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A Thermostable Alkaline Lipase from a Local Isolate *Bacillus subtilis* EH 37: Characterization, Partial Purification, and Application in Organic Synthesis

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Abstract A mesophilic bacterial culture producing a novel thermostable alkaline lipase was isolated from oil rich soil sample and identified as *Bacillus subtilis* EH 37. The lipase was partially purified by ammonium sulfate precipitation and hydrophobic interaction chromatography with 17.8-fold purification and 41.9 U/ml specific activity. The partially purified enzyme exhibited maximum activity at pH 8.0 and at 60 °C. It retained 100% of activity at 50 °C and 60 °C for 60 min. The presence of Ca^{+2}, Mg^{+2}, and Zn^{2+} exhibited stimulatory effect on lipase activity, whereas Fe^{+3} and Co^{+2} reduced its activity. The enzyme retained more than 80% of its initial activity upon exposure to organic solvents, exhibited 107% and 115% activity in the presence of 15% isopropyl alcohol and 30% n-hexane, respectively. The EH 37 lipase also proved to be an efficient catalyst in synthesis of ethyl caprylate in organic solvent, thus providing a concept of application of *B. subtilis* lipase in non-aqueous catalysis.

Keywords Lipase · Esterification · Thermostable

Introduction

Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) are one of the most important classes of hydrolytic enzymes that catalyze both the hydrolysis and the synthesis of esters [1]. Microbial lipases are widely diversified in their properties and substrate specificity, which make them attractive tools for industrial applications. Due to their

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specificity, unwanted side products that normally appear in chemically catalyzed reactions are reduced or completely eliminated. However, an enzyme, i.e., lipase should posses certain characteristics if it has to be used for industrial processes. It must be reasonably thermostable and maintain their activity in organic solvents if they are to be used in synthetic reactions. Thermostability is essential for resisting the chemical modifications caused by high temperatures employed in various industrial lipase catalyzed reactions due to the high melting points of the substrates (lipids). Stability in organic solvents is required since low-water systems based on organic solvents are necessary in order to provide conditions that favor the synthetic reaction over the normal hydrolytic reaction. These systems can offer other advantages for synthetic reactions such as high solubility of substrates and products that are non-polar, provision of oil–water interface required for lipase activity, modification of the specificity of the enzyme, and thermo stabilization of enzyme [2].

Among the numerous lipases described in the literature, only the enzymes belonging to few species have been demonstrated to have adequate stability and biosynthetic capabilities to allow routine use in organic reactions, and hence, they may be considered industrially relevant enzymes [3]. Therefore, attempt to isolate microorganisms that produce thermostable lipase always gains attention since this enzyme can be used in numerous biotechnological processes.

The drawbacks of the industrial application of lipases such as their high cost of production and low stability can be overcome by exploring new sources of immobilization and activation of the biocatalyst [4].

Attractive properties of *Bacillus subtilis* like its capability to secrete homologous and heterologous proteins in appreciable quantities into the growth medium and classified as generally regarded as safe organism by US Food and Drug Administration have made it an important expression host to produce proteins of commercial interest [3]. However, reports of thermostable lipases from *Bacillus* sp. that are active in alkaline (pH 9.0–10.0) conditions are very few [5].

Here, we have successfully isolated an alkaline thermostable lipase-producing bacteria identified as *B. subtilis* EH 37. In this paper, we report isolation of the bacterial culture *B. subtilis* EH 37, partial purification of the lipase, and its characterization. Further, we have demonstrated the potential of this lipase as a catalyst for synthesis of ethyl caprylate, an important ester used in food industry.

Materials and Methods

Phenyl Sepharose® 6 was procured from Sigma-Aldrich (Germany), tributyrin oil and bovine serum albumin were obtained from Himedia (India), olive oil was procured from Figaro (Spain), and gum arabic from Titan Biotech (India). All other solvents and chemicals used during the experiment were of analytical grade.

