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Immobilized Enzyme Lipase Catalyzed Transesterification of Olive Oil in Packed Column Reactor

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Transesterification reaction was performed using triglycerides and short-chain alcohol by immobilized lipase. Olive oil was used as a substrate for the transesterification reaction. The long-chain fatty acid ester, which is the product of this reaction, can be used as a diesel fuel which doesn't produce sulfur oxide and minimize the soot particulate. Immobilized enzyme lipase from *Pseudomonas aeruginosa* showed the highest activity in this reaction. Immobilization of lipase was carried out using sodium alginate beads as a carrier. The activity of immobilized lipase was highly increased in comparison with free lipase. Immobilized enzyme could be repeatedly used without troublesome method of separation and the decrease in its activity was not largely observed. The reaction was carried out in a packed column with beads packed to a height of 12 cm. The conversion is very good and the separation of the product is also simple in this enzymatic route. Since the lipase is immobilized it can be used for several cycles and the process is also economical.

Key Words: Transesterification, Olive oil, Immobilization, Enzyme, Lipase, Packed column reactor, *Pseudomonas aeruginosa*.

INTRODUCTION

In recent times, the world has been confronted with an energy crisis due to depletion of resources and increased environmental problems. The situation has led to the search of an alternate fuel, which should not only be sustainable but also environmental friendly. For developing countries fuel of bio origin such as alcohol, vegetable oils, biomass, biogas, synthetic fuels etc are becoming important. Such fuels can be used directly, while others need some sort of modification before they are used as substitute for conventional fuels[1-3]. Diesel fuel is largely utilized in the transport, agriculture, commercial, domestic and industrial sectors for the generation of power/mechanical energy and the substitution of even a small fraction of total consumption by alternative fuels will have a significant impact on the economy and the environment. Of the alternative fuels, biodiesel obtained from vegetable oil holds good promises as an eco-friendly alternative to diesel fuel[4,5]. The vegetable oils usually contain free fatty acids, phospholipids, sterols, water, odourants and other impurities. Because of these, the oil cannot be used as fuel directly[6]. To overcome these problems the oil requires slight chemical modification mainly transesterification[7,8], pyrolysis[9,10] and emulsification[11]. Among these, the transesterification is the key and foremost important step to produce the cleaner and environmentally safe fuel from vegetable oils[12,13]. Biodiesel is the monoalkyl esters of long chain fatty acid derived from renewable feed stocks, such as vegetable oils or animal fats, for use in compression ignition engine[14]. Biodiesel, which is considered as a possible substitute of conventional diesel is commonly, composed of fatty acids methyl esters that can be prepared from triglycerides in vegetable oils by transesterification with methanol[15,16]. The resulting biodiesel is quite similar to conventional diesel fuel in its main characteristics[17,18].

Transesterification or alcoholysis is the displacement of alcohol from an ester by another in a process similar to hydrolysis, except that alcohol is used instead of water[19,20]. This process has been widely used to reduce the high viscosity of triglycerides. The transesterification reaction is represented by the general equation[21].

$$\underset{\text{Ester}}{RCOOR'} + \underset{\text{Alcohol}}{R''OH} \underset{}{\overset{\text{Catalyst}}{\rightleftharpoons}} \underset{\text{Ester}}{RCOOR''} + \underset{\text{Alcohol}}{R'OH}$$

If methanol is used in this process it is called methanolysis. Transesterification is one of the reversible reactions and proceeds essentially by mixing the reactants[22,23]. However, the presence of a catalyst (a strong acid or base) accelerates the conversion. There are different ways of production with different kinds of raw material, refine, crude or frying oils[24]. There are different types of catalyst, basic ones such as sodium

or potassium hydroxides, acids such as sulfuric acid, ion exchange resins, lipases and supercritical fluids. Transesterification as an industrial process is usually carried out by heating an excess of the alcohol with vegetable oil under different reaction conditions in the presence of an inorganic catalyst. The most commonly used catalysts are alkali hydroxides and alcholates. Alcoholysis is also possible under acidic conditions, but this process requires higher reaction temperatures. A disadvantage of both alkali and acid catalyzed procedures is that the homogeneous catalysts are removed with the glycerol layer after the reaction and cannot be reused. In technical alcoholysis, the purification of glycerol as a secondary product is an important step and it is getting more difficult when large amounts of inorganic materials have to be removed. The disadvantages caused by chemical catalysts are largely prevented by the lipases which allow mild reaction conditions and easy recovery of glycerol without purification or chemical waste produced[25,26]. In this work of transesterification we use the enzyme catalyst lipase obtained from the microorganism *Pseudomonas aeruginosa*. The vegetable oil is olive oil[27]. The reaction is carried out in a packed column of 45 cm height and 2.54 cm diameter. Since the enzyme is costly we immobilize the enzyme and are reused several times. This has made the process very economical.

EXPERIMENTAL

Pseudomonas aeruginosa was selected to extract lipase. The mother culture was brought from NCIM.

Media and culture conditions: The strain from mother culture was inoculated in a 3 mL nutrient broth. The medium was left for overnight incubation at 37 ºC at 170 rpm. The next day culture growth was observed and it was sub cultured into another 250 mL nutrient broth and again kept for overnight incubation. After the culture was taken from the incubator, it was terminated by keeping in the refrigerator. Then it was streaked in nutrient agar plate. The nutrient agar was pour plated in the petriplates and allowed to cool. Then the culture was taken from the refrigerator and kept in the incubator for overnight.

Mineral media was used for further processes. A single colony from the plate was taken and inoculated in 3 mL mineral medium and incubated overnight at 37 ºC. The next day it was subcultured in a 250 m culture flask and the growth curve was plotted for the *pseudomonas* sp[28,29].

Confirmation of strain using gram staining: The strain was confirmed using gram staining procedure. *Pseudomonas aeruginosa* is a gram negative bacteria. The microorganisms that are stained by the gram's method are commonly classified as gram-positive or gram non-negative and appear purple brown the microscopic examination. Others are referred to as gram negative, which appear red. A gram stain is a differential stain requiring a primary stain (crystal violet), which is followed by an iodine solution, then washed by alcohol (95 % ethanol). After decolourizing step, saffranin which is the counter strain, was applied to the smear. Gram reactions are only reliable for cultures of 24 h or less.

From an overnight fresh isolated culture, a loopful culture was spread in a thin film and over a microscope slide, air dried and fixed by passing the slide through the flame. Then it was stained with primary stain for 1 min, after that the slides were washed with distilled water and iodine solution and is left for 60-180 s. The colour resulted after the addition of iodine was then removed by the alcohol wash. After rinsing with water, it was stained with saffranin solution; the counter stain for 30 s. The slides were then washed, dried and visualized under the microscope.

Growth curve for *Pseudomonas aeruginosa*: The growth curve of *Pseudomonas aeruginosa* was plotted. The culture was taken and for every 1 h optical density was measured at 600 nm. The graph was plotted with time in x-axis and the optical density at y-axis. The culture was collected at the log phase and terminated. Lipase was extracted using acetone precipitation of the terminated culture.

Tributyrin agar diffusion assay: The presence of lipase in the culture was checked using tributyrin agar diffusion assay. In this assay tributyrin is added as a substrate for pseudomonas to produce lipase. Tributyrin agar was prepared and pour plated. Then a small amount of culture was pipetted in the plate. The plate was left for incubation overnight at 37 ºC. Next day a zone of clearance was observed which confirmed the presence of lipase in the culture[30].

Acetone precipitation: Culture, acetone and TCA were taken in the ratio 1:8:1. 3 mL of culture was taken and, respectively all other components were taken. Acetone was chilled at -20 ºC for 1 h. When each component was added it was mixed thoroughly. Then the mixture was centrifuged at 10500 rpm for 15 min in the Hitachi centrifuge at 4 ºC. Then the supernatant was discarded and the falcon was kept at room temperature and vortexed at every 20 min gap for 1 h then the mix was centrifuged at 13500 rpm for 20 min in mini centrifuge. The supernatant was saved and the pellet was discarded. The supernatant contains the lipase enzyme along with other enzymes and it was saved at -80 ºC for further use[31,32].

Lipolytic activity: The lipolytic activity of the crude enzyme was found using *p*-nitrophenyl palmitate (*p*-NPP) test. *p*-Nitrophenyl palmitate when reacted with lipase yields *p*-nitrophenol as product. *p*-Nitrophenol standard curve was plotted using various molar concentrations of *p*-nitrophenol against their respective optical density at 410 nm. 50 µL of enzyme was taken in a test tube. 75 µL of *p*-nitrophenyl palmitate was added to it. Then the solution was made up to 3 mL using phosphate buffer (50 mM). The reaction mixture was incubated at 45 ºC for 20 min. Dark yellow colour was formed and the reaction was terminated using chilled acetone (1 mL). Optical density at 410 nm was noted. It was plotted in the graph and the enzyme activity was calculated. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 mol of *p*-nitrophenol per minute[33].

Enzyme immobilization using sodium alginate beads: There are many methods available for immobilizing lipase. Among them forming beads using sodium alginate was the best technique being followed. 3 % sodium alginate powder was made using distilled water. It was stirred well using magnetic pellet. The solution was left still for 0.5 h so that all the bubbles get dissolved. Then 1 mL crude enzyme was pipette into the solution and stirred well. The solution is left till the bubbles dissolve. The solution was taken in a 5 mL syringe

and the beads were dropped from a height of 20 cm. The diameter of the bead was measured. The beads formed were dropped in a beaker containing calcium chloride solution (0.1 M). The beads were stored at room temperature for further use[34,35].

Reactor setup: A glass column of 45 cm height and 2.54 cm diameter was taken as the reactor in which the beads were packed without calcium chloride.

Transesterification: Methanol and olive oil, which is the substrate, were taken in different ratios and mixed well. The solution was added to the column and stoppered. The reaction between olive oil and methanol was catalyzed by lipase which is immobilized in the alginate beads. The ester group was replaced in the olive oil and it becomes a mono alkyl methyl ester. At regular intervals of 0.5 h the optical density at 715 nm was measured for the solution, which is collected from the column. The decrease in optical density indicates the decrease in olive oil concentration. A graph was plotted for optical density *versus* time. The reaction was repeated for three times with three set of solutions to check the change in activity of lipase. At the end of the reaction the column was fully drained and washed with calcium chloride (0.1 M) before the next reaction. Three sets of products were collected[36-39].

RESULTS AND DISCUSSION

Growth curve for *pseudomonas aeruginosa*: The optical density and time values are given in the Table-1 and the growth curve is shown in Fig. 1.

TABLE-1
GROWTH CURVE-*Pseudomonas aeruginosa* AT 600 nm

Time (h)	Optical density	Time (h)	Optical density
0	0.010	7.0	0.245
2	0.025	8.0	0.257
4	0.105	15.5	0.510
5	0.145	24.0	0.526
6	0.217	25.0	0.590

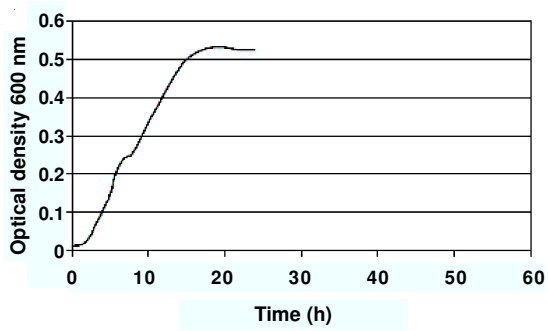

Fig. 1. Growth curve for *Pseudomonas aeruginosa* at 600 nm

Gram staining: The staining procedure showed filamentous pink coloured colonies. So the organism is gram negative and its morphological characteristics showed it is pseudomonas species.

