partial fulfilment of the requirement for the degree of

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in

Chemistry

Submitted by

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Certificate

This to certified that the thesis entitled ***"Transesterification of rice bran oil using Biocatalyst"*** *being submitted in the partial fulfilment of requirements for the award of degree of* ***Master of Science in Chemistry*** *submitted in* ***School of Chemistry and Biochemistry, Thapar University, Patiala*** *is a bonafide work carried under the supervision of* ***Dr. Ranjana Prakash*** *Assistant Professor,School of Chemistry and Biochemistry,Thapar University, Patiala and that no part of this project has been submitted for the award of any other degree.*

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Declaration

I hereby declared that the work presented in this thesis entitled ***"Transesterification of rice bran oil using Biocatalyst"*** *submitted in the partial fulfilment of requirements for the award of degree of* ***Master of Science in Chemistry*** *submitted in* ***School of Chemistry and Biochemistry, Thapar University, Patiala*** *is an authentic record of my own work carried out under is a bonafide work carried out under our guidance and supervision and that no part of this project has been submitted for the award of any other degree.*

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Needless to say errors and omissions are solelymine.

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Summary

The work presents the findings on the whole cell catalyzed transesterification of used-rice bran oil with dry biomass of *Aspergillus* species. The effect of chain length of alcohol on the extent of transesterification was examined using primary and other alcohols as acyl donors. The results indicated that increase in chain-length significantly favours the reaction towards enhanced yield of alkyl esters especially with acyl donors ranging from butanol to heptanol. The study also resulted in deriving an equation for the NMR based quantification of alkyl esters, generated through transesterification of oil using primary alcohols beyond ethanol. The results obtained through the modified equation significantly correlated with the yield of alkyl esters determined through GC analysis.

Contents

1.0 Introduction

Two preliminary considerations, 21st-century man's demand for goods and services has no precedent in history and the state of health of the economy is founded precisely on this constant increase in consumption. Every human activity, from large scale industrial production to individual small everyday actions, releases chemical substances which sooner or later are discharged into the biosphere. If we put these two issues together it is apparent that human beings, due both to their increasing numbers and also their increasing demands seem destined over time to exert more and more aggressive pressure on the environment.

This is exactly what we mean by the "dirty face" of development. If mankind were able to learn from his mistakes, then emerging countries would set off down "clean paths" of development. Unfortunately, virtuous behaviour is also the most expensive; today, the main goal for three billion Indian and Chinese people is to speed up their economic development as much as possible without losing time and money addressing environmental and health issues. Adopting industrialisation as the quickest route to rapid economic development brings with it the large-scale importation of technologies, often obsolete and dirty, from more developed countries.

These days, there are lot of problems that the humans are facing with regards to air pollution. More is the number of vehicles used everyday all around the world, bigger will be the problem. The blame goes to the emission of harmful gases from diesel engines of automobiles and industrial equipments those uses fuel.

Diesel exhaust is one of the world’s most pervasive sources of toxic air pollution. World’s millions of diesel dependent mechanical systems - buses, trucks, trains, ships, and construction equipment - emit pollutants that lead to 21,000 premature deaths each year and create a cancer risk that is seven times greater than the combined risk of all 181 other air toxics tracked by the EPA of United States of America. Scientific studies link pollutants in diesel exhaust to a myriad of public

health effects, including asthma attacks, heart attacks, stroke, cancer, and premature death. Exposure to diesel emissions is nearly inescapable. Diesel pollution is also an Environmental Justice issue. Children and seniors are most vulnerable to the health effects of diesel pollution.

Black carbon, a component of diesel pollution, is also one of the largest drivers of climate change. Black carbon is a form of particulate matter emitted by diesels (and other sources) that warms the atmosphere by absorbing sunlight and radiating heat into the air (like a blacktop road). It can also darken snow and ice, and directly accelerate melting. The United States has the highest per-capita emissions of black carbon in the world, with more than half coming from diesel engines.

Due to rapid economic expansion, India has one of the world's fastest growing energy markets and is expected to be the second-largest contributor to the increase in global energy demand by 2035, accounting for 18% of the rise in global energy consumption. About 70% of India's energy generation capacity is from fossil fuels, with coal accounting for 40% of India's total energy consumption followed by crude oil and natural gas at 24% and 6% respectively. India is largely dependent on fossil fuel imports to meet its energy demands by 2030, India's dependence on energy imports is expected to exceed 53% of the country's total energy consumption. In 2009-10, the country imported 159.26 million tonnes of crude oil which amount to 80% of its domestic crude oil consumption and 31% of the country's total imports are oil imports. The former President of India, Dr. Abdul Kalam is one of the strong advocators for production of bio-diesel. In one of his recent speech, the Former President said that out of the 6,00,000 km^2 of waste land that is available in India over 3,00,000 km^2 is suitable for Jatropha cultivation.

Aside from performing well just as ordinary diesel fuels do, biodiesel is more economic to use and has been proven to have same performance as regular fuel engines. It also does not only last longer but also have higher rate of lubrication with lower concentration levels, which makes it blend better and emitting less pollution than other fuels, especially diesel. Biodiesel consists of long chain fatty acid ester produced by transesterification reaction of vegetable oils with short chain and long chain alcohols using some catalyst.

With these advantages that make biodiesel more useful than other regular and diesel fuel. Biodiesel is a form of diesel fuel manufactured from vegetable oils, animal fats, or recycled restaurant greases. It is safe, biodegradable, and produces less air pollutants than petroleum-based diesel. Biodiesel can be used in its pure form (B100) or blended with petroleum diesel. Common blends include B2 (2% biodiesel), B5, and B20, B2 and B5 can be used safely in most diesel engines. Biodiesel does not contain sulphur or aromatic compounds and its use results in lower emissions of carbon monoxides, hydrocarbons, and suspended particulate matter.

Biodiesel production can be carried out by transestrification reaction catalysed either chemically or enzymatically. Chemically catalysed process, including alkali and acid. Transeterification by acid catalysis is much slower; it is more suitable for oils and fats that have relatively high free fatty acid (FFA) and water contents. It has been reported that acid-catalyzed transesterification can be used when the starting materials are low-grade fats or have a high FFA content. Acid-catalyzed reaction commonly requires high temperature. For alkali-catalyzed transesterification, the starting materials (oil or fats) must be dry and free of FFA. The presence of minor amount of FFA and moisture in the reaction mixture produces soap, which lowers the yield of esters due to its interference in process of transesterification. So chemically catalysed process, including alkali and acid both have their own limitation towards the catalysis of waste oil.

Biocatalysts, particularly lipase, can be used in place of alkali or acid to catalyse transesterification reaction. Lipase act as the biocatalysts for the hydrolysis of TAG (triacylglyceride) to free fatty acids, monoacylglycerol (MAG), diacylglycerols (DAG), and glycerol. The main advantages of lipase catalysis are selectivity and stereo specificity. Lipases can catalyse process of transesterification in presence of free fatty acids and moisture with no soap formation. Despite numerous advantages, enzymatic processes have some drawbacks such as: low reaction rate and low enzyme stability in the presence of excess methanol, high cost of pure lipases. Pure lipases can be isolated from bacteria (*Pseudomonas* sp.), yeast (*Candida* sp. and *Mucor* sp.) and fungi. The isolation of pure lipase is tedious and

expensive. Pure lipases can be used as catalyst in the transesterification reaction but it loses its activity due to the high percentage of the oil. Whole cell capable of producing lipase in specific culture conditions can also be used as catalyst for transesterification. Whole cell as biocatalyst with cell bound lipase activity is an alternative way to reduce the cost of lipase, since it can avoid the complicated processes of isolation, purification, and immobilization of extracellular lipase, which decrease the large part of the enzyme cost (Li et al., 2007; Fukuda et al., 2008).

In addition, free fatty acids content in the oil can also be completely converted to alkyl esters in presence of whole cell catalyst and alcohol. Suitable alcohols such as methanol, ethanol, propanol, butanol, pentanol, etc., can be used for the conversion of oil to alkyl esters. Most of the studies using lipase as a catalyst reported in literature were carried out using methanol for alcoholysis. There are limited reports on the use of other alcohols as acyl donors in whole cell catalysed transesterification.

In present study, different types of alcohols (ethanol to decanol and other different alcohols)has been used for whole cell catalysed transesterification of vegetable oil to determine the effect of chain length of alcohol on alkyl ester production.

2.0 Review of literature

Biodiesel

In recent years one of the important areas of research in the field of oil technology is the generation of biodiesel from vegetable oil and animal oil. Biodiesel can be defined as the monoalkyl ester of fatty acids from vegetable oils and animal fats. Biodiesel is a promising alternative diesel fuel obtained from the transesterification of vegetable oil or animal fat with an alcohol. (Fukuda, 2001; Ma, 1999).

Biodiesel and other bio fuels are produced from renewable agriculture crops that assimilate carbon dioxide from the atmosphere. Biodiesel reduces the emission of carbon monoxide, ozone-forming, hydrocarbons, hazardous diesel particulate, and acid rain causing sulphur dioxide, smoke and soot. Biodiesel is the only alternative fuel that runs in any conventional, unmodified diesel engine. Also, it does not require any changes in storage facilities, which exists for storing petroleum-based diesel. Biodiesel can be used alone or mixed in any ratio with petroleum diesel fuel - (http://earthsci.org/energy/biofuels/biofuels.html). The most common blend is a mix of 20% biodiesel with 80% petroleum diesel “B20”. Biodiesel has positive performance attributes such as increased cetane number, high fuel lubricity, and high oxygen content, which may makes it a preferred blending stock with future ultra clean diesel.

In the early literature, there are several terminologies for these ester-forming reactions, namely: alcoholysis, acidolysis and ester interchange; but recently, it is more common to use the term "transesterification" to describe the reaction, which when carried out with an alcohol in the presence of an acid or base catalyst is known as alcoholysis. Depending on the specific alcohol used, alcoholysis is referred to as methanolysis, ethanolysis, propanolysis, and butanolysis, etc.

Transesterification

Biodiesel is produced from any fat or oil through process called transesterification. Presently industrial production of biodiesel fuel is performed by alcoholysis of waste oil using alkaline catalyst. A by-product, glycerol, thus contains the alkali and hence as to be treated as a waste material.

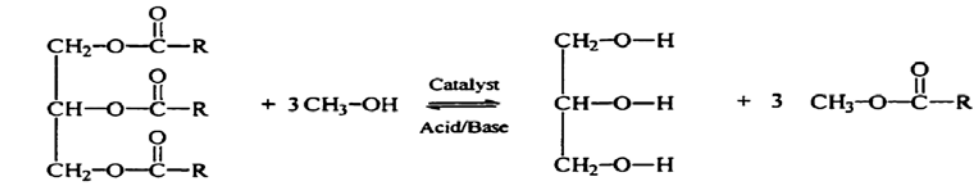
The reaction can be catalyzed by either base or acid. The overall chemistry of transesterification with methanol is represented in equation 1. It involves the interchange of the alkoxide group between an ester and an alcohol to give a new ester and a new alcohol. The overall reaction, as exemplified by synthesis of methyl ester, in equation 1 consists of a number of consecutive and reversible reactions as follows (Fukuda et al., 2001):

a) The formation of diglycerides (equation 2)

b) The formation of monoglycerides (equation 3)

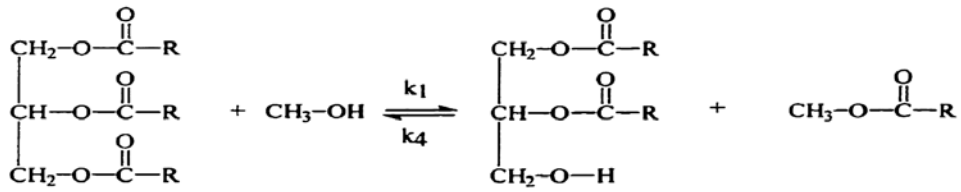
c) The formation of glycerol (equation 4)

eq.1



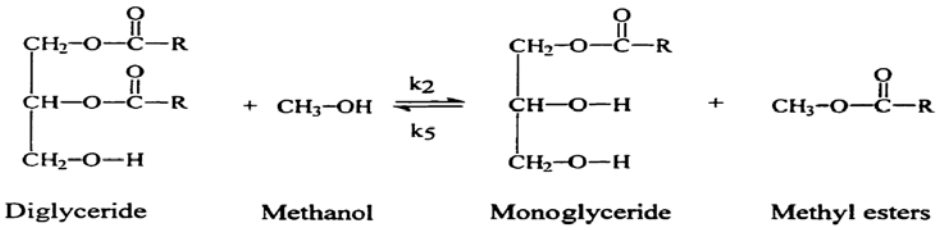
Triglyceride (oil) Methanol Glycerol Mixture of methyl esters

eq.2

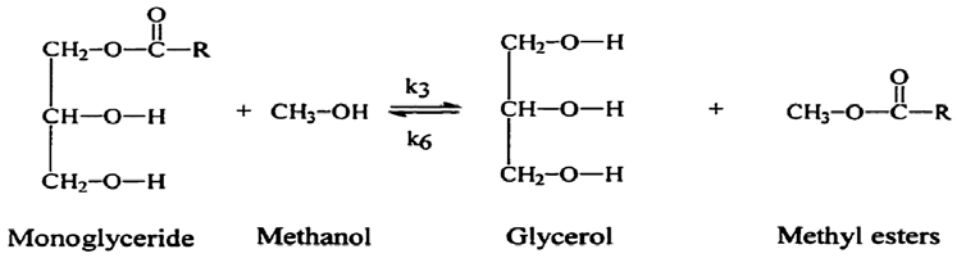


Triglyceride Methanol Diglyceride Methyl esters

eq.3



eq.4



Base-catalyzed transesterification

The base-catalyzed transesterification of vegetable oils and fats to form alkyl ester is faster than the acid-catalyzed reaction. Base-catalyzed reaction proceeds rapidly at ambient temperature, whereas the acid-catalyzed reaction commonly uses temperature above 100°C (Sridharan, 1974), depending on the boiling point of the alcohol. This base-catalyzed reaction normally works well when the substrates contain low free fatty acids and are substantially anhydrous; i.e. the starting material should have free fatty acid content less than 0.5% (acid value less than 1) (Wright et al., 1944) and water content less than 0.3%. Metal hydroxides and alkoxides are both employed for base-catalysis and are known to be effective. Being relatively cheap, they are widely used for commercial processes. For typical base catalyzed alcoholysis of oils and fats, the catalyst is first dissolved in the alcohol such as methanol or ethanol and then mixed with oil. The solubility of alcohol in oil varies depending on the size of the alkyl group of the alcohol and the reaction temperature. The solubility of methanol in oil is fairly low at room temperature even at moderate temperature such as 60°C(Mao, 1995). Therefore, very vigorous mixing is employed to initiate the fust phase of the reaction. The triglyceride is converted stepwise to