Bacterial Strain

Screening and Isolation of Lipase-producing Bacteria

The potent lipase producer was isolated from soil sample collected from various oil spilling sites of Vallabah Vidyanagar, Gujarat, India. The *s*ample was subjected to enrichment culture technique by inoculating 1 g of soil sample into 100 mL tributyrin broth (1% (*v*/*v*) tributyrin

oil, 0.5% (*w*/*v*) tryptone, and 0.3% (*w*/*v*) yeast extract) in Erlenmeyer flasks. The flasks were incubated at 37 °C on orbital shaker (150 rpm) for 10 days. The samples were serially diluted and plated onto 1% tributyrin agar plates. The colonies showing zone of clearance after incubation for 48 h at 37 °C were selected. Lipase assay for each isolate was performed, and the isolate showing maximum lipase activity was selected for further experimentation.

Identification of Isolated Bacterium Using 16S rRNA Gene Sequence

The isolate was identified using 16S rRNA gene sequence. Genomic DNA of isolate was extracted as described by Ausubel et al. [6]. The genomic DNA was diluted properly to (20–50 ng) and used as template (30 μl) in polymerase chain reaction (PCR) using universal Eubacteria primers 8F (5′AGAGTTTGATCCTGGCTCAG-3) and 1492R (5′-GGTTA CCTTGTTACGACTT-3′) custom synthesized (BIORON, GmbH, Ludwigshafen, Germany). The amplification of 16S rRNA gene was done in BioRad PCR cycler (USA). Each PCR cycle (35 cycles in total) consisted of a 2-min denaturation step at 94 °C, followed by a 1-min annealing step at 55 °C, and a 1.5-min elongation step at 72 °C, with an initial denaturation step at 94 °C for 5 min, and a final extension step at 72 °C for 15 min. PCR products were resolved on a 1.2% (*w*/*v*) low-melting-point agarose gel in 1X TAE buffer, with a 1-Kb ladder (BIORON, GmbH, Ludwigshafen, Germany) and visualized with ethidium bromide staining in Gel documentation (Alpha-Inotech, USA). The amplified PCR product was subjected to sequencing by automated DNA Analyzer 3730 using ABI PRISM® BigDye™ cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The nearly complete sequence (>95%) was submitted to Genbank at National Center for Biotechnology Information. BLAST (n) program at NCBI server was used to identify and download nearest neighbor sequence from BLAST database [6].

Growth and Lipase Production Profile

The 500-mL Erlenmeyer flasks containing 200 ml tributyrin broth were inoculated with an overnight grown culture of EH 37 to obtain an initial culture density of ($OD_{600\ nm}$) 0.05 and incubated on orbital shaker (150 rpm) at 37 °C. The samples were withdrawn at regular time interval of 24 h and analyzed for cell growth and enzyme activity. The enzyme activity was estimated from the supernatant obtained upon centrifugation of 10 ml medium. The cell pellet obtained upon centrifugation was resuspended in 10 ml of distilled water and its absorbance measured at 600 nm and reported as growth of the culture.

Partial Purification of Lipase

Ammonium Sulfate Precipitation

The calculated amount of solid ammonium sulfate was added to cell free supernatant with constant stirring at 4 °C to achieve 80% saturation**.** The precipitates thus obtained were harvested by centrifugation at 13,000×g for 30 min and resuspended in minimum volume of 25 mM sodium phosphate buffer (pH 7.2). This enzyme solution was subjected to dialysis for 24 h at 4 °C against the same buffer, with three intermittent changes of the buffer. Further, the filtrate was concentrated using 30 KDa centricon filter at 4 °C for 60 min at 800×g. Lipase activity and protein estimation were determined for both the dialyzed and the concentrated filtrate sample.

