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Immobilization and stabilization of *Pseudomonas aeruginosa* SRT9 lipase on tri(4-formyl phenoxy) cyanurate

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Abstract−Lipase was extracted and purified from *Pseudomonas aeruginosa* SRT9. Culture conditions were optimized and highest lipase production amounting to 147.36 U/ml was obtained after 20 h incubation. The extracellular lipase was purified on Mono QHR5/5 column, resulting in a purification factor of 98-fold with specific activity of 12307.81 U/mg. Lipase was immobilized on tri (4-formyl phenoxy) cyanurate to form Schiff's base. An immobilization yield of 85% was obtained. The native and immobilized lipases were used for catalyzing the hydrolysis of olive oil in aqueous medium. Comparative study revealed that immobilized lipase exhibited a shift in optimal pH from 6.9 (free lipase) to 7.5 and shift in optimal temperature from 55 °C to 70 °C. The immobilized lipase showed 20-25% increase in thermal stability and retained 75% of its initial activity after 7 cycles. It showed good stability in organic solvents especially in 30% acetone and methanol. Enzyme activity was decreased by ~60% when incubated with 30% butanol. The kinetic studies revealed increase in K_M value from 0.043 mM (native) to 0.10 mM for immobilized lipase. It showed decrease in the V_{max} of immobilized enzyme (142.8 μmol min^{-1} mg^{-1}), suggesting enzyme activity decrease in the course of covalent binding. The immobilized lipase retained its initial activity for more than 30 days when stored at 4 °C in Tris-HCl buffer pH 7.0 without any significant loss in enzyme activity.

Key words: *Pseudomonas aeruginosa* Lipase, Multipoint Binding, Immobilization, Olive Oil Hydrolysis

INTRODUCTION

Lipases (EC 3.1.1.3), triacylglycerol hydrolases, are enzymes that catalyze the hydrolysis of triglycerides at the oil-water interface. To date, a large number of lipases from bacteria and fungi have been extensively studied, both from the biochemical and from the genetic point of view [1-3]. The most productive species belongs to genera *Geotrichum*, *Penicillium*, *Aspergillus* and *Rhizomucor* [4,5]. Lipases from unicellular bacteria, mainly those produced by various species of the genus *Pseudomonas*, have proven to be useful both in organic reactions and in the detergent industry [6]. Many of them have been purified, characterized and their encoding genes cloned. Microbial lipases have potential applications in a number of industrial processes such as synthesis of triglycerides, esterification of terpenic alcohols, resolution of secondary alcohols, as cosmetic ingredients or surfactants [7-11].

Among the high number of lipases described in the literature only the enzymes belonging to a few species have been demonstrated to have adequate stability and biosynthetic capabilities to allow routine use in organic reaction, and hence, considered as industrially relevant enzymes [12,13]. Therefore, to improve the efficiency of certain chemical processes there is a need for the immobilization of enzymes by different techniques based on the consideration of the stability and reuse of biocatalyst [14]. The choice of the support is of paramount importance in the development of an efficient biocatalytic process. Among various immobilization techniques, immobