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CONTINUOUS HYDROLYSIS OF OLIVE OIL USING INDIGENOUSLY DEVELOPED IMMOBILIZED LIPASE WITH HIGHER EFFICIENCY

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ABSTRACT

Lipase producing bacteria was isolated and screened from waste water near east Kolkata waterbodies (West Bengal). After screening, the media showed maximum lipase activity were selected for further studies. The Maximum specific activity of lipase was calculated as 2.44 mg/min.mg protein for selected media. Qualitative and quantitative estimation of lipase confirmed the presence of lipase in the fractions obtained from 60% to 70% ammonium sulphate cut offs. Hydrolysis of olive oil was enhanced with increasing water concentration and maximum free fatty acid was obtained in 0.13% of water (v/v of oil). Purified lipase was immobilized in different matrices and egg shell immobilized lipase was found to be best in terms of its hydrolysis efficiency. Continuous process with immobilized lipase indicated that it was stable for 216 min with better hydrolysis efficiency as compared to batch process. The present study provides a benchmark for indigenously developed immobilized lipase system for different applications.

KEY WORDS: **Lipase; Purification; Immobilization; Eggshell; Continuous hydrolysis.**

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INTRODUCTION

One of the most significant evolutionary enzymes in the biochemical industry till date invented is triacylglycerol lipase (EC 3.1.1.3). This serine hydrolase has contributed several roles potentially in various industrial segments as a hydrolyzer at the oil-water interface. Having unique identity, lipase has a wide range of applications in biotechnology. The main function of this enzyme is to work on the acylglycerols to liberate fatty acids and glycerol and also hydrolyze long-chain water soluble triglycerides into diglycerides, monoglycerides as well as free fatty acids. Many commercially available lipases are obtained from microbial origin rather than plant or animal sources due to their wide availability and low price. Some of the potential lipase producing organisms are unicellular species that belongs to the genera *Geotrichum, Penicillium, Aspergillus* and *Rhizomucor* and various species of *Pseudomonas*. Owning an exceptional diversification ability, this lipase producing microorganisms are found in industrial wastes, olive oil processing factories, dairy factories, oil-contaminated soil, oilseeds and decaying food, compost heaps, coal tips and hot springs. Having 4% share in the market of enzyme, this lipase possesses as the worth profitable catalytic substance due to enlarging arcade of application of biotechnology worldwide. With variety of lipase available in the market, this group of enzyme constitutes versatility of applications and manufacturing capability by the aid of human beings. Apart from the aforesaid applications, lipase is also used in biotechnological fields, such as synthesis of biodiesel or biopolymers, the production of pharmaceuticals, agrochemicals and neutraceuticals, flavour compounds in the food industry, detergent industry, leather tanning and beverages. Although the enzyme has numerous applications in different types of industries, the availability with specific characteristics is still a restrictive option. Thus, to explore the lipases with divergent properties is considered for weighty research explorations.[1] Downstream processing of extracellular lipase produced by these microorganisms is still a major cost intensive factor in an industrial production process.[2] Enzyme immobilization is one of the significant key processes used to fix the soluble enzyme into a matrix to reduce its cost by reusing it in industrial as well as laboratory scale. Immobilization is done by various methods like adsorption, entrapment, cross-linking and covalent interactions. Continuous process provides higher productivity that in turn reduces cost of the product produced by immobilized lipase.[3] The aim of the present study is to produce and purify lipase from an indigenous microorganism and immobilizing purified lipase in low cost matrices and to improve productivity by running continuous process for olive oil hydrolysis.

MATERIALS AND METHODS

2.1. *Chemicals*

All chemicals were of analytical grade. Tributyrin and Polyvinyl Alcohol (PVA) were purchased from Sigma and Hi-Media Laboratories, India respectively. Cellulose acetate membrane filters (0.22µm) were procured from Millipore India Pvt. Ltd., Mumbai, India. TLC plate (F_{254} silica, aluminium support) was procured from Merck India. Standard Lipase was purchased from Sigma India. The Olive oil used as substrate in this study was purchased from local market. Bradford Reagent Kit was purchased from Genei, Bangalore, India.