Hydrophobic Interaction Chromatography

The concentrated lipase solution was applied on a hydrophobic interaction chromatography column using phenyl Sepharose® 6 (1.5 cm×6 cm), which was pre-equilibrated with 0.6 M ammonium sulfate dissolved in 25 mM phosphate buffer (pH 7.2). The bound enzyme was eluted by ammonium sulfate dissolved in 25 mM phosphate buffer (pH 7.2) at a flow rate of 1 mL/min through negative linear gradient. The resultant fractions were subjected for determination of lipase activity and protein content.

Characterization

Effect of pH on Lipase Activity and Stability

Optimum pH of the extracellular lipase was determined by measuring the enzyme activity over a pH value ranging from 4.0 to 12.0. Buffers (50 mM) used were acetate buffer for pH 4.0–5.0, sodium phosphate buffer for pH 6.0–8.0, glycine NaOH buffer for pH 9.0–10.0, and phosphate NaOH buffer for pH 11.0–12.0.

Effect of Temperature on Lipase Activity and Stability

The effect of temperature on the activity of partially purified lipase was determined by monitoring the enzyme activity at different temperatures in the range of 30 to 80 °C at buffer pH 8.0. The thermal stability of lipase was studied from 50 to 70 °C at pH 8.0, and the residual activity was measured at every 15 min interval for a total period of 2 h.

Effect of Metal Ions on Lipase Activity

To determine the effect of various metal ions viz $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CoCl_2$, and $FeCl_3$, the partially purified lipase was pre-incubated with these agents at 1 and 10 mM concentration for 1 h and then assayed for residual lipase activity.

Effect of Organic Solvent on Lipase Activity

To study the effect of organic solvents, the partially purified lipase was incubated for 1 h with various organic solvents like ethanol, methanol, isopropyl alcohol, dimethyl formamide, dimethyl sulfoxide, n-hexane, and acetone to final concentrations of 15% and 30% (*v*/*v*) and then assayed for residual lipase activity.

Synthesis of Ethyl Caprylate Under Water Restricted Environment Using Free and Immobilized EH 37 Lipase

Ethyl caprylate was synthesized by the condensation of ethanol and caprylic acid using free as well as immobilized lipase as described by Dandavate and Madamwar [7]. For immobilization, the partially purified lipase was first entrapped in a surfactant solution comprising of 0.1 M sodium bis(2-ethylhexyl) sulfosuccinate (AOT) in isooctane by vigorously mixing the two solutions to create a clear reverse micellar system of AOT/buffered lipase/isooctane (1 mL). This system was then immobilized by the addition of 14% gelatin (1.5 mL) maintained at 55 °C and vigorous mixing to obtain micro-emulsion-based

organogel (MBGs). The Wo value, which relates to the water activity in MBGs was adjusted to an optimized value of 60. The gel was poured into plastic petri plates, dried overnight, and cut into small pieces. The reaction mixture consisted of 20 mL isooctane and equimolar concentration (100 mM) of ethanol and caprylic acid. The esterification reaction was initiated by addition of the MBGs to the reaction mixture in glass-stoppered flasks kept on orbital shaker at 37 °C and 150 rpm. Five hundred microliters of the reaction mixture was withdrawn every 24 h and analyzed by gas chromatography. Equal amount of partially purified lipase was used as free and for MBG preparation (41.9 units). Ester identification and quantification was done by comparing retention time and the peak area of the sample with a standard (0.1 M ethyl caprylate).

Analytical Procedures

Lipase Assay

The pH stat method: Lipase activity was measured under controlled temperature using a pH 718 STAT Titrino Titrator (Metrohm, Switzerland). The buffer used for the activity determination comprised of 10% (*w*/*v*) gum arabic, 0.2% (*w*/*v*) bile salt, 20% (*w*/*v*) $CaCl_2$, 5% (*v*/*v*) olive oil, as substrate and 0.1 M NaOH as the titrant. The insoluble triglycerides were dispersed by vigorous stirring. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of fatty acid per min at 60 °C.

Protein Estimation

Soluble protein was estimated by Lowry's method using bovine serum albumin as standard [8].

