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Lipase Catalyzed Transesterification of Cottonseed Oil

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Transesterification of vegetable oil is a route to produce fatty acid alkyl ester, which can be used as substitute to petroleum based diesel fuel. Various approaches such as acid, alkali or enzyme catalysis can be used to carry out this process. Among various enzymes, transesterification of vegetable with alcohols commonly uses lipase as catalyst. This study presents the investigations carried out to examine the extent of transesterification through enzyme catalysis. Lipase from *Candida rugosa* was used to examine the extent of transesterification (methanolysis) of the fresh and the waste cooking oil (cottonseed) oil using ethanol or methanol with reference to time. The products thus obtained were characterized using TLC and ^{1}H-NMR spectroscopy.

Key words: Lipase, *Candida rugosa*, tranesterification, cotton seed oil.

There is an increasing interest and awareness, on alternative energy sources such as biodiesel, hydrogen and bio-ethanol to reduce the global dependency on petroleum based fuels. The use of biodiesel is not new as Rudolph Diesel in 1911, first used vegetable oil (groundnut/peanut oil) to power the diesel engine (1). The use of 100% pure vegetable or animal fats to power diesel has several drawbacks such as high viscosity, low power output, thickening or gelling of the lubricating oil, oxidative stability, and low volatility resulting in carbon deposits due to incomplete combustion. Amongst various approaches to circumvent some of these problems, transesterification with short-chain alcohols to form longer-chain fatty acid alkyl esters (FAAE) has been extensively employed. Transesterification is the simplest and the best route to produce biodiesel, in large quantity, that approximate physical characteristics of fossil diesel with little or no deposit formation after combustion in diesel engines. Although several researchers have reviewed biodiesel production through chemical and enzymatic reactions[1-11], very few studies were devoted to the enzymatic approach to biodiesel[3,6,7,12]. Commercially the production of biodiesel utilizes alkaline catalysts to convert vegetable oil or fat to fatty acid methyl esters, (FAME). The basic catalysts employed are hydroxides of sodium or potassium as they are relatively inexpensive[13,14]. However, they form soap with high free fatty acid oils, which consumes

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the catalyst and decreases the yield, thereby making purification and isolation of the FAME difficult. Other basic catalysts employed are sodium or potassium alkoxides, as well as their carbonates. Vegetable oils with high free fatty acid contents are better esterified with acid catalysts. No soap is formed with acid catalysts, but higher temperature and higher alcohol/oil molar ratios may be needed. Acid catalysts are normally avoided and result in slower reactions and a low yield of FAME. In general, using chemical catalysts results in a FAME yield > 98%.

As an alternative to chemical catalysts, transesterification can be carried out with enzymes[15-24]. Of late there has been increasing interest in using lipases as the biocatalyst to convert vegetable oils and fats to FAME. Lipase catalyzed reaction are efficient, highly selective, involve less energy consumption and side products. The recent research involving lipase catalyzed reactions has revolved around discovering best enzyme sources, and optimizing the reaction conditions such as substrate molar ratio, solvent[20] or no solvent[21], temperature, water content[19,20,23], free fatty acid level[18], percent conversion, acyl migration[25], and substrate flow rate in packed bed bioreactors. This has helped to improve the yield of biodiesel comparable to base-catalyzed reactions and for possible industrial scale-up. Other advantages that enzymes have over their chemical counterparts is former's ability to improve the reaction yields by immobilization and genetic engineering. The genetically engineered lipases can accommodate new substrates and higher temperatures. The removal of immobilized enzyme can be done by simple filtration, a step that can be further avoided in case of the use of packed bed of immobilized enzyme. A major problem is inactivation of enzyme in presence of methanol which is used as substrate. The step-wise addition of methanol or use of cosolvents such as *t*-butanol[26] also facilitates preventing lipase inactivation.

NMR spectroscopy has become one of the most powerful techniques to investigate and identify the structure of chemical compounds and dynamics or molecular systems in almost all branches of chemistry[27-28]. The first reports pertinent to use of this technique include yield determination of methanolysis of vegetable oils or animal fat, and studies on kinetics and mechanism of biodiesel production[29]. Present study relates to transesterification of oil using lipase sourced from *Candida rugosa* Type VII. The study here draws a comparison between relative rate of tranesterification (at different time intervals) using different alcohols (methanol or ethanol) with used and refined cottonseed oil.