2.2. *Sample collection, screening and morphology determination*

The bacterial strain used for the production of extracellular lipase was isolated from water collected from a local agro-industrial waste near Kolkata (West Bengal, India). In order to screen the microorganisms with lipolytic activity, the collected water sample was serially diluted and was spread on a LB agar plate supplemented with Rhodamine B (0.9% w/v) and olive oil (1% v/v). The plates were incubated at 37 °C for 2 days until the strains with lipolytic activity showed shiny orange halos under ultraviolet light (350 nm). Selected bacterial colonies were streak on LB agar plate, and were sub-cultured in order to attain a pure colony. The gram straining process was performed to view the morphology of the isolated strain under compound microscope at 100 X magnification. Result is shown in section 3.1.

2.*3. Media composition and screening*

From literature sources, six different production media was carefully chosen as being the most suitable for the lipase production and denoted as M1, M2, M3, M4, M5 and M6. The media composition is summarized in Table 1. Protocols standardized for each media was maintained to produce lipase using isolated strain. After 48 hrs of incubation, the zone of hydrolysis observed in tributyrin-agar plate indicates the presence of lipases in the media. The media produced lipase with maximum activity was selected for further experiments. Result is shown in section 3.2.

Table 1
Summary of different production media

Media composition	Parameters/ Conditions	Yield/ Productivity	References
M1: Peptone, Tween- 80, $MgSO_4$. $7H_2O$, Beef extract, Na_2HPO_4, $MnSO_4$. $4H_2O$, Yeast extract, $C_2H_3NaO_2$, Glucose, $(NH_4)_2PO_4$	pH 5.0, 30 °C, 48 h	37 U/ml	4
M2: Glucose, Yeast extract, Peptone, K_2HPO_4, NaCl, $FeSO_4$, $MgSO_4$, KCl, NH_4Cl, Olive oil	30 °C, 144 h	N/A	5
M3: Tryptone, Yeast extract, Gum Arabic, $NaNO_3$, $MgSO_4$, Glucose	pH 7, 25 °C, 72 h	4580 U/l	3
M4: Peptone, Olive oil, Yeast extract, NaCl, Na_2HPO_4, NaH_2PO_4, $MgSO_4$. $7H_2O$	pH 7.4, 30 °C, 220rpm, 24 h	10 U/ml	6
M5: Dextrose, Peptone, Yeast extract, $(NH_4)_2SO_4$, $MgSO_4$. $7H_2O$, $FeSO_4$. $7H_2O$, NaCl	pH 6.5, 28 °C, 120 h	4205 U/l	7
M6: Peptone, Yeast extract, Beef extract, $MgSO_4.7H_2O$, K_2HPO_4, $CaCl_2$	pH 7.2, 28 °C, 220 rpm, 24 h	26 U/ml	8

2.4. Growth kinetics

Growth characteristic of the bacterial strain was studied in the LB media. 10 ml of fresh overnight culture was inoculated in 100 ml of sterilized LB media and incubated for 24 hrs at 37 °C in the shaker incubator providing gentle shaking. Samples were taken out at half an hour interval. Optical density (OD) of each sample was measured by a colorimeter at 660 nm. The plate count technique was used to determine the number of viable colonies. The sample was serially diluted with distilled water before plating. All plates were kept in an incubator at 37 °C for overnight. On the next day, the colonies were counted and plotted in graph to obtain the growth pattern of the strain. The result is shown in section 3.3.

2.5. Growth profile of lipase producing bacteria in different production media

After selecting the best suitable production media (section 3.2), the growth profile of bacteria in the media that showed maximum lipase activity was determined. The growth kinetics in production media was carried out for 10 h at 37 °C with gentle shaking. Every half an hour intervals, 1 ml of samples were withdrawn and kept in the 4 °C. The optical densities of those samples were measured at 660 nm. The growth curve was prepared by plotting log colony forming unit (CFU) value with respect to time. The result is shown in section 3.3.

2.6. Quantitative estimation of protein

The product was centrifuged at 5000 rpm for 10 min to separate cells from supernatant. As it was reported that lipase is an extracellular enzyme, the cell pellet was discarded and cell free supernatant was used to estimate protein and lipase activity. For the quantitative estimation of protein, Bradford protein estimation method was performed using Bovine Serum Albumin (BSA) as standard.