Quantification of Ester

Ethyl caprylate was estimated by gas chromatography (PerkinElmer, Model Clarus 500, Germany) equipped with flame ionization detector and 30 m Rtx-R-20 (Cross bond 80% dimethyl-20% diphenyl polysiloxane) capillary column. Nitrogen served as carrier gas at a split flow rate of 90 ml/min and column temperature ranging from 40 °C to 280 °C. The temperature was programmed with a rise of 40 °C to 210 °C at the rate of 6°/min and from 210 °C to 280 °C at the rate of 15°/min. The temperatures of injector and detector were 250 °C and 280 °C, respectively.

Results and Discussion

Isolation and Identification of Lipase-producing Organism

The use of medium supplemented with emulsified triglycerides is a standard methodology for the selection of lipase-producing microorganisms [3]. Out of all isolates showing clearance zone on tributyrin agar plate, the isolate EH 37 showed the highest lipase activity (9 U/ml), and hence used for further studies. Microscopic examination indicated that the isolate is a gram-positive rod, and biochemical analysis indicated it as *B. subtilis* (data not shown). Also, the DNA sequencing and BLAST analysis of 16S rRNA gene sequence of the strain EH 37 showed maximum sequence homology (100%) with the complete

sequence of *B. subtilis,* and hence in the present study, it is referred as *B. subtilis* EH 37. The sequence has been deposited in Gene Bank with accession Number F J 373271.

Growth and Lipase Production Profile

Maximum lipase activity of 9 U/ml was observed on the third day of production with the optical density 1.750 at 600 nm. On the fourth day, increase in lipase activity was negligible, and after the fourth day, decline in lipase activity was observed (Fig. 1). The decrease of lipase production at the later stage could be possibly due to pH inactivation, proteolysis, or both. Maximum lipase activity on the third day of fermentation has also been reported from *Bacillus licheniformis B 42* [9]. It was observed that EH 37 exhibited maximum lipase production at 37 °C, 150 rpm, and initial medium pH 6.5 (data not shown). Therefore, these conditions were employed for cultures used in future experimentation.

Partial Purification of Lipase

A high state of purity is generally not required in areas such as food processing, detergent, paper, and pulp industry. However, it may be necessary to exclude certain other unwanted impurities such as proteins and enzymes that may exert an antagonistic affect on the desired enzyme's activity [10]. The extracellular lipase from free fraction of liquid culture was subjected to ammonium sulfate precipitation (80% saturation), ultrafiltration, and phenyl Sepharose® 6 column chromatography in sequence resulting in partial purification of 17.8-fold with specific activity of about 41.9 U/mg (Table 1). Compared to other reports, good yield and purification was achieved; thermostable alkaline lipase from *Bacillus coagulans* BTS-3 was purified by ammonium sulfate precipitation and DEAE-Sepharose column chromatography with 4.8 U/ml specific activities and 40-fold purification [5]. Kambourova et al. [11] reported 11.94 specific activity with 19.25 purification fold of lipase from thermophilic *Bacillus stearothermophilus* MC7 employing ultrafiltration, sephadex G 200, and DEAE-cellulose (column chromatography) [11].

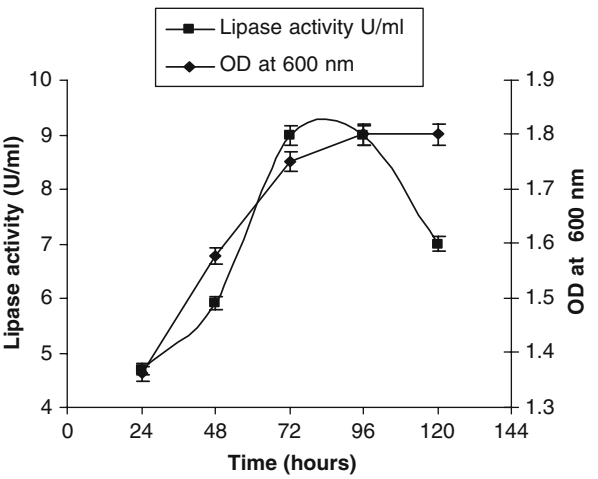

Fig. 1 Lipase production and growth profile of *Bacillus subtilis* EH 37. The production was carried out at 37 °C under shaking condition (150 rpm) in presence of tributyrin oil with initial pH of medium 6.5

Table 1 Summary of purification steps.