MATERIAL AND METHODS

Used cottonseed oil was collected from restaurant waste oil stock. Methanol and ethanol were obtained from MERCK. *Candida rugosa* lipase Type VII was purchased from Sigma-Aldrich. 10.0001

Silica gel of TLC (Thin layer chromatography) grade was from Loba Chemie Pvt. Ltd. Hexane, Ethyl actetate and Acetic acid used as solvent were purchased from SDFine.

Studies on Enzyme catalyzed reactions

Lipase sourced from *Candida rugosa* Type VII was prepared as an enzyme solution by dissolving 0.5g powder in 5.0 ml water and the mixture was stirred for 1 hr. After centrifugation at 3500g for 10 min, the supernatant was recovered and used as such in reaction. The reaction mixture consisted of 10g cottonseed oil (used/refined), 0.4g alcohol (methanol/ethanol) and 1ml enzyme solution in 50ml round-bottom flasks. The reaction mixture was then incubated at 35°C in rotary shaker operated at 120 rpm.

Determining extent of transesterification reaction

Progress of the tranesterification reaction was monitored as explained here. 100mL of reaction mixture was mixed in 1mL hexane and the spots from organic layer were marked on glass plates coated with thin layer of silica G. Mobile phase used was hexane:ethylacetate : acetic acid :: 90:10:1 and TLC plates were exposed to iodine vapour for developing.

Quantification of all the products was done using ^{1}H-NMR (Bruker advance II) 400MHz NMR spectrometer and the methyl ester was calculated by using the formula

Calculation of methyl ester content

This was done by using eq. (1) (30)

$$C = 100 \times [2I_{Me} / 3ICH_2 2acyl] \quad ...(1)$$

Where
I_{Me} = integration of peak appearing at 3.05 – 3.65 ppm and
ICH_2acyl = integration of peak appearing at 2.30 ppm.

RESULTS AND DISCUSSION

TLC was used to monitor the progress of the transesterification reaction at different time intervals. The product, methyl ester at 2, 8 and 24 hrs was isolated and the extent of transesterification determined by means of ^{1}H-NMR spectroscopy. This was done for both used and un-used cottonseed oil. NMR spectra of pure cottonseed oil and the corresponding transesterified product with methanol are depicted in Fig. 1.

Double doublet (4.25-4.35 ppm) appearing in Figure 1 (inset) due to glyceryl methylenic hydrogens are replaced by singlet (3.66ppm) due to methyl proton of ester moiety in Fig. 1 indicating transesterified product. Also, if the transesterification is not complete and oil

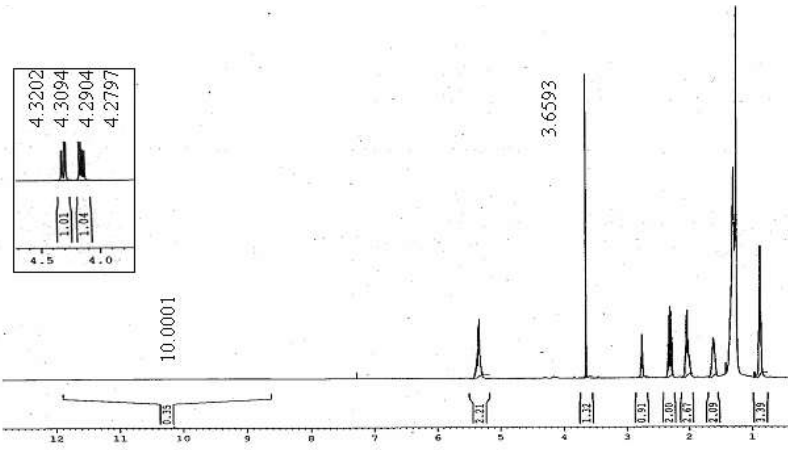

Fig. 1. ^{1}H-NMR spectra of un-used cottonseed oil (inset) and corresponding tranesterified product with methanol and used cottonseed oil as reactants