2.7. Lipase activity assay

Lipase activity in the culture broth was determined by titrimetry using tributyrin as substrate. One unit of lipase activity is defined as the amount of enzyme required to release one µmol of fatty acid per minute under standard assay conditions such as pH 7 and temperature 37 °C. The protocol of this method illustrated that 2% of Polyvinyl Alcohol (PVA) was dissolved in 10 ml of distilled water and heated for proper mixing, which was denoted as solution1. Solution 2 consisted of 10 ml of tributyrin mixed with 9 ml of polyvinyl alcohol (solution 1). One ml reaction mixture consists of 500 µl of solution 2, 400 µl of phosphate buffer (pH 7, 100 mM) and 100 µl enzyme sample. The mixture was incubated at 37 °C in a shaking water bath for 30 min. At the end of the incubation, the reaction was stopped by addition of 2 ml of acetone: ethanol mixture (1:1) and the liberated butyric acid was titrated with 0.1 N NaOH.

2.8. Precipitation of protein using ammonium sulphate

After centrifugation, the cell free supernatant was then treated with ammonium sulphate salt for protein precipitation. This method was taken into seven parts depending upon the ammonium sulphate saturation percentage, such as 30%, 40%, 50%, 60%, 70%, 80% and 90%. Each precipitation was done in cold condition to prevent denaturation of proteins. After completion of each ammonium sulphate cut, the protein solutions were centrifuged at 10000 rpm for 20 min at 4 °C. From each step the pellet was collected by re-suspending them into phosphate buffer (100 mM, pH 7) and supernatant were used for next cut off. After 90% cut off, the supernatant was discarded.[9] Each precipitate dissolved into buffer was dialyzed against the same buffer using a cellulose membrane dialysis bag (14 kDa) to bring out the salt attached with the desired protein. The dialysis was carried out in cold temperature for continuous 2–3 days with changing with fresh buffer at least 2-4 times. Quantitative analysis of lipase activity was done with each dialyzed sample as described in sections 2.7. The total protein amount was estimated by Bradford assay.[10] Result is shown in section 3.5.

2.9. Hydrolysis of oil

As hydrolysis of oil by lipase is influenced by amount of water present in the reaction mixture, an experiment was carried out using different amount (0.5 ml, 1 ml, 1.5 ml and 2 ml) of phosphate buffer (100 mM, pH 7) with 15 ml of oil and 1 mg enzyme. The reactions were carried out at 37 °C temperature with gentle shaking. After 10 hrs of reaction, the acid value of hydrolyzed oil samples were determined (section 3.6) and qualitative analysis was done by thin layer chromatography method (TLC).

2.10. Qualitative estimation of fatty acid by TLC

Thin layer chromatography was performed to determine the free fatty acids released from olive oil.[2] Hexane, ethyl acetate and acetic acid in the ratio of 9:1:0.1 was used as mobile phase. The different glycerides along with FFA were visualized by putting the TLC plate in a closed chamber saturated with iodine vapour and compared with standards. The amount of FFA released can be qualitatively estimated from the zone of spots developed in the TLC plate.

2.11. Acid value determination

The acid value (AV) is performed to quantitatively determine the amount of free fatty acids (FFA) present in the sample. The FFA liberated after hydrolysis of oil was determined in present study. More FFA in the sample indicates more lipase activity. Acid value is defined as the weight of KOH in mg needed to neutralize the organic acids present per gm of fat or oil. The acid value was determined by the process reported in our earlier publications[11] using the following equation (Eqn. 1):