***p*-Nitrophenylpalmitate assay:** The *p*-nitrophenylpalmitate assay was performed. Standard curve was plotted for *p*-nitrophenol at various molar concentrations. The readings at various molar concentrations were noted. They are listed below in Table-2.

TABLE-2
STANDARD PLOT DATA FOR
p-NITROPHENYLPALMITATE ASSAY AT 410 nm

Concentration (µg/mL)	Optical density (nm)	Concentration (µg/mL)	Optical density (nm)
0	0.240	12	1.160
4	0.457	14	1.295
6	0.656	16	1.458
8	0.834	18	1.587
10	0.994	20	1.713

p-Nitrophenylpalmitate was reacted with lipase and *p*-nitrophenol was got as product and the optical density was measured at 410 nm. The reading obtained was plotted in the graph which is shown in Fig. 2. The concentration of *p*-nitrophenol was found and thereby the activity of enzyme for 1 mL was calculated. The reading obtained from the reaction was 1.34 at 410 nm. This reading was plotted in the graph and extended in x axis and the corresponding concentration of *p*-nitrophenol was noted. It was found to be 14 µg/mL. The enzyme activity (1 U) was calculated and found to be 2.161 U mg^{-1} protein.

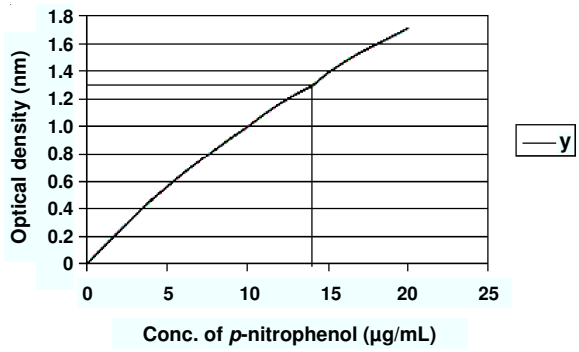

Fig. 2. *p*-Nitrophenol standard curve at 410 nm

Immobilization of lipase using sodium alginate: The lipase enzyme was immobilized as sodium alginate beads which have a diameter of 2.5 mm. The beads were saved in 0.1 M calcium chloride solution. The picture of sodium alginate is shown in Figs. 3 and 4.

Fig. 3. Immobilized enzyme lipase beads

Reactor setup: The beads were loaded in the column and the reactants were added to it. The reaction starts and the optical density of the mixture at 715 nm was noted at regular intervals. There was a steady decrease in the readings, which indicated the decrease in the concentration of olive oil, which

Fig. 4. Beads in the column

is being converted into monoalkyl methyl ester. The bed height, bead diameter and the reactor inner diameter were also noted and the values are given below. Bed height: 12 cm, column height: 45 cm, column diameter: 2.54 cm, bead diameter: 2.5 mm (Fig. 5).

Fig. 5. Reactor setup

Transesterification results: Transesterification was carried packed column reactor by using olive oil and methanol catalyzed by immobilized lipase beads. A standard plot of olive oil concentration *vs*. optical density was made by measuring the optical density for different concentrations of olive oil (0-100 %). The tabulation is given in Table-3 and the standard plot is shown in Fig. 6.

TABLE-3
STANDARD PLOT DATA OF OLIVE OIL CONCENTRATION WITH OPTICAL DENSITY

Conc. of olive oil (%)	Optical density	Conc. of olive oil (%)	Optical density
0	0	60	0.202
10	0.060	70	0.270
20	0.100	80	0.303
30	0.135	90	0.348
40	10.730	100	0.415
50	0.185	–	–

The experiments were carried out for olive oil concentrations of 40, 60 and 80 %. As the reaction proceeds there was a decrease in olive oil content which confirms the biodiesel production. Samples were taken at regular intervals and the optical density was measured at 715 nm. The results were given in Tables 4-6.