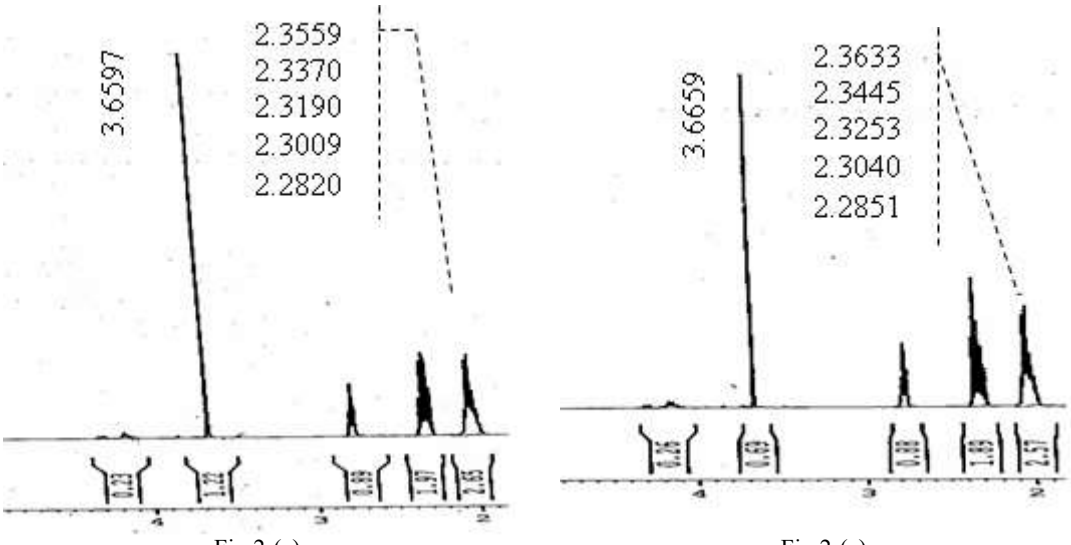

Fig 2 (a) Fig 2 (a)

Fig. 2. ^{1}H-NMR spectra of products after (a) 8 h & (b) 24 h with used cooking oil and methanol as reactants

breaks down into corresponding fatty acid and glycerol we observe broad singlet due to OH group of acid around 9.5-10.5 ppm. The NMR signal for glycerol is not observed in spectra because it goes in water layer due to its high solubility during extraction of methyl ester with hexane. Percentage conversion of oil into resulting methyl ester can be calculated by comparing integral value of singlet at 3.66 ppm with that of acyl protons appearing 2.1 ppm. This is because (i) acyl groups, being adjacent to carbonyl of acid appear in the region where there is no overlap of any other type of protons and (ii) the number of acyl protons in oil and ester remain same. However, since methyl and acyl have 3 and 2 protons respectively, we calculate the integral value of single protons by dividing the total integral value by number of protons involved.

In case of used oil, spectrum (Fig 1) after 2hr of reaction showed complete disappearance of glyceridic peaks (4.25-4.35 ppm) indicating conversion of oil into methyl ester (3.66 ppm) and free fatty acid (10.00 ppm). Interestingly, these peaks later re-appeared at 8 (Fig 2a) and 24h (Fig

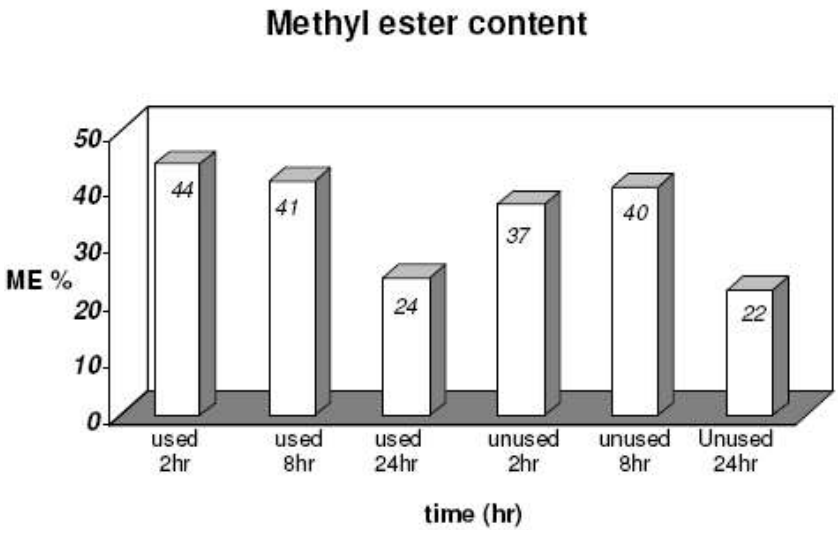

Fig. 3: Methyl ester content of used and unused cottonseed oil in course of time as quantified using ^{1}H-NMR