$$AV = (56.1 \times V \times N)/M \quad \text{... (Eqn. 1)}$$

Where, V= titration volume, N= normality of KOH, M= mass of oil sample

2.12. Immobilization of lipase to increase lipase reusability

In the present study, the immobilization of lipase was done to reuse the enzyme. From various applications standpoint, the immobilization of lipase should be physically robust and preferably applicable in both batch and fixed bed continuous processes. Various low cost materials, namely calcium carbonate, calcium alginate and egg shell were used as matrices for immobilizing the enzyme. The protocols for immobilization of lipase in each matrix are described elsewhere.[12] In brief, for adsorption, 5 ml enzyme solution was incubated with 1 g $CaCO_3$ or powdered egg shell at 37 °C for 30 min. In another set, 0.2% glutaraldehyde was added to facilitate adsorption of lipase into $CaCO_3$. For entrapment, 2% sodium alginate solution was mixed with 5 ml partially purified enzyme and drop wise, added to 2% chilled $CaCl_2$ solution to form alginate beads. After immobilization, the immobilized enzyme preparation was washed with buffer and used for further bioconversion studies. The result is shown in section 3.7.

2.13. Continuous hydrolysis of oil

It was reported that continuous process using immobilized lipase provides better productivity and stability than batch process.[13] To check the stability of immobilized enzyme, continuous hydrolysis was performed in a packed bed reactor. A schematic diagram of reaction setup is provided in Fig. 1. The reactor volume of 10 ml was packed with immobilized enzyme and oil water suspension was continuously flowed through the bed. Samples were collected from the bottom and residence time was measured. Acid values of each fraction were determined to analyze the hydrolysis efficiency of the immobilized enzyme.[6] The result is shown in section 3.8.

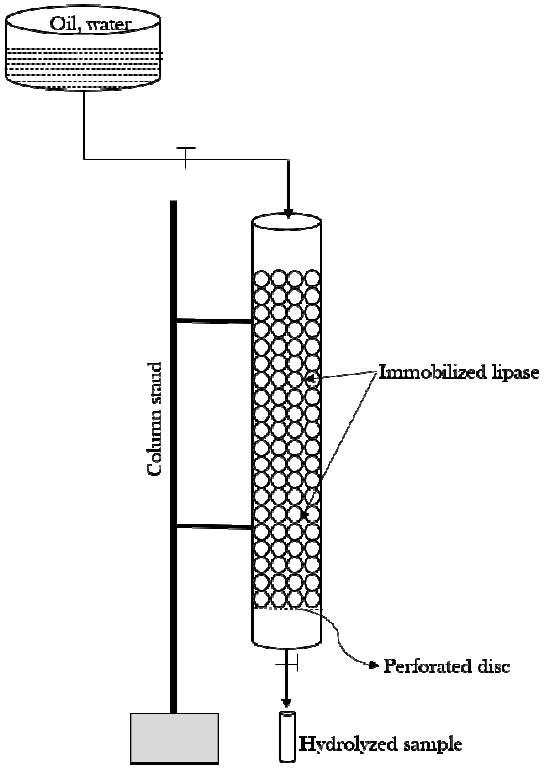

Figure 1
Continuous hydrolysis of oil using immobilized lipase in a packed bed reactor

RESULTS AND DISCUSSIONS

3.1. Screening and isolation of microorganism

The lipase producing bacteria isolated from different water samples were detected using rhodamine dye as an indicator. The bacterial colonies grow in LB agar plates with rhodamine dye and olive oil after 24 hrs of incubation were observed under ultraviolet light in trans-illuminator.[14] It is evident from the figure (Fig. 2) that some of the colonies grown in the plate showed shiny orange halos. The rhodamine is a dye that binds with lipids. Bacteria that uptake olive oil, fluoresces in the presence of rhodamine dye under UV light exhibiting the shiny orange halos and considered lipase producer.[15] Positive bacterial colonies were selected and sub-cultured in LB agar plate to get pure colonies.

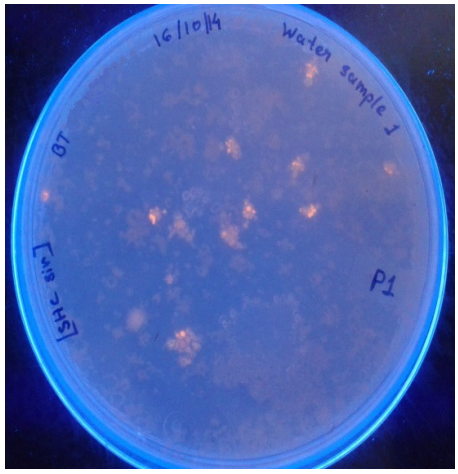

Figure 2
Screening of lipase producing bacteria on LB-agar- Rhodamine B-olive oil plate