diglyceride, monoglyceride and finally to glycerol. The glycerol starts to form after the addition of alcohol and slowly settles at the bottom of the reaction vessel due to gravity. The lower glycerol-rich phase containing a small amount of alcohol and probably some monoglyceride is separated for purification; the upper fatty ester-rich phase contains the remainder of glycerol, most of the unreacted alcohol, catalyst, and mono-, di, and triglycerides. The excess alcohol is removed by distillation, and the ester-rich phase is washed with water to pH 7. Soap is usually a by-product. The fatty acids neutralize alkaline catalyst and form soaps. Although the amount of base catalyst may be adjusted for the acid values of the substrate, the resulting soap will cause an increase in viscosity or formation of gels and interfere with separation of glycerol (Freedman et al., 1984). This base-catalyzed transesterification is exothermic and a rise of 1 to 2°C above the reaction temperature occurs immediately after the addition of catalyst solution to the oil (Feuge and Gros, 1949).

2.2.2 Acid-catalyzed transesterification

Acid-catalyzed transesterification is slower than base-catalyzed transesterification. The reaction temperature is usually above 100°C and reaction time ranges from 3 to 48 hours except when the reaction is conducted under very high temperature and pressure (Allen et al., 1945). The procedure of acid-catalyzed transesterification is different from base-catalyzed one. The reaction is refluxed at or near the boiling point of the mixture of co-solvent and alcohol. Sulphuric acid or hydrochloric acid are usually employed as catalysts. Glycerol settles down at the bottom of the reaction vessel by gravity. Glycerol is separated and excess methanol removed by distillation. The organic phase is washed with water and dried by an evaporator (Boocock et al., 1996). Acid-catalyzed transesterification is very useful when the starting materials are low grade fats or have a large amount of free fatty acid content.

Problem associated with chemical transesterification of vegetable oil

- High reaction temperature (Fukuda et al., 2001).
- Reduced ester yield due to soap formation due to presence of free fatty acid in oil in case of base catalysed transesterification (Freedman et al., 1984).

- Difficulty in purification of glycerol, as a pure by-product (Mittelbach, 1990).
- Hinderance in reusability of homogenous chemical catalyst due its association with glycerol layer.
- Low yield of ethyl ester with conventional alcoholysis (Foidl et al., 1996).

In view of these disadvantages associated with the chemical transesterification for biodiesel production, enzymatic alcholysis of oil/fat is now considered desirable and promising in nature.

Enzymatic catalysis

To overcome problem associated with chemical catalyst for production of biodiesel, enzymatic process using lipase have been developed. Alcoholysis by lipase is considered to be one of the most effective reactions for the production of biodiesel. Lipase (E.C.3.1.1.3) hydrolyzes triglycerides and can be isolated from variety of micro-organisms, plants and animals. Lipases have great potential as biocatalysts for biodiesel production. Advantages of using lipase include ease of product recovery; low energy and temperature requirements; ease of enzyme recovery; mild reaction conditions of pH, temperature, and pressure; regeneration and reuse of the enzyme several times; use of reactors for continuous production; thermal stability at a relatively low temperature of operation; operational stability of the enzyme; flexibility of accepting various substrates and alcohols; and reaction in solvent and solvent-free systems (Ranganathan et al., 2007; Marchetti et al., 2007; Akoh et al., 2007). Further, immobilization of these enzymes allow reuse, confers stability, and higher enzyme loading for faster reaction. Because of all these obvious advantages, lipase received much attention in the fields of organic chemistry, biotechnology, food technology, industrial and pharmaceutical chemistry (Jaeger and Eggert, 2002; Sharma et al., 2001). New applications are being developed based on the ability of lipases to catalyze synthesis reactions in systems with very low water concentrations (Abbas and Comeau, 2003; Gubicza et al., 2001). Some studies have been attempted to understand the functional properties of lipases in processes that involve modification of the properties of fats and oils (Gandhi et al., 1997). Owing to their ubiquitous nature, broad substrate specificities, requirement of mild reaction

conditions and environmental friendliness (Zheng and Yan, 2004), lipases have been widely used for enzymatic bio-transformation of oils/fats and ester synthesis (Deng et al., 2003).

Despite numerous advantages, enzymatic processes have drawbacks such as: low reaction rate, enzyme cost for industrial scale use in comparison to alkali catalyst, low enzyme stability in the presence of excess methanol (Bajaj et al., 2010; Fjerbaek et al., 2008). In order to make enzymatic transesterification competitive on industrial scale there are several issues that have to be addressed such as solvent engineering, lipases immobilization, selection of acyl acceptor and reactor system.

There are two categories of enzymatic biocatalysts viz., extracellular lipases (i.e. the enzyme has previously been recovered from the cultivation broth and then purified); and intracellular lipases present either within the cell or attached to the cell wall. Intracellular lipases were dominantly isolated and produced from organisms such as *Candida rugosa*, *Candida utilis*, *Candida antarctica* and *Pseudomonas cepacia*. Among lipases of plant, animal and microbial origins, most commonly used are ones sourced from microorganisms. Using microorganisms, it is possible to achieve a higher yield of enzymes with desired properties. In addition, the enzymatic yield is independent of potential seasonal variations and it is possible to achieve rapid growth of microorganism in low-cost culture media (Gupta et al., 2004).

Reaction system for lipase is a two-phase system consisting of aqueous phase with dissolved enzyme and an organic phase with dissolved substrate. Lipases have a specific reaction mechanism due to the fact that active site of the enzyme is covered by amphiphilic peptide loop that acts like a lid. This disables the substrate molecule to bind to the enzyme active site, resulting with negligible activity of lipases in aqueous solutions (in the absence of an interphase or organic solvent). When contact occurs with a lipid/water interface, lid undergoes a conformational rearrangement which renders the active site accessible to the substrate (Schmid and Verger, 1998).

One of the main obstacles for industrial application of lipases is the high cost of biocatalysts. Therefore, immobilization of lipases, which allows their reusability, is a necessity to make them more attractive for industrial biodiesel processes. The

aim of immobilization is to enhance lipases properties such as thermo stability and activity in non-aqueous media, and to improve handling, recovery and recycling of biocatalyst (Knežević et al., 2004). Recycling of immobilized enzymes greatly reduces the cost of the production, so the most promising immobilization supports and methods could make the enzymatic biodiesel production competitive to chemical processes. By definition, the immobilization of enzymes is localization or confinement of an enzyme onto a solid support or on a carrier matrix. There are a number of enzyme supporting matrices that can be used for immobilization and the selection depend on several factors: thermal stability, chemical durability, mechanical strength, lipase type, type of the reaction system, ease of regeneration, loading capacity and cost (Jagannathan and Abang, 2008).