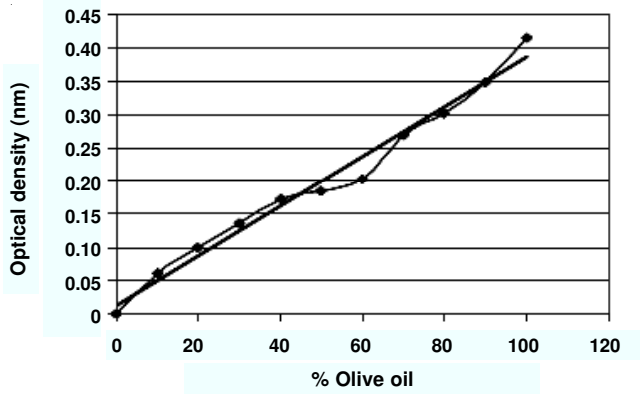

Fig. 6. Standard plot graph of olive oil conc. with optical density

TABLE-4
SPECTROMETRIC ANALYSIS AT 715 nm FOR REACTION 1 (40 %)

Time (h)	Optical density	Time (h)	Optical density
0	0.170	4.0	0.096
1.5	0.161	4.5	0.081
2.0	0.149	5.0	0.068
2.5	0.130	5.5	0.054
3.5	0.109	–	–

TABLE-5
SPECTROMETRIC ANALYSIS AT 715 nm FOR REACTION 2 (60 %)

Time (h)	Optical density	Time (h)	Optical density
0	0.303	1.5	0.185
0.5	0.263	2.0	0.161
1.0	0.221	2.5	0.130

TABLE-6
SPECTROMETRIC ANALYSIS AT 715 nm FOR REACTION 2 (80 %)

Time (h)	Optical density	Time (h)	Optical density
0	0.303	1.5	0.185
0.5	0.263	2.0	0.161
1.0	0.221	2.5	0.130

The percentage biodiesel conversion for the three concentrations were tabulated in Tables 7-9. The strain grew well in mineral medium than in nutrient broth. The tributyrin agar diffusion assay did not respond in either very high pH or very low pH. It responded only in pH 6. It did not respond for high concentration of tributyrin also it responded only in 0.4 % tributyrin. This was because higher concentration of the substrate started to act as an inhibitor for the microorganism growth. The *p*-nitrophenylpalmitate assay was done for 4 sets of enzyme extracts and all the extracts showed almost the same activity. The enzyme was mixed with sodium alginate and the beads were formed perfectly at a diameter of 2.5 mm. The transesterification reaction was performed in a column as a packed bed reactor. It showed decrease in optical density which indicates the decrease in the olive oil concentration which in turn indicated the formation of mono alkyl methyl ester that is biodiesel. The repeated use of the enzyme immobilized beads did not show any significant loss in activity. This showed that the beads could be reused without any problem. The viscosity of product was calculated and it was nearly equal to the viscosity of the biodiesel which was found in literature.

TABLE-7
PERCENTAGE BIODIESEL CONVERSION FOR REACTION 1 (40 %)

Time (h)	Biodiesel production (%)	Conversion (%)
1.5	3	7.5
2.0	6	15.0
2.5	11	27.5
3.5	17	42.5
4.0	21	52.5
4.5	25	62.5
5.0	27	67.5
5.5	31	77.5

TABLE-8
PERCENTAGE BIODIESEL CONVERSION FOR REACTION 2 (60 %)

Time (h)	Biodiesel production (%)	Conversion (%)
2.0	32	53.30
2.5	40	66.67
3.5	46	76.67
4.0	50	83.30
Over night	53	88.30

TABLE-9
PERCENTAGE BIODIESEL CONVERSION FOR REACTION 3 (80 %)

Time (h)	Biodiesel production (%)	Conversion (%)
0.5	11	13.75
1.0	17	21.25
1.5	30	37.50
2.0	43	53.75
2.5	51	63.75

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