3.2. Media screening
From a qualitative estimation of lipase on tributyrin agar plate indicated that media 1–5 (M1–M5) produce lipase with different activities (Fig. 3). M6 media did not show any significant lipase activity on the same plate. Among these media, M1 and M4 showed maximum lipase activity and selected for further experiments.[7]

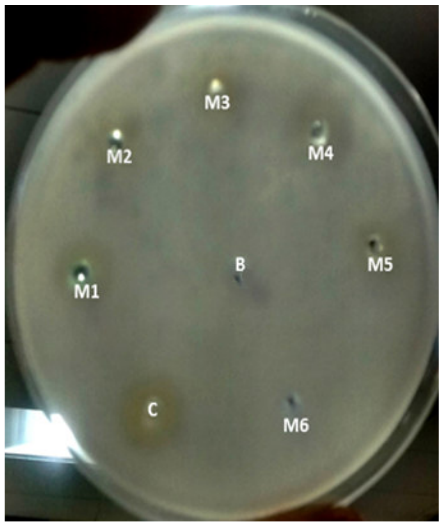

Figure 3
Selection of different production media in tributyrin agar plate.M1 to M6 indicate media 1 to 6. B: blank, C: control lipase

3.3. Growth profile of bacteria in production media
The selected M1 and M4 production media were also for visualizing the rate of growth of the bacteria in the particular media compositions and conditions. The graph demonstrated the comparable study about the increasing number of colony forming units (CFU) in logarithmic value (log N) in production media than the number of cells in the LB media (Figure 4). As lipase produce maximum at end log phase, in production media, rate of enzyme production was faster as compare to LB media.[16]

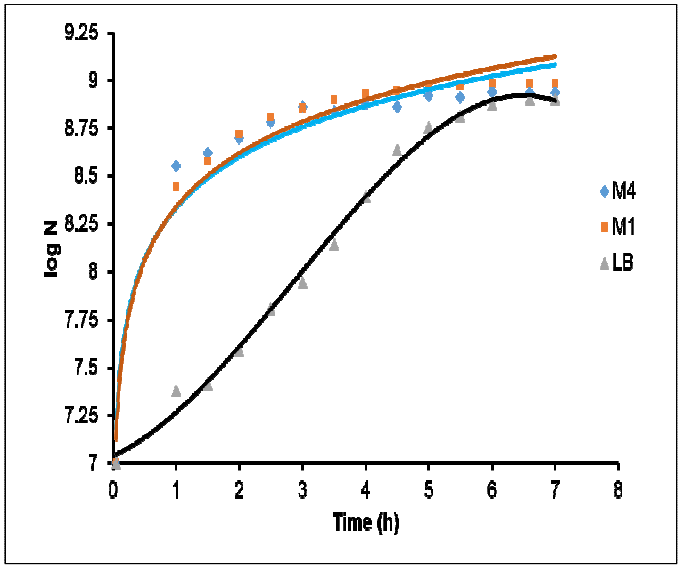

Figure 4
Comparative analysis of bacterial growth in M1, M4 and LB media

3.4. Protein estimation and lipase activity assay

The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10 fold concentration range. Protein estimation using Bradford assay and lipase activity data is represented in Table 2. This is the total protein present in the sample and lipase activity before purification.[17] From the table it is clear that specific activity of lipase is more using M1 as compared to M4 as production medium.

Table 2
specific activities of crude lipase produce in two different medium

Media	Protein amount (µg)	Lipase activity (mg/min)	Specific activity (mg/min.mg protein)
M1	37.5	0.0916	2.44
M4	36	0.0717	1.99

3.5.Partial purification of lipase by salt precipitation

For both M1 and M4 medium, specific activity of lipase increased with increasing ammonium sulphate cut indicated that different amount of lipase precipitate at different fractions during salt precipitation. It was observed that purified lipase activity is little less as compared to un-purified one. This was due to the fact that during purification (salt precipitation, centrifugation and dialysis), the enzyme was subjected to transferred from one vial to another and that might lead to some loss of enzyme activity.[18] From specific activities of proteins obtained in different fractions (Fig. 5) it was observed that as the number of interfering proteins decreased, specific activity increased.[19]