Whole cell biocatalysis

In addition to use of pure lipase, microbial strains that exhibit lipolytic activity have been exploited as whole cell catalysts for transesterification reaction, by various research groups. Transesterification reaction was carried out using Jatropha oil with methanol using immobilized cell of *Pseudomonas fluorescens* MTCC 103. The various parameters affecting biodiesel yield were studied and the maximum yield of 72% was obtained at optimum condition of 40°C, pH 7, reaction time of 48h, 3g of beads containing immobilized enzymes and 1:4 molar ratio of oil to alcohol (Devanesan et al.,2007). Recently, similar observations were reported use of *Bacillus subtilis* as a whole cell catalyst (Utoff et al.,2009).Utilizing *Rhizopus oryzae* cells immobilized within biomass supporting particles (BSPs) as a whole cell biocatalyst, Ban et al.(2001) investigated the culture conditions for lipase production and on methanolysis. When methanolysis was carried out with stepwise addition of methanol using BSP-immobilized cells, in the presence of 10-20% water, methyl ester content in the reaction mixture was observed to reach 80-90% without any organic solvent pretreatment. This level of methyl ester production was observed to almost same as that achieved using extra-cellular lipase (Kaieda et al., 1999). Further, studies on immobilization of *R.oryzae* cells, through cross-linking treatment with 0.1% gluteraldehyde solution was examined indicated high efficiency in methyl

ester generation during six batch cycles, with the methyl ester content reaching 70-83% within 72h in each cycle (Ban et al.,2001).

However, there are limited reports on whole cell bio-catalyzed transesterification especially using fungi. The reports are mostly associated with *Rhizopus* sp., which indicated generation up to 90% of methyl ester in the presence of 10-20% water. Due to the 1,3-regiospecificity of lipases from *Rhizopus*, only limited conversion of triglycerides to alkyl esters in transesterification reactions of fats and oils is possible.

Feed stock for biodiesel production

There are many possible raw materials with a potential to obtain biodiesel. Generally, the main feedstock for biodiesel production include vegetable oils such as sunflower oil (Dizge et al., 2009; Modi et al., 2007), soybean oil, rapeseed oil (Li et al., 2006),jatropha oil (Shah and Gupta, 2007; Tamalampudi et al. 2008) and cotton seed oil (Royon et al., 2007).

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

resulting in 90% methyl ester yield (Tiwari et al., 2007; Berchmans and Hirata, 2008).

Blend of Jatropha and palm biodiesel has been studied by Sarin et al. (2007) for their physico-chemical properties and their optimum mixture achievements at low temperature with improved oxidation stability. Interestingly, Jatropha seeds themselves contain lipase activity required to catalyse transesterification reaction (Staubmann et al., 1999). Biodiesel production from jatropha oil in solvent free system was worked out by Shah et al.(2004) using three different lipase (*Chromobacteriumviscosum*, *Candida rugosa*, and Porcine pancreas). However, appreciable yields were obtained only form *Chromobacterium viscosum* in which the lipase was immobilized on celite-545 using enzyme preparation with process time of 8h at 40 °C. Moreover, addition of water to free (1%, w/v) and immobilized (0.5%, w/v) enzyme preparation further enhanced the yields to73 and 92%, respectively. Lipase producing whole cell of *R. oryzae* immobilized onto BSP also proved as promising biocatalysts for producing low cost biodiesel (Tamalampudi et al., 2007).

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

In near future, considering many available oils such as cottonseed, jatropha (Wood, 2005; Sarin et al., 2007) algae and coconut as potential raw materials for biodiesel production (Frohlich and Rice, 2005) is seeming crucial not only from

economic evaluation point of view but also from environmental protection stand point (Tashtoush et al., 2004, Nelson et al., 2006).

Sharma and Singh (2008) have reported use of Karanja oil in biodiesel production due to furanoflavones, chromenoflavones, flavones and furanodiketones which promote it as non-edible. They produced biodiesel form karanja oil using NaOH and KOH and reported a maximum of 89.5% yield achieved at 8:1 molar ratio for acid esterification and 9:1 molar ratio for alkaline esterification, 0.5 wt.% catalyst (NaOH/KOH) using mechanical stirrer. The two plant species soapnut (*Sapindus* mukorossi) and jatropha (*J. curcas, L.*) non-edible oils as the feedstock for biodiesel production have been worked out and compared by Chhetri et al. (2008) which reveals soapnut oil to have an average of 9.1% free FA, 84.43% triglycerides, 4.88% sterol and 1.59% others. Jatropha oil contains approximately 14% free FA, approximately 5% more than soapnut oil. Biodiesel produced from soapnut oil has been reported to contain approximately 85% of unsaturated FA while jatropha oil biodiesel was found to have approximately 80% unsaturated FA. The dominating FA in both soapnut and jatropha biodiesel was found to be oleic acid. Both oils yielded nearly 97% FAME conversion. Jatropha being grown in marginal and waste lands had shown no possibility of land use competing with food production. Similarly the use of soapnut seeds for biodiesel production entitles waste–to–energy scheme. Prospects of FAE of some of 26 non-tradition plant seed oils including jatropha have been studied by Azam et al.(2005) to use as a potential biodiesel in India. The one among them which are found most suitable for use as biodiesel and meet the major specification of biodiesel for use in diesel engine include *Azadirachta indica*, *Calophyllum inophyllum*, *J. curcas* and *Pongamia pinnata.* Nevertheless over 75 oil bearing plants containing nearly 30% or more oil in their seed, fruit or nut have also been reported. To the list are nearly 300 different species of trees which produce oil bearing seeds (Subramanian et al., 2005). Hence, non-edible oil sources from different plants have significant potential for biodiesel production as an alternative to petro diesel.

Use of Rice Bran oil as feed stock

Rice oil, also called as rice bran oil, has been extensively used across Asia such China, Korea, Taiwan, Japan, Pakistan, Thailand to a certain extent in India (Orthoefer, 2005). Rice bran oil (RBO) is one of the most nutritious oils due to its favourable fatty acid composition and a unique combination of naturally occurring biologically active and antioxidant compounds (Lai et al., 2005; Orthoefer, 2005). However, crude RBO has been difficulty to refine because of its high content of free fatty acids (FFA), unsaponified matter and extreme dark colour. RBO contains relatively lower content of triacylglycerol compared to other vegetable oils and high contents of partial glycerides, glycolipids, wax esters and unsaponifiable constituents (Ju and Vali, 2005). The viscosity of RBO is twice that of common vegetable oils. Another major drawback in RBO is its high free fatty acid (FFA) content, as RBO has very short half life due to decomposition of lipids to FFA by lipases. Rice bran itself has several types of lipases and cleaves the 1.3-site of tryacylglycerol. The estimated potential yield of RBO is about eight million metric tonnes if all rice bran produced in the world were to be harnessed for oil extraction (Arumugan et al., 2004). Until recently, rice bran was mostly used as animal feed and most the oil produced being used for industrial purposes (as boiler fuel) (Arumugan et al., 2004) with less preference towards its use as edible oil (Harnandez et al., 2000).

Crude RBO, is therefore, a low-cost feedstock for alkyl ester production as compared to traditional oils derived from cereals or seed sources. Defatted rice bran is a rich source of protein, carbohydrates and phytochemicals such as phytic acid and myoinositol, which have high commercial value. The generation of biodiesel from rice bran oil can further be made cost effective through alcoholysis followed by distillation of fatty acid alkyl esters (FAAE) which will facilitate in concentration of valuable compounds in residual lipid fraction (Lai et al., 2005). These compounds such as myo-inositol, gammo-oryzanol, tocol, policosanol, phytosterols and fatty acid setryl esters, have many beneficial biological effects and have wide applications in food, pharmaceutical and cosmetic industries. Purification and isolation of these compounds not only generates by-products with commercial potential and makes

alkyl ester (biodiesel) production more remunerative, but also reduces the effluent load and thus makes biodiesel production environmentally benign (Lai et al., 2005).