Purification method	Volume (ml)	Total activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Crude extract	250	10,500	4,468	2.35	1	100
Ammonium sulfate precipitation	40	2,407	225	10.7	4.6	23
Ultrafiltration	25	2,130	78	27.3	11.6	20
HIC	8	1,633	39	41.9	17.8	16

Characterization

Effect of pH on Lipase Activity and Stability

The activities of lipases are highly pH dependent, and any alteration in the pH reaction mixture is likely to affect their catalytic potential. The pH influences the structure of proteins and hence governs their catalytic activity [12].

The optimum pH for lipase activity of *B. subtilis* EH 37 was found to be 8.0, and it retained 90% of its maximum activity at pH 9.0 (Fig. 2). The data is in agreement with literature information suggesting that *Bacillus* lipases generally have pH optima of 7.0–9.0 [13].

Effect of Temperature on Lipase Activity and Stability

Initial activity was determined in the temperature range of 30 °C to 80 °C. The optimum temperature was found to be 60 °C, and the lipase activity significantly increased with increase in temperature from 30 °C to 60 °C but further increase in temperature adversely affected the lipase activity (Fig. 3).

The lipase was highly stable at 50 °C and 60 °C, retaining 100% of its activity for 60 min and more than 75% for 75 min, whereas at 70 °C, the enzyme retained about 100% activity for 30 min (Fig. 4). The activity and stability in this temperature range is not common for lipases of mesophilic *Bacillus* such as *B. subtilis* [2]. In fact, the characteristics observed in the present work for lipases of *B. subtilis* EH 37 are similar to those found for thermophilic *Bacillus* species such as *Bacillus thermoleovorans CCR11* [14], *B. stearothermophilus* MC7

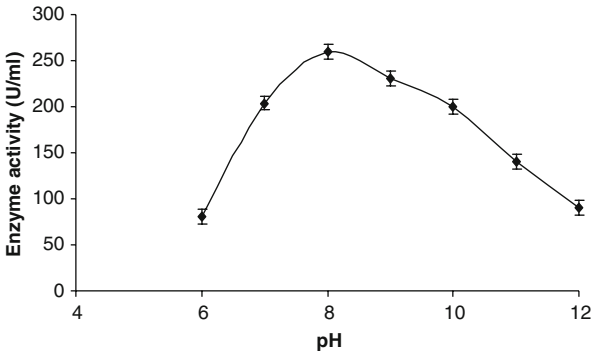

Fig. 2 Effect of pH on lipase activity of *Bacillus subtilis* EH37 after 1 h incubation at 30 °C

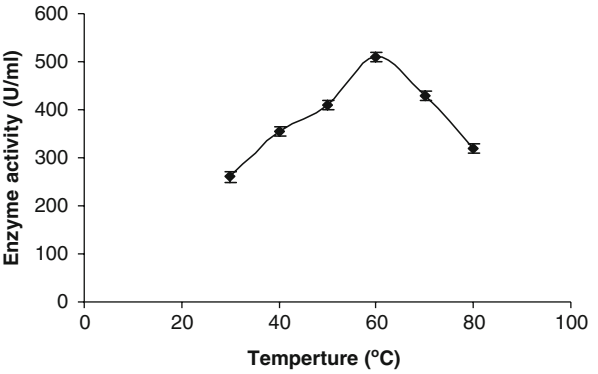

Fig. 3 Effect of temperature on lipase activity of *Bacillus subtilis* EH37 after 1 h incubation at pH 8.0

[11], and *B. coagulans* BTS-3 [5]. However, there are less reports showing thermostable lipase from *Bacillus* sp. retaining 100% of its activity at 60 °C for 1 h [15]. Many reports exhibited less activity under the same conditions [1, 14].