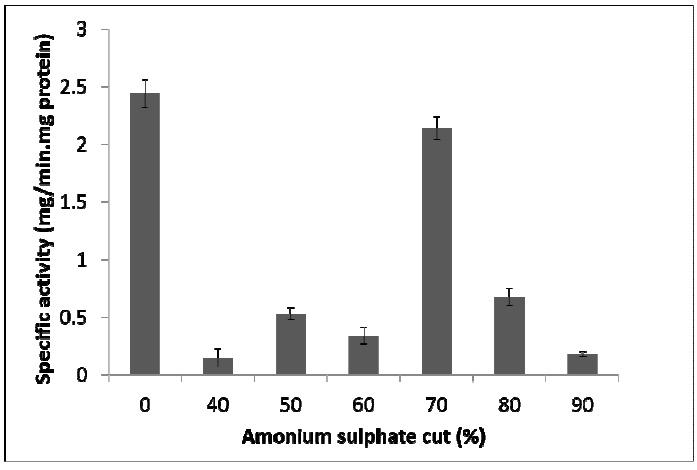

Figure 5
Change in specific activity of lipase with different ammonium sulphate cut

3.6.Effect of water amount in oil hydrolysis using free enzyme

The hydrolyzed products of oil by the partially purified protein with varying concentration of water are summarized in Fig. 6. It was observed that hydrolyzed product of oil increased with the increased amount of water. Minimum product was formed without presence of water. This is mainly due to the fact that lipase acts

on oil water interface.[20] Increasing water amount increases interfacial area, which facilitate hydrolysis. Enzyme activity in terms of hydrolysing efficiency was not decreased with increase in water amount.

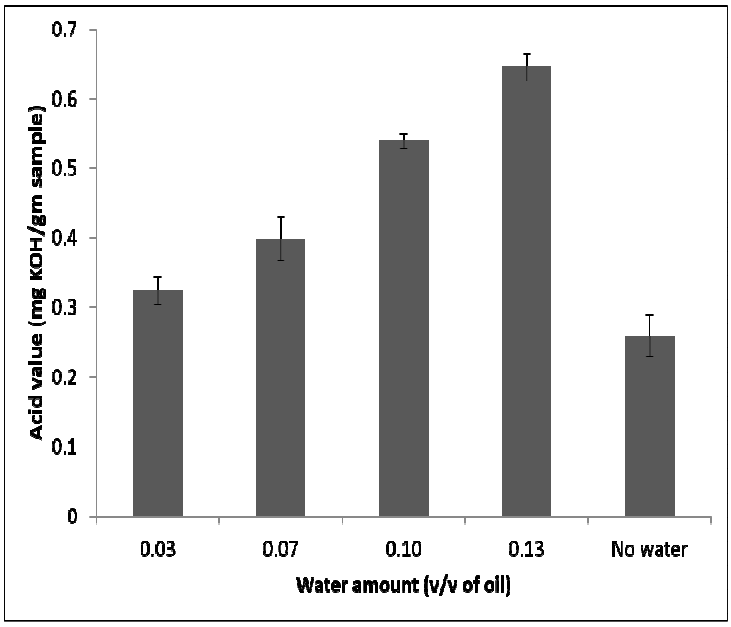

Figure 6
Effect of water amount on hydrolysis of oil by lipase

3.7. Immobilization of lipase in different matrices

Quantitative estimation of FFA was done using acid value determination. It was observed that after immobilization, amount of FFA released was decreased as compared to free enzyme (Fig. 7). This reduction might be due to the hindrance exerted by matrices on enzyme during immobilization.[8] As there is maximum resistance in case of entrapment, minimum hydrolyzed product was observed in case of lipase immobilized into calcium alginate beads.[21] Glutaraldehyde improves cross linking between enzyme and matrix helps in efficient immobilization.[22] The experimental result also indicated little increase in FFA in case of $CaCO_3$ with glutaraldehyde. As egg shell is a low cost waste material[23] and produced almost equal amount of FFA as compared to $CaCO_3$, further experiments were carried out using ES immobilized lipase.