The most common method for production of alkyl esters is alkali-catalyzed reaction of vegetable oil with alcohol. However, the important pre-requisite for this reaction are that both water and FFA content in substrate oil must be below 0.5% (Feuge and Gros, 1949; Bradshaw and Meuly, 1944), whereas RBO may contain up to 80% FFA (Lai et al., 2005). The FFA content is also dependent upon the storage conditions and history of the bran, thus making it unsuitable for biodiesel generation.

The alternate method to alkali catalysis in either lipase or acid catalyzed transesterification (Zhang et al., 2003). Although acid catalysis can be used with RBO, this catalytic process has certain obvious disadvantages hindering its use at industrial scale. As an alternative to chemical catalysis, lipase catalyzed methanolysis can be effectively used for transesterification reaction with RBO, although there are limited reports in these aspects. Lai et al. (2005) investigated the use of two immobilized lipases, Novozym 435 and Lipozyme IM 60 as biocatalyst for biodiesel generation using RBO. The results show that at a reaction time of 2 h, approximately 60% fatty acid methyl esters (FAME) content was obtained with both lipases. The FAME content further increased to 98% in case of Novozym 435 and to 81% in case of Lipozyme at 7 h and 4 h respectively. When dewaxed/degummed RBO with high FFA content was used, the FAME yield was more than 96% in 6 h with both the types of enzymes. These results of Lai et al. (2005) demonstrate the effective use of enzyme catalysis for FFA rich RBO as substrate for generation of alkyl esters.

Used frying oil as feed stock

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 cost (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 (Connemann and Fischer, 1998; 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 and Cvengrosova,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 and Cvengrosova,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 color, and (v) an increase in the tendency of fat to foam. (Cvengros and Cvengrosova, 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). 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 (Bastidaet al**.,** 2001).

Influence of post-frying physico-chemical changes on transesterification reaction

Although used-frying oil is a good and cost-effective substrate for generation of alkyl esters, there are several changes in physicochemical parameters such as such as free fatty acid value, peroxide value, saponification value, unsaponifiable matter etc. that take place during frying of cooking oil.

Extended heating (abuse) of vegetable oils leads to their oxidation (degradation) thereby resulting in formation of oxides such as hydroperoxides and epoxides which have adverse consequences pertaining to health exhibited in the form of growth retardation or increased size of liver. Thus are disposed to public sewers causing a number of problems. But proper recycling of WCO can prevent its inappropriate disposal problems. Recycled WCO is mainly used in producing animal feeds and a small proportion of it also helps in manufacturing soaps and biodegradable lubricants, however certain health risks including undesirable level of contaminants, particularly PAHs (polycyclic aromatic hydrocarbons), PCBs (polychlorinated biphenyls), dioxins and dioxin related substances have been traced in using these recycled cooking oil in animal feeding (Riera et al., 2000). Increase in viscosity and specific heat, change the surface tension and colour, increase in tendency of fat to foam are some commonly observed physical changes (Cvengros and Cvengrosova, 2004). During frying the three major types of reaction that occur are thermolytic, oxidation and hydrolytic (Nawar, 1984; Mittelbach and Enzelsberger, 1999).

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

happens between acyl groups within same molecule, in case of glycerides (Nawar, 1984).

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

Primary oxidation of oils is generally determined by measuring peroxide value. Initially, there were little changes in the peroxide values of the fish oil. Peroxides are the primary reaction products formed in the initial stages of oxidation of oil and therefore give an indication of the process of lipid peroxidation. Lipid peroxidation depends on the reaction between unsaturated fatty acids and oxygen. The commonly consumed oils have higher peroxide values e.g. palm, coconut, groundnut, and melon oils contain 4.0, 10.0, 18.2, and 2.0 meq/kg, respectively (Weiss, 1983).

Factors that affect biocatalyzed transesterification

There are several factors which affect the transesterification reaction such as the type of alcohol, time duration of incubation, oil-water molar ratio, etc.

Several studies have focused their attention on branched and long chain alcohols. Experiment showed that increase of the number of carbon atoms increased the cetane number as well as heat content of the fuel. Also, fatty acid esters of secondary or branched chain alcohols can be used as fuel additives since they decrease the solidification point, and consequently, the high cloud point and pour

point (Salis et al., 2005; Watanabe et al., 2007). Kose et al. (2002) dealt with the alcoholysis of cotton seed oil with primary and secondary alcohols by using Novozyme 435 lipase. They analyzed the effect of alcohol types on alcoholysis on cotton seed oil indicating that the highest yield was obtained with iso-amyl alcohol. Ognjanović et al. (2009) investigated the influence of methanol, 2-propanol and n-butanol on biodiesel synthesis. Operational stability of lipase from *C. antarctica* was investigated in a three-step addition of alcohol in a solvent-free system. With all three acyl acceptors a high initial yield was achieved but lipase exhibited poor activity during the repeated experiments.

The main drawback in industrial implementation of lipases into the solvent-free biodiesel synthesis is low enzyme stability in the presence of excess methanol, since several studies reported that a high methanol concentration could lead to serious inactivation of lipase (Kose et al. 2002; Royon et al. 2007). Methanol is the most popular alcohol used in transesterification process because of its relatively low price in comparison to other alcohols. The replacement of methanol with less polar alcohols resulted in only slight increase in retained activity, but the significant inactivation of lipase still occurs. This might be due to the inactivation effect caused by alcohol and the negative effect caused by product glycerol adsorbed on the surface of the immobilized lipase (Ognjanović et al., 2009). Ethanolysis proceeds at slower rate than methanolysis because of the higher reactivity of the methoxide anion in comparison to ethoxide. As the length of the carbon chain of the alkoxide anion increases, a corresponding decrease in the nucleophilicity occurs, resulting in a reduction in the reactivity of ethoxide in comparison to methoxide. An example of this phenomenon is the transesterification (at 25°C) of canola oil with a 1:1 mixture of ethanol and methanol (to provide an overall molar ratio of alcohol to oil of 6:1) that result in 50% more methyl than ethyl ester (Kulkarni et al., 2007). But when butanol is used in the transesterification process it shows more reactivity towards the lipase catalyst because butanol is completely miscible with vegetable oil and animal fats. It is significantly less polar than methanol and ethanol (Boocock et al., 1996). Consequently, transesterification reactions employing butanol are monophasic throughout (Zhou and Boocock, 2006).

With reference to the oil to alcohol ratio, alcohol in excess of the stoichiometric molar ratio of 1:3 (oil: methanol) is generally used to ensure higher biodiesel alkyl ester yield. An increase in the number of moles of alcohol with respect to the triglycerides results in an increase in the production of esters.

The enzymatic transesterification is generally performed at lower temperature than the chemical reaction to prevent loss of lipase activity. Optimum temperature determined for various lipases used for biodiesel synthesis, ranges between 30°C and 55°C. Reaction temperature may vary from 23 to 50°C. Optimal temperature for methanolysis of sunflower oil is 50°C when *T. lanuginose* is used as biocatalyst, but when *R. miehei* is used in the same reaction, temperature optimum lower than 40°C has been reported (Soumanou and Bornscheuer, 2003). In general, increasing the temperature leads to an increase the reaction rate of biodiesel production. When optimum is reached, further increase in temperature, leads to decreased catalytic activity of the enzyme caused due to denaturation and inactivation. The researchers have shown that immobilization of enzymes shift temperature optimum to higher values in comparison to free enzymes. It seems that immobilization provides a more rigid external backbone for lipase molecule, leading to the increase of the temperature optima and higher reaction rates.

Lipases need an optimal small amount of water to maintain the activity in the organic media. Nevertheless, increased water concentration has an unfavourable effect on the equilibrium conversion, since it promotes reverse reaction of hydrolysis. The amount of water in the system should be a compromise between minimizing hydrolysis and maximizing lipase activity for the transesterification reaction and it should be determined for a particular reaction system (Noureddini et al., 2005). Many studies have shown that immobilized enzymes show highest activity in low water system. Tamalampudi et al. (2008) showed that, in biodiesel synthesis using lipase from *C. antarctica* (CALB), the rate of methanolysis decreased with increase of the water content, reaching the FAME content of 75% when no water was added in the system. Similar results were achieved using the same lipase in the transesterification of sunflower oil, where yield of over 90% was achieved in an anhydrous reaction medium (Ognjanović et al., 2009). It has been shown that many

immobilized lipases contain sufficient amount of water to preserve the catalytic conformation.

Lacunae

Although the importance of rice bran oil as a potential substrate for alkyl ester generation and the use of used oil to reduce the cost of ester generation, is well understood, limitations on use of pure enzymes with reference to cost implications and reusability potential has reduced the possibilities of the use of this process at industrial scale.

Keeping this in view, the present study was focussed to explore the use of dried biomass of a whole cell catalyst for transesterification of refined and used-frying rice bran oil for generation of alkyl esters. The influence of different alcohols as acyl donors were studied for transesterification of rice bran oil.

3.0 Materials and Methods

Refined rice bran oil was procured from open 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, Bi-ammonium hydrogen ortho-phosphate ($(NH_4)_2HPO_4$), potassium hydroxide(KOH), hydrochloric acid (HCl), sodium thiosulphate ($Na_2S_2O_3$), starch, potassium iodide (KI), ethyl alcohol and phenolphthalein were purchased from SD Fine-Chem limited, India. All the reagents used were analytical grade.

Preparation of used frying oil

Frying oil was generated using 7 litres rice bran oil after deep frying. The first sample was collected after frying of 200 breads and second sample was collected after frying of approximately 10-12 kg vegetables in batches in the same oil.

Determination of free fatty acids (FFA)

The FFA value of pure oil and collected fried oil samples were determined by using standard method outlined by AOCS Ca5a-40 (AOCS, 1989) with certain modifications. 5.0 gm of oil (pure or fried) was dissolved in 50ml ethanol and titrated with 0.1N KOH by using phenolphthalein as indicator. End point of titration was change in colour from colourless to pink which is stable for 10 seconds**.** The FFA values were determined by the following formula:

$$\%FFA = [56.1^{*} \times \text{Volume used of KOH} \times 0.1N]/ [\text{Weight of sample}]$$

* Molecular weight of potassium hydroxide

Determination of the saponification value

The saponification value of the frying oil samples were estimated by the method of AOAC (2000). Approximately 2 g of oil sample was taken in a round bottom (RB) flask and 25 ml alcoholic KOH solution was added. Alcoholic KOH was prepared by mixing of 1.2 L of absolute ethanol with 10 gm of KOH and 6 gm

of aluminium foil and refluxing was done for 30 min. 1 L of alcohol was collected by distillation after discarding first 50 ml. 40 g of KOH was dissolved in 1L alcohol by keeping it in ice bath. The solution was kept overnight and clear solution thus formed was transferred in a bottle and kept for further use.

Round bottom flask containing oil and alcoholic KOH was refluxed for 45 min on heating mental for completion of saponifiaction. Clear and homogenous solution indicates that saponification completed. After cooling, solution was titrated against 0.5 N HCl using phenolphthalein as indicator. Alcoholic KOH was used as blank/control. The saponification value was calculated as follows:

[Saponification value =56.1 (B-S) N/W]

B= Volume (ml) of standard HCl utilized for the blank.

S = Volume (ml) of standard HCl utilized for the sample.

N = Normality of the standard HCl.

W = Weight in gm of the oil/fat taken for the test.

Preparation of Biomass

The given spores of *Aspergillus sp*. inoculated aseptically in 500ml Erlenmeyer flask containing 200 ml of sterile PDB and incubated at 30°C, 120 rpm for 3 days. 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) supplemented with mycological peptone (0.5% w/v), $(NH_4)_2HPO_4$ (0.5%w/v) and virgin cotton seed oil (30% v/v). Mycological peptone and $(NH_4)_2HPO_4$ were used to supplement nitrogen and cotton seed oil was used as main carbon source for fungal growth. The active culture obtained from PDB was inoculated into 200ml minimal media containing nitrogen and carbon source. Culture flask was incubated at 30°C, 120 rpm for 5 days. Fungal biomass was separated by filtering through Whatman filter (No.1) paper, washed with hexane to remove the excess oil and dried with blotting paper. The partially dried biomass was crushed in liquid nitrogen to make homogenous powder using pestle mortar.

Transesterification reaction

1 g of dried biomass was taken in RB containing 10 ml of used frying oil (containing highest FFA among all samples). 3 ml of alcohol was added and mixture was stirred for 36 h on magnetic stirrer at 30° C. Reaction mixture was washed 3 times with 10 ml of hexane to separate out the product formed. Hexane was pooled in clean RB and hexane was removed by rota-evaporator. The progress of the reaction was checked regularly by thin layer chromatography. Different type of alcohols (*primary*:methanol, ethanol, propanol , butanol, pentanol, hexanol, heptanol, octanol, nonanol and decanol; and *secondary*: 2-methyl-propane-1-ol, propane-2-ol and tertiary 2-methyl propane-2-ol) were tried to check the effect of chain length of alcohol and extent of transesterification.

Identification and quantification of alkyl esters

The product (ester) obtained was analysed using 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).

GC Analysis

The product was quantified by gas chromatography using methyl heptadecanoate as a standard. Percentage of alkyl ester of fatty acid present in sample was determined according to EN ISO 5508 with internal calibration (methyl heptadecanoate, 10 mg/ml). Sample was prepared by weighing 250 mg of alkyl ester in a 10 ml vial, followed by the addition of 5 ml methyl heptadecanoate (10 mg/ml). 1.0 µl of sample was injected into GC-5765 (Nucon, India) equipped with a flame ionization detector. A fused silica capillary column(0.25-mm internal diameter, 30-m length and 0.25-µm film thickness, wall coated with EC wax:polythene glycol) was used to separate FAEE. The flow rates of nitrogen as carrier gas and hydrogen gas were 30 ml/min while that of zero air was 300 ml/min. The injector and detector temperature were maintained at 230 and 240°C respectively. The oven initial temperature (160 °C) hold time was 1 minute and final oven temperature was 240°C. The rate of increase in temperature was 4°C/min and complete program duration was 45 min. Split injection ratio 1:30 and split flow rate 30 ml/min were maintained. The

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

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

Wherein,

Σ A = total peak area from the alkyl esters of oil;
A_{EI} = peak area corresponding to methyl heptadecanoate;
C_{EI} = the concentration in milligram per millilitre of the methyl heptadecanoate solution;
V_{EI} = was the volume in millilitres of methyl heptadecanoate solution being used; and
m = was the mass in milligrams of the sample.