Effect of Metal Ions on Lipase Activity

Metal ions and salts are of importance for thermostability of enzymes from thermophilic organisms. A number of enzymes require the presence of metal ions, such as calcium ions for the maintenance of their stable and active structures [12]. Thus, to find out whether the different metal ions stabilize or destabilize the enzyme, lipase activity was determined in the presence of several metal ions and additives. Table 2 shows that the residual lipase activity increased upon exposure to most metal ions at low concentrations, i.e., 1 mM, except for $FeCl_3$ and $CoCl_2$, which reduced the activity to 95.8% and 95.0%, respectively. Also, as the concentration of all the metal ions used in the study were increased by tenfold, i.e., 10 mM, not much decrease in the lipase activity was observed as the enzyme retained more than 85% of its activity. Another distinguishing feature of this lipase was that it showed high activity in presence of Zn^{+2} ions which is unusual for this class of lipases as Zn^{+2} has shown to be destabilizing for *Bacillus* sp. lipases [1, 5]. It has been suggested that the effect of metal ions could be attributed to a change in the solubility and behavior of the ionized fatty acids at interfaces, and from change in the catalytic properties of the enzyme itself [14]. The stimulatory effect of Ca^{+2} and Mg^{+2} on lipase activity has been observed by

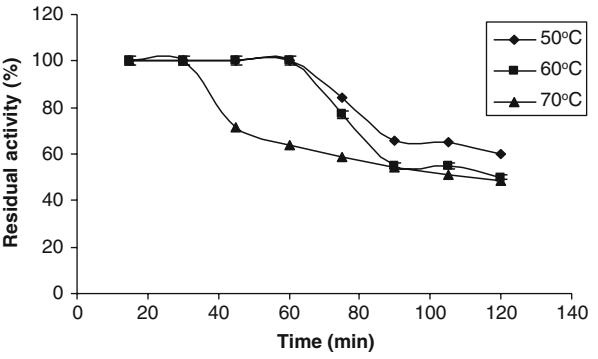

Fig. 4 Thermostability of partially purified lipase of *Bacillus subtilis* EH37

Table 2 Effect of different ions on lipase activity of partially purified lipase of *Bacillus subtilis* EH 37 at pH 8.0 and 60 °C.

Inhibitors	Concentration (mM)	Residual activity (%)
$CaCl_2$	1.00	116.0±1.2
	10.0	94.50±1.2
$MgCl_2$	1.00	107.0±0.9
	10.0	90.00±0.8
$FeCl_3$	1.00	95.80±1.1
	10.0	85.00±1.1
$CoCl_2$	1.00	95.00±1.1
	10.0	86.00±1.1
$ZnCl_2$	1.00	102.0±1.2
	10.0	91.00±1.2

several researchers and has been attributed to structural alterations rather than catalytic activity [16].

Effect of Organic Solvents on Lipase Activity

Lipases are diverse in their sensitivity to solvents, but there is general agreement that polar (water miscible) solvents are more destabilizing than non-polar solvents [14]. Table 3 shows the effect of different organic solvents on lipase activity. In this study, lipase from *B. subtilis* EH 37 exhibited high stability in the presence of 15% and 30% of different organic solvents and showed negligible decrease in activity in presence of most of the organic solvents. Except for n-hexane, the enzyme showed higher activity in lower

Table 3 Effect of different solvents on activity of partially purified lipase of *Bacillus subtilis* EH 37 at pH 8.0 and 60 °C.

