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Biodiesel Preparation from Non-Edible *Jatropha curcas* L. Plant Oil using Lipase as Biocatalyst

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Abstract Three lipases from *Candida rugosa*, *Chromobacterium viscosum* and *Aspergillus niger* were explored as biocatalyst for biodiesel preparation from *Jatropha* oil. Among the three lipase tested, lipase from *Chromobacterium viscosum* showed highest activity and specific activity of 53.7 and 1193.0 3μ mol of free fatty acid liberated / min / ml and μ mol free fatty acid liberated / min / mg protein, respectively. The percent conversion of oil into ethyl ester was maximum i.e. 62.3% with addition of 26 mg of *Chromobacterium viscosum* lipase as biocatalyst. The studies on effect of ethanol to oil molar ratio, reaction temperature and reaction time on lipase catalyzed transesterification revealed that 68.3% conversion of oil into ethyl ester (biodiesel) was reported with ethanol to oil molar ratio of 4 : 1, 26 mg *Chromobacterium viscosum* lipase at 40°C after 48 h of reaction time.

Keywords Lipase, Transesterification, Biodiesel, Biocatalyst, Oil.

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Introduction

Energy security and sustainable development are of immense concerns due to their high demand, volatility and global demand [1]. Increasing population and modernization race have escalated energy requirements and per capita energy consumption which is directly proportional to the economic growth of a country. The largest contributors of energy are fossil fuels (67.9%) and other resources include nuclear (10.9%), hydro power (16.2%) and other renewable (5%) [2]. Among them, fossil fuels on the globe are depleting rapidly, thereby, causing dramatic decrease in their prices which will be economically inappropriate. On the other hand, use of fossil fuels has adverse impact on the environment because of the emissions of the harmful gases like CO_2, SOx, and NOx usually known as greenhouse gases [3]. With prevailing threats of green house gases and exhaustion of conventional energy sources, it becomes important to search renewable sources of energy and to develop technologies for efficient extraction of power from them [1].

Biodiesel, as one of the renewable energy source, is getting increasing attention worldwide as an important substitute for petroleum based diesel due to environmental concerns and depletion of vital resources like petroleum and coal [4, 5]. Methyl and ethyl esters of fatty acid known as biodiesel are considered to be non-toxic, bio-degradable and an excellent replacement of petroleum diesel that can be used in diesel engines with few or no modifications [6]. Biodiesel has a higher cetane number than petroleum

diesel fuel, no aromatics, and contains 10 to 11% oxygen by weight. These characteristics of biodiesel reduce the emissions of carbon monoxide (CO), hydrocarbons (HC), and particulate matter (PM) in the exhaust gas compared with diesel fuel [7]. Biodiesel is a product obtained from transesterification of triglycerides or esterification of fatty acids with monoalcohols [8]. This reaction is carried out by chemical or enzyme catalytic method. The enzymatic method has recently gained attention due to some drawbacks associated with chemical catalysis i.e. high energy and reactant consumption, complex treatment of glycerol and high amount of waste water from catalyst [9].

Lipases constitute a diverse and ubiquitous family of enzymes produced by plant, animals and microorganisms. The lipases from microorganisms are the most used biocatalyst for biotechnological applications and organic chemistry [10]. Most of the lipases are able to convert triglycerides, diglycerides, mono glycerides and free fatty acid to fatty acid alkyl esters (biodiesel) in addition to fat hydrolysis [11, 12]. The advantages of using lipases in biodiesel production are their ability to work under mild conditions, no soap formation, capitulate high quality of glycerol, oil with high free fatty acids can be catalyzed with complete conversion to alkyl esters, low alcohol to oil ratio and downstream processing easier [13]. One of major problem with enzymatic transesterification is the high amount of glycerol that accumulates in the reaction mixture and inhibits the lipase activity thereby, decreasing the yield of biodiesel considerably as compared to conventional transesterification. In the present study, three lipases were used as biocatalyst for transesterification of non-edible *Jatropha curcas* plant oil to identify the most efficient lipase as biocatalyst for biodiesel production.

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Materials and Methods

Candida rugosa lipase was procured from Sigma Chemicals Co. ; *Chromobacterium viscosum* lipase was procured from Calbiochem, Merck Millipore. Lipase from *Aspergillus niger* was purchased from Hi Media, Mumbai India.

Determination of lipase activity

The required amount of lipase was dissolved in 20 ml of 0.1 M sodium phosphate buffer (pH 8.0) and lipase activity was assayed titrimetrically by using olive oil as substrate. To the reaction mixture, added 5 ml of oil emulsion, 3 ml of 0.1 M sodium phosphate buffer (pH 8.0) and 1 ml of lipase and reaction mixture was incubated at 37°Cfor 30 min. The reaction was stopped by adding 15 ml of acetone: ethanol (1 : 1). The liberated fatty acids were titrated with 0.05 N potassium hydroxide solution using phenolphthalein as indicator [14]. One unit (1U) of enzyme activity is defined as the amount of enzyme that produces 1 µmol of free fatty acids per min under assay conditions.

Lipase catalyzed transesterification

The transesterification of crude *Jatropha* oil was carried out by standard method [15] with some modifications. The reaction was carried out in three 50 ml screw capped bottles containing each 10 ml of *Jatropha* oil. To this, 1.4 ml of 0.1 M sodium phosphate buffer (pH 8.0) and 1.4 ml of ethanol was added. The required amount of lipase was added separately in each bottle and reaction was carried out in an incubator shaker at a temperature of 35°C for 48 h at 160 rpm. One third of total amount of ethanol was added at 0, 24 and 36 h in the transesterification reaction mixture. Upon the completion of reaction, the product was centrifuged in order to separate the upper layer which was ethyl ester (biodiesel). The different concentration of *Candida rugosa* (50 to 500 mg), *Chromobacterium viscosum* (50 to 500 mg) and *Aspergillus niger* (13 to 52 mg) lipases were used as biocatalyst to identify the most efficient lipase for conversion of crude *Jatropha* oil into ethyl ester (biodiesel). The most efficient lipase was further selected as biocatalyst in transesterification reaction and the effect of ethanol to oil molar ratio (3 : 1 to 6 : 1), reaction time (12 to 72 h) and reaction temperature (20 to 60°C) on maximum conversion of oil into ethyl ester (biodiesel) was studied.

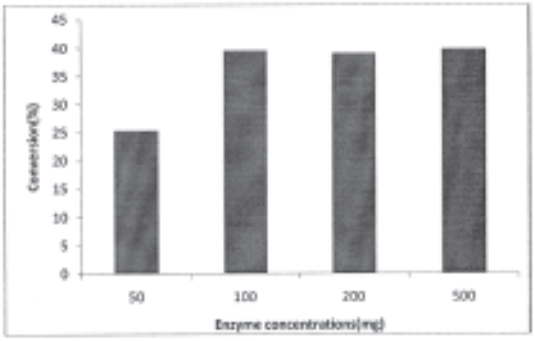

Fig. 1. Effect of addition of different amount of *Candida rugosa* lipase on per cent conversion of *Jatropha* oil into ethyl ester.

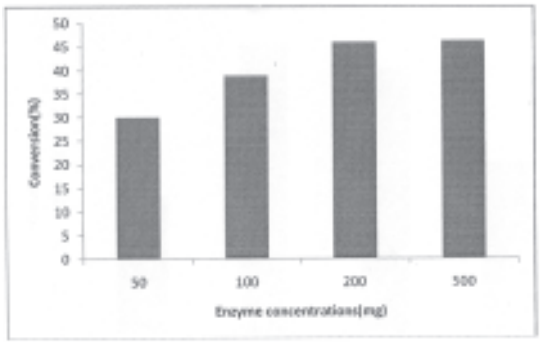

Fig. 2. Effect of addition of different amount of *Aspergillus niger* lipase on per cent conversion of *Jatropha* oil into ethyl ester.

Determination of percent conversion of oil into ethyl ester

The estimation of per cent conversion of oil into biodiesel was done by the method of Kates [16]. The method basically involved the estimation of glycerides present in the form of unconverted oil in the prepared ester. Total glycerides in the oil and unconverted glycerides in the ester (biodiesel) were estimated in terms of glycerol, which was obtained after saponification of the test sample.

Saponification was carried out by adding 0.8 ml of 33% aqueous potassium hydroxide and 20 ml of 95% ethyl alcohol to 0.5 ml of oil/ester sample in a round-bottomed flask. The mixture was refluxed for 90 minutes on boiling water bath. Immediately after refluxing, 20 ml of 2N HCl was added. Thereafter, the mixture was cooled and 40 ml of petroleum ether (40–60°C) was added. The solution was thoroughly mixed again and allowed to settle down for about 20 min. The upper layer of the petroleum ether containing free fatty acid was discarded. The lower layer of ethanol water containing glycerol was evaporated to half the volume in order to remove the excess alcohol. Then, the volume of the solution containing glycerol was made up to 50 ml with distilled water.