^{1}H NMR Analysis

The alkyl esters were analyzed further using ^{1}H-NMR (Bruker-Advance II-400 with 5mm BBO probes) with $CDCl_3$ as solvent and tetra methyl silane as internal standard. Methyl ester content in the reaction mixture was quantified by using the equation proposed by Gelbard et al. (1995) wherein the signals at 4.1–4.3 ppm are caused by the protons attached to the glycerol moiety of mono–, di–, or triacylglycerols. The strong singlet at 3.6 ppm indicates methyl ester ($–CO_2\ CH_3$) formation. The signals at 2.3 ppm result from the protons on the CH_2groups adjacent to the methyl or glyceryl ester moieties ($–CH_2\ CO_2\ CH_3$for methyl esters).

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

Wherein:

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

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

$A_{\alpha\text{-}CH2}$ - integration value of the methylene protons.

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

Ethyl ester quantification by ^{1}H NMR spectroscopy is more complex than methyl ester quantification due to a superimposition of the glyceryl methylenic

hydrogens in oil and the -OCH_2 from ethyl ester in biodiesel where partial conversion was obtained. However, in the reaction where peak due to glyceryl methylenic hydrogens in oil at 4.25-4.35 ppm completely disappeared, the process of transesterification was considered to be nearly complete. In case of incomplete transesterification, ethyl ester quantification was carried using the equation proposed by Ghesti et al. (2007).

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

Where

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

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

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

The alkyl esters produced by the reaction with various other primary alcohols *viz.*, propanol to decanol were quantified by proposing a modified formula earlier derived by Gelbard et al. (1995) for methyl esters as given below wherein the triplet at 4.0-4.1 ppm of methylenic protons indicates alkyl ester (–CO_2 **CH_2** $(CH_2)_x$ CH_3) formation. The signals at 2.3 ppm result from the protons on the CH_2groups adjacent to the alkyl or glyceryl ester moieties (–**CH_2**CO_2 $CH_2(CH_2)_x$ $CH_3$for alkyl esters).

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

Wherein:

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

$AE_{\alpha\text{-}CH_2}$ - integration value of the methylene protons of the alkyl esters (the triplet peak).

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

Statistical analysis

Data was analyzed using Graphpad Prism 5.0 for linear regression and correlation analysis where ever applicable.

4.0 Results and Discussion

The present study aimed at determining the role of different alcohols on the biocatalyzed transesterification of used-rice bran oil having high FFA content.

Free fatty acid content

Frying of oil leads to the generation of FFA due to hydrolysis of oil in the presence of moisture released during frying. The results showed that there was significant increase in FFA content after 1st and 2nd frying. The observations on FFA generation due to frying indicated increase in its content on frying, with reference to pure rice bran oil (*Table-1*).

Table 1. FFA content in pure and used rice-bran oil. Data are mean±SD (*n=3*). Values followed by the different letter are statistically different at *P≤ 0.05* (student t-test).

S No.	Oil Samples	Free fatty acid value (%)
1.	Pure rice bran oil	0.16 ± 0.05^{a}
2.	Oil after 1st frying	0.73 ± 0.05^{b}
3.	Oil after 2nd frying	0.93 ± 0.05^{c}

During deep-fat frying, the fat is continuously being exposed to elevated temperatures (150-180°C) in the presence of the substrates air and water. A complex series of reactions such as hydrolysis, oxidation, polymerization, isomerization, and cyclization takes place during the deep-fat frying as a result, used cooking oil contains compounds such as polymers, volatiles, FFA and other degradation products (Lee et al. 2002).Hydrolytic action on triglycerides by steam produced during food preparation forms FFA, glycerol, mono and diglycerides (Mittelbach and Enzelsberger, 1999). This change in composition of oil can be quantified by measuring the monoglycerides or diglycerides content. Earlier studies carried out by Prakash et al. (2010) on cottonseed oil, indicated that the FFA content increased from 0.2% to 8.3% over time from 10 min to 7 h of frying. The cumulative effect of these thermolytic, oxidative and hydrolytic chemical reactions is the formation of

undesirable by-products. Repetitive heating also leads to enhanced polar content of the oil badly affecting the quality of the oil (Guesta et al., 1993). Bastida et al.(2001) have reported normal polar content of fresh unused oil between 0.4 and 6.4 mg/100 g (Bastida et al., 2001). 20 fryings with sunflower oil increases its polar content 640% and with olive oil by 480% as per study conducted on sunflower oil, olive oil and a mixture of the two oils (Bastida et al., 2001). In study on sunflower, Bastida et al. (2001) also reported that thermo-oxidative and hydrolytic changes in the frying oil could be minimized if fresh oil is frequently added throughout uptil 20 repeated fryings. European countries have set maximum 25% polar content of edible oils to be normal beyond which the oil needs to be discarded.In general, deep-fat frying decreases the content of unsaturated fatty acids in frying fat and oil.

Saponification value

Saponification value indicates the average molecular weight of a fat or oil. The saponification value may be defined as the number of milligrams of KOH required to neutralize the fatty acids obtained by complete hydrolysis of one gram of oil or fat. Thus saponification value gives us information whether an oil or fat contains high proportion of lower or higher fatty acids. In present work due to frying of rice bran oil, FFA content increased after each frying therefore saponification value also increases significantly (*Table-2*).

Table-2. **Saponification value of rice bran oil after frying.**
Data are mean±SD (*n=3*). Values followed by the different letter are statistically different at *P≤ 0.05* (student t-test).

S No.	Oil samples	Saponification value
1.	Pure rice bran oil	186.6 ± 0.48^{a}
2.	Rice bran oil after 1st frying	195.0 ± 0.08^{b}
3.	Rice bran oil after 2nd frying	203.4 ± 0.07^{c}

As reported by Pearson (1976), oil with higher saponification values contain high proportion of lower fatty acids. The saponification values obtained for some vegetable oils range from 188 – 196 mg KOH gm^{-1}(Pearson, 1976). However, there

are some vegetable oils with higher saponification values such as coconut oil (253.0 mg KOH gm^{-1}), palm kernel oil (247.0 mg KOH gm^{-1}) and butter fat (225.0 mg KOH gm^{-1}) (Aremu et al., 2006).

Biocatalyzed alcoholysis of used rice-bran oil

Chemically, transesterification (also called alcoholysis) means taking a triglyceride molecule or a complex fatty acid, neutralizing the free fatty acids, removing the glycerin and creating an alcohol ester. A catalyst is usually used to improve the reaction rate and yield. Theoretically, the transesterification reaction is an equilibrium reaction. In this reaction, however, marginally higher amount of alcohol is used to shift the reaction equilibrium to produce more alkyl esters as a desired product. Alcohols are primary or secondary monohydric aliphatic alcohols having 1‑8 carbon atoms. Methanol and ethanol most commonly used in production of biodiesel due to their cost-effective nature. However, other higher alcohols are also being considered as acyl donors due possible generation of fuels with different properties in comparison to methyl and ethyl esters (Knothe, 2005).