Solvents	Concentration (mM)	Residual activity (%)
Ethanol	15	88.00±1.4
	30	80.00±1.3
Methanol	15	82.00±1.2
	30	76.00±1.2
Isopropyl alcohol	15	107.00±1.5
	30	86.60±1.3
Dimethylformamide	15	84.70±1.1
	30	76.00±1.1
Dimethyl sulfoxide	15	100.0±1.2
	30	81.00±1.2
n-Hexane	15	98.0±0.7
	30	115.0±0.9
Acetone	15	102.0±1.3
	30	99.80±1.0

concentration (15%) than in higher (30%). This may be attributed to the fact that a thin layer of water molecules remains tightly bound to the enzyme acting as a protective sheath along the enzyme's hydrophilic surfaces and allowing retention of the native conformation [14]. Polar solvents tend to strip this thin water layer and distort the enzyme's conformation resulting in lower activities, while non-polar solvents do exactly the opposite. Since n-hexane is a highly non-polar solvent, its presence in high concentration enhanced the activity as shown in Table 3. Other possibilities could also be considered that can explain the stimulatory effect of solvents on enzyme activity; the solvent may modify the oil–water interface to make enzymatic action easier without causing protein denaturation. Secondly, enhancement in enzyme activity could be due to disaggregation of lipase, or solvents may induce some structural change in the enzyme.

Synthesis of Ethyl Caprylate Under Water Restricted Environment Using Free and Immobilized EH 37 Lipase

The biosynthesis of esters is currently of much commercial interest because of the increasing popularity and demand for "natural" products amongst consumers [17, 18]. This indicates that biocatalysts have an advantage over their chemical counterparts. It has also been observed that fatty acid esters synthesized using enzymes often have better odor and flavor characteristics when compared to those produced chemically [18].

The ester produced here was ethyl caprylate, which has a "fruity-flowery" fragrance. This ester is used as a constituent in various fruity flavors such as peach, apple, banana, and pineapple aroma. It has been discovered as a flavor-enhancing compound in fermentation industry and commonly associated with wines and whiskey [19, 20].

The partially purified lipase from EH 37 exhibited significant esterification efficiency with 55.8% for synthesis of ethyl caprylate. When immobilized in AOT-based organogels, it exhibited maximum of 1.34-fold (approximately 75%) higher esterification efficiency for synthesis of ethyl caprylate when compared to free enzyme (Fig. 5). Hence, EH 37 enzyme may prove to be promising in esterification and improvement in purification may increase its efficiency as a biocatalyst.

The immobilization of lipase in MBGs has been reported to improve the catalytic of enzyme by providing protection against inhibitory effect of organic solvents as well as aid in reusability of lipase facilitating easy recovery by simple filtration [7].

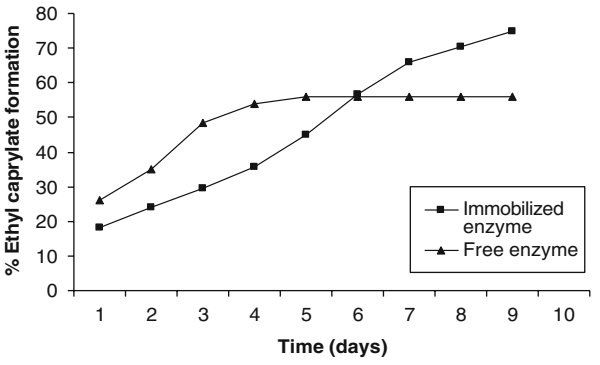

Fig. 5 Profile of ethyl caprylate synthesis catalyzed by free and immobilized EH 37 lipase

Conclusion

A mesophilic organism *B. subtilis* EH 37 producing lipase with interesting characteristics was isolated from oil rich soil. It produced an alkaline thermostable lipase, which was purified 17.8-fold by ammonium sulfate precipitation and hydrophobic interaction chromatography to give a final specific activity of 41.9 U/ml. It exhibited unusual features, similar to those found in thermophilic *Bacillus* species. The distinguishing features included its stability in organic solvents and enhanced activity in presence of Zn^{+2} ions which have been generally associated with decrease in lipase activity. Its ability to catalyze the esterification reaction in free as well as immobilized state highlights its promising applicability in organic synthesis.

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