Glycerol in the above solution was estimated by the method of Kates [16]. To 0.2 ml of the sample, 1.8 ml of distilled water and 0.1 ml of 10 N H_2SO_4 was added, followed by the addition of 0.5 ml of 0.1 M sodium periodate ($NaIO_4$). This mixture was mixed thoroughly and left at room temperature for 5 minutes. Thereafter, 0.5 ml of 10% sodium bisulfite ($NaHSO_3$) was added, mixed and placed a 0.5 ml aliquot in a glass-stoppered tube, followed by addition of 5 ml of chromotropic acid reagent (0.18%, 100 mg dissolved in 10 ml distilled water and added to it 45 ml of 24 N H_2SO_4). The contents were mixed thoroughly and the tubes were placed in the boiling water bath for approximately 30 min. The tubes were cooled and the absorbance of solution was recorded at 570 nm. Standard glycerol (0.1 to 0.6 µmole) and blank containing distilled water was also run simultaneously (Fig. 2). Total glycerides in oil and unconverted glycerides left in the ester were estimated. Finally, the per cent conversion of oil into ester (biodiesel) was calculated by subtracting unconverted glycerides from the total glycerides.

Table 1. Determination of lipase hydrolytic activity from different sources.

Sources	Lipase hydrolytic activity (µ moles of free fatty acid liberated/min/ml)	Specific activity (µ moles of free fatty acid liberated/min/ mg protein)
Candida rugosa	21.3	507.1
Chromobacterium viscosum	53.7	1193.0
Aspergillus niger	1.3	130.0

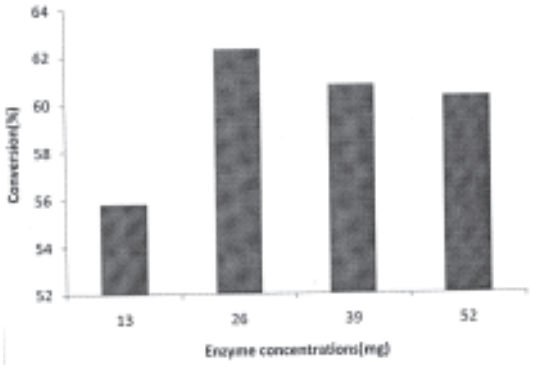

Fig. 3. Effect of addition of different amount of *Chromobacterium viscosum* lipase on per cent conversion of *Jatropha* oil into ethyl ester.

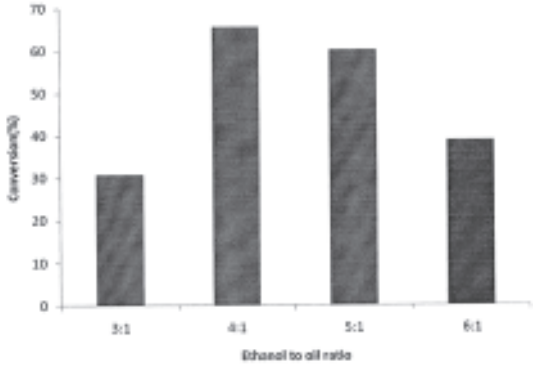

Fig. 4. Effect of ethanol to oil molar ratio on transesterification reaction catalyzed by *Chromobacterium viscosum* lipase.

Results and Discussion

Lipases as biocatalyst for transesterification of *Jatropha* oil

The lipase activity of three lipases was determined by olive oil emulsion method. The total and specific activity of three lipase from *Candida rugosa*, *Chromobacterium viscosum* and *Aspergillus niger* was reported to be 21.3, 53.7 and 1.3 μ mol of free fatty acid liberated / min / ml and 507.1, 1193.0 and 130.0 μ mol free fatty acid liberated / min / mg protein, respectively (Table 1). The lipase from *Chromobacterium viscosum* showed highest lipase activity as compared to lipases from *Aspergillus niger* and *Candida rugosa*.

The effect of addition of different concentrations viz. 50, 100, 200 and 500 mg of *Candida rugosa* and *Aspergillus niger* lipase as biocatalyst into transesterification reaction mixture was studied. The results revealed that maximum conversion of oil into ethyl ester was reported to be 39.8 and 46.2% with 500 mg each of *Candida rugosa* and *Aspergillus niger* lipase, respectively (Figs. 1 and 2). However, maximum conversion of oil into ethyl ester achieved was 62.3% with addition of 26 mg of *Chromobacterium viscosum* lipase as biocatalyst (Fig. 3). The higher conversion efficiency of *Chromobacterium viscosum* lipase may be due to high specific activity of this enzyme as compared to other two enzymes as shown in Table 1.

Biodiesel production using *Chromobacterium viscosum* lipase as biocatalyst

Among three lipases studied, *Chromobacterium viscosum* lipase was considered best for transesterification of *Jatropha* oil into ethyl ester. The *Chromobacterium viscosum* lipase was further selected for transesterification reaction. The effect of various factors i.e. ethanol to oil molar ratio, reaction time and reaction temperature on transesterification reaction using *Chromobacterium viscosum* lipase as biocatalyst was studied to achieve maximum conversion of oil into ethyl ester.

Effect of ethanol to oil molar ratio

The optimum level of ethanol concentration for maximum conversion of oil into ethyl ester using *Chro-*

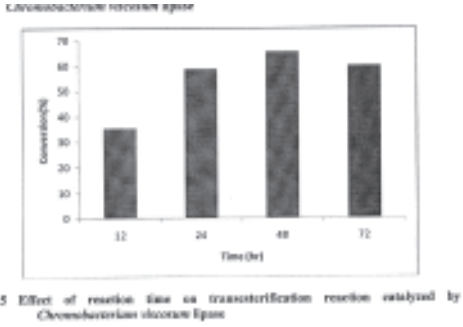

Fig.5 Effect of reaction time on transesterification reaction catalyzed by *Chromobacterium viscosum* lipase

Fig. 5. Effect of reaction time on transesterification reaction catalyzed by *Chromobacterium viscosum* lipase.

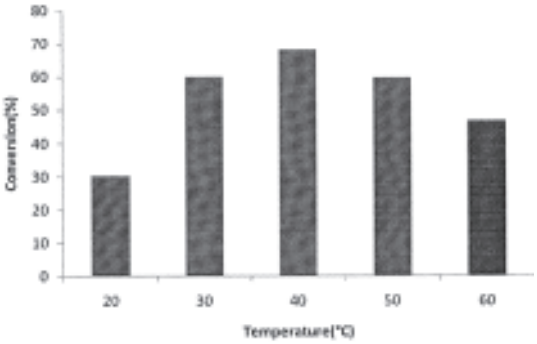

Fig. 6. Effect of reaction temperature on transesterification reaction catalyzed by *Chromobacterium viscosum* lipase.

mobacterium viscosum lipase as biocatalyst was investigated (Table 1). The transesterification reaction was carried out at 35°C with 26 mg of lipase for 24 h with varying ethanol to oil molar ratio from 3 : 1 to 6 : 1. The ethanol in excess of stoichiometric molar ratio of 3 : 1 (ethanol to oil) was added to achieve maximum conversion of oil into ethyl ester. The increase in ethanol to oil molar ratio from 3 : 1 to 4 : 1 resulted in an increase in percent conversion of oil to ethyl ester from 30.8 to 65.6%. Further increase in ethanol to oil molar ratio from 4 : 1 to 6 : 1 resulted in decrease in conversion of oil into ethyl ester i.e. 38.8%. Garlapati et al. [17], however, reported that maximum 62.23% molar conversion of *Simarauba glauca* oil was achieved with methanol to oil ratio molar ratio of 1 : 1 and further increase in methanol to oil ratio resulted in a decrease in methyl ester content. This may be due to the inhibitory effect of methanol on lipase activity [17].

Effect of reaction time and temperature

The effect of reaction time on lipase catalyzed transesterification was studied. The transesterification reaction was carried out at 4 : 1 ethanol to oil molar ratio at 35°C with 26 mg of lipase for 24 h. The samples were with drawn regularly after 12, 24, 48 and 72 h. The percent conversion of oil into ethyl ester increased from 35.4 to 65.4 from 12 to 48 h of reaction and then decreased to 60.0% at 72h.

Generally, reaction temperature has an important effect on activity and thermostability of enzyme biocatalyst. High temperature can denature the enzyme, whereas it takes long time to reach to equilibrium at low temperature. The effect of temperature on transesterification of *Jatropha* oil was studied (Table 1). The transesterification reaction was carried out at 4:1 ethanol to oil molar ratio with 26 mg of lipase for 24 h at different temperature (20 to 60°C). The maximum conversion of oil into ethyl ester i.e. 68.3% was achieved at 40°C. However, percent conversion of oil into biodiesel decreased from 68.3 to 46.8% with further increase in temperature from 40 to 60°C. Similarly Ebrahimi et al. [8] reported that methyl ester yield was maximum at 45°C and the yield of ester decreased considerably when temperature was increased further. The high temperatures (greater than 45°C) had a negative impact on the lipase activity primarily due to denaturation of enzyme.

Conclusion

Based upon the above studies, the maximum 68.3% conversion of oil into ethyl ester was achieved with ethanol to oil molar ratio of 4:1, 26 mg *Chromobacterium viscosum* lipase at 40°C after 48 h of reaction time.

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