Among the alcohols that can be used in the transesterification reaction are methanol, ethanol, propanol, butanol and amyl alcohol (Sprules and Price, 1950). Methanol and ethanol are used most frequently. Ethanol is a preferred alcohol in the transesterification process compared to methanol because it is derived from agricultural products and is renewable and biologically less objectionable in the environment, however methanol is used because of its low cost and its physical and chemical advantages (polar and shortest chain alcohol).

The present work is based on whole-cell catalyzed transesterification reaction of used rice bran oil in the presence of alcohols of different chain lengths, preliminary examination by TLC (Thin layer chromatography) indicate the formation of ester in each case.*Table-3*presents the results thus obtained from GC analysis (Annexure 1) showing noticeable influence of the chain length of alcohol on extent of transesterification.

***Table-3*. Effect of different alcohols on extent of transesterification. Data are mean±SD (*n=3*).**

S.No.	Alcohol used for transesterification	% conversion	
		GC	^{1}H NMR
	Primary alcohols		
1	Methanol	8.13±0.06	10.17
2	Ethanol	7.90±0.58	12.76
3	Propanol	3.84±0.26	4.61
4	Butanol	73.79±4.27	69.19
5	Pentanol	75.55±2.30	77.22
6	Hexanol	59.96±1.40	67.57
7	Heptanol	75.41±2.49	74.08
8	Octanol	54.91±4.79	57.48
9	Nonanol	50.62±1.08	47.85
10	Decanol	31.49±1.00	48.90
	Other alcohols		
12	2-methyl-propane-1-ol	67.26±2.72	--
14	Propane-2-ol	5.61±0.66	--
13	2-methyl-propane-2-ol	20.55±1.45	--

The ^{1}HNMR results further confirmed the formation of alkyl esters (Annexure 2). The ^{1}H NMR of methyl ester indicated singlet in the region of 3.60 ppm due to the proton of methyl ester. In case of ethyl ester, appearance of quartet of $-OCH_2$ at 4.10 to 4.20 ppm confirmed the formation of ester. With reference to other alkyl esters, ^{1}H NMR of alkyl ester obtained after transesterification indicated the appearance of triplet at integration value at around 4.10 ppm.

The modified equation proposed in the present study, as outlined in the methodology, could facilitate quantification of different alkyl esters using ^{1}H NMR. To the best of our knowledge, there is no equation available in literature to quantify alkyl ester based on ^{1}H NMR, for acyl donors other than methanol and ethanol. The objective behind proposing this formula was to exploit the efficacy of NMR technique due to obvious advantages such as faster and easily adaptable analysis; non-destructive measurements; and ease with smaller amount of samples (Ghesti et al., 2007). In the present study, the percent conversion obtained by GC significantly correlated ($R^2 = 0.95$) with the percent conversion obtained by ^{1}H NMR (Fig. 1).

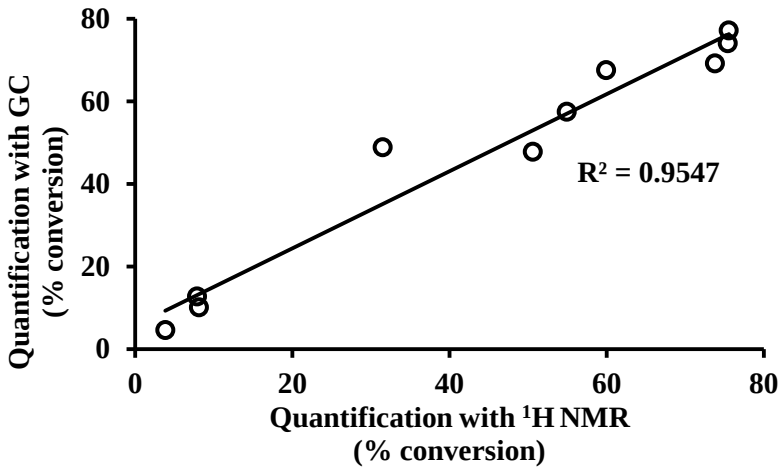


Fig 1. Correlation between the alkyl ester yields quantified using GC and NMR

In the case of transesterification reaction employing methanol, methanolysis takes place between two immiscible liquids (Doell et al. 2008; Stavarache et al. 2008). On formation of diacyl and monoacyl glyceridic intermediates form in sufficient quantities, they serve as surfactants that improve mass transfer of triacylglycerides into methanol phase (Moser, 2009). Ethanolysis reaction proceeds at a slower rate than methanolysis because of the higher reactivity of the methoxide anion in comparison to ethoxide. As the length of the carbon chain of the alkoxide anion increases, a corresponding decrease in nucleophilicity occurs, resulting in reduced reactivity of ethoxide in comparison to methoxide (Sridharan and Mathai, 1974). Butanol is completely miscible with vegetable oils and animal fats because it is significantly less polar than methanol and ethanol (Boocock et al. 1996). Consequently, transesterification reaction employing butanol is monophasic (Zhou and Boocock, 2006a, b). The monophasic nature of butanolysis reaction influences the rate and extent of the reaction and there is no mass transfer limitation, since all reactants and catalysts are contained in a single phase. As a result, initial rate of the butanolysis is faster than that of methanolysis (Schwab et al. 1987). The weaker nucleophilicity of butoxide versus methoxide is another factor that affects the extent of reaction although butanolysis proceeds at faster rate than methanolysis (Schwab et al. 1987). These observations obtained in case of chemical catalysis, also support the

findings in the present study wherein the extent of transesterification was observably low till propanol beyond which it significantly increased with butanol as acyl donor.

Observations reported by Romero et al. (2011) indicated that there was no noticeable influence on the esterification effect when propanol, butanol, hexanol and octanol were used as acyl donors. However, the findings in the present study vary with the above results, as the extent of transesterification enhanced only from butanol to pentanol followed by decrease in hexanol and octanol. The decrease in hexanol is attributed due to experimental artifact as the overall trend indicated increase in extent of transesterification upto heptanol beyond which there was a decreasing trend till decanol. Romero et al. (2002) studied the esterification of acetic anhydride using several alcohols (ethanol to decanol). One of the main conclusions derived was that esterification rate increased with the carbon number of the alcohol. Methanol has a serious negative effect on enzymatic activity. A molar ratio of methanol to oil of above 1:1 leads to serious inactivation of the enzyme. However, when methyl acetate was used as the acyl acceptor, a yield of 92% of methyl ester could be obtained with a molar ratio of methyl acetate to oil of 12:1, and methyl acetate showed no negative effect on enzymatic activity. Additionally, with crude soybean oil as the oil source and methanol as acyl acceptor, a much lower methyl ester yield was obtained than that with refined soybean oil, while with methyl acetate as acyl acceptor, an equally high yield of methyl ester (92%) was achieved for both soybean oils (Wei et al. 2004).

Conclusion

In conclusion, this study presents the observations on the effect of chain length of alcohol (acyl donor) on the process of biocatalyzed transesterification. The study also proposes a derivation based on NMR analysis that can be applied to quantify alkyl esters obtained with primary alcohols beyond ethanol, the observations of which significantly correlated with GC analysis using more simplified equation than the one reported.

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