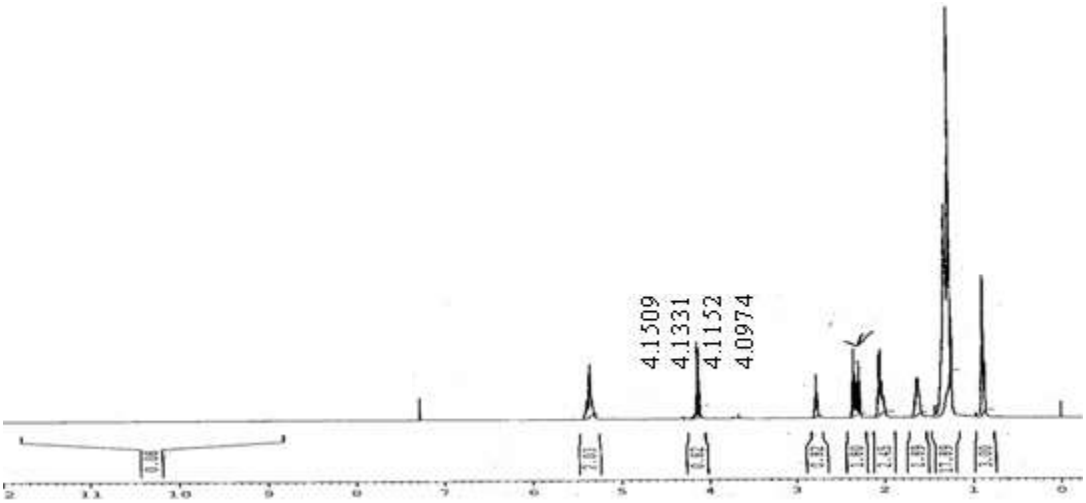

Fig. 4. ^{1}H-NMR spectrum showing incomplete transesterification with waste cooking oil and ethanol as reactants

2b), which is presumed to be due to reversible nature of the reaction.

Freedman *et al*.[31] proposed reversible nature of transesterification reaction for production of biodiesel which was further confirmed by Morgenstern *et al.* [32] by calculating initial rates of FAME formation and calculating an activation energy of 27.2 kJ mol^{-1} for the rate-determining step (di and monoglycerides). Calculation of methyl ester content, formed after 2hr (44 %), 8hr (41%) and 24hr (24%) confirmed the reversibility of the reaction (Fig. 3).

Similar studies were done by replacing methanol with ethanol to observe the influence of the nature of alcohol on transesterification. Substitution of double doublet (Fig. 1) at 4.25-4.35 ppm with quartet at 4.10-4.20 ppm (Fig. 4) due to ethoxy hydrogen's indicated formation of ethyl ester. Appearance of broad singlet at 10.15 ppm point to the presence of free fatty acids there by indicating that transesterification was not complete here as well.

However, in contrast to the observation obtained with methanol, no reversibility was observed here even after 24 hr. Quantification was not done in case of ethyl ester due to lack of suitable validated formula. Available formula[30] uses integration value of glyceridic peak is not suitable for estimation of ethyl ester in this case due to the presence of free fatty acids.

In case of unused oil, yield of methyl ester was lower as compared to used oil. Calculation of methyl ester content based on ^{1}H-NMR using equation (1) showed 37 %, 24 % and 22 % yields after 2, 8 and 24 h respectively. Observation of decreased yields in case of unused oil after 2 h point to reversible nature of *Candida rugosa* sourced lipase-catalyzed transesterification. The results with ethanol in case of fresh cottonseed oil were similar to that with used oil.

CONCLUSIONS

Present work demonstrates a greener way to synthesize biodiesel from cottonseed oil and methanol or ethanol using *Candida rugosa* sourced lipase. Although, the yields were low, the study establishes that i) used cottonseed oil can facilitate higher yields of tranesterified products than fresh one ii) the reversible nature of reaction with methanol and iii) reaction with ethanol cleaves the triglycerides leaving free fatty acids. Work is in progress to increase the yield of methyl ester with other oils using same lipase.

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