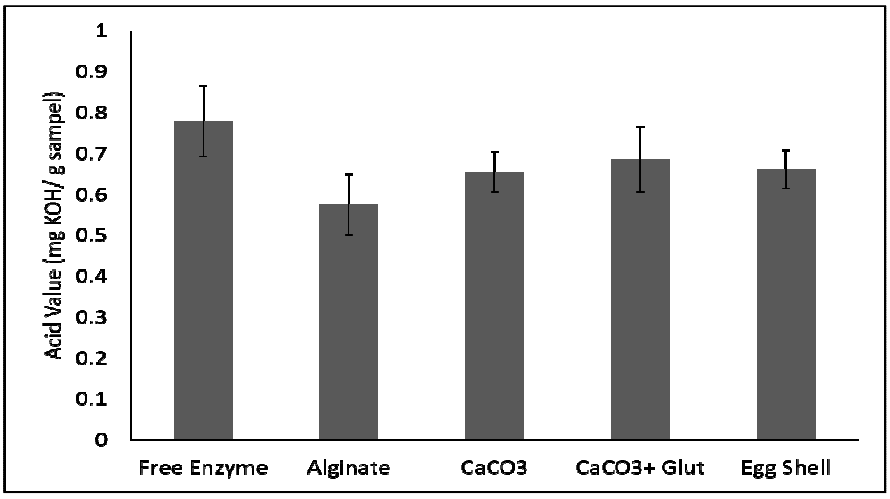

Figure 7
Effect of lipase immobilization on different matrices on hydrolysis of oil; glut: glutaraldehyde

3.8.Continuous hydrolysis using lipase immobilized on egg shell matrix

It was observed that acid value first decrease and then almost stable for 9 fractions (Fig. 8). The residence time calculated from experiments was 24 min. The result indicated that the immobilized lipase could hydrolyze oil almost for 216 min without considerable loss of efficiency. The data indicated that immobilized lipase was stable and could be used for hydrolysis of oil. Gradual loss of efficiency was observed that might be due to leaching of lipase from matrix or inactivation of lipase due to oil or glycerol layer formed over matrix. The hydrolysis efficiency of continuous process was better as compared to batch process.[24]

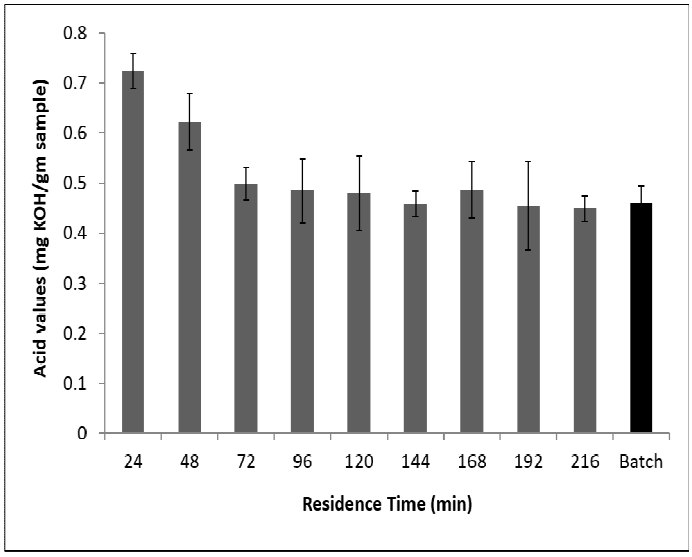

Figure 8
Continuous hydrolysis of oil using immobilized lipase

CONCLUSION

The present study indicates that the lipase was produced and partially purified from indigenous microorganism successfully. The hydrolysis efficiency was increased with increasing water concentration in the reaction mixture. Among different low cost matrices, egg shell showed best suitable for immobilization of the purified enzyme. The immobilized lipase was found to be stable for several hours after use. Continuous process using immobilized lipase provided a better hydrolysis efficiency than batch process. Therefore the importance of cost effective production of bacterial lipase along with reusable cheap and widely available egg shell matrix with enhancing hydrolyzing efficiency can be concluded in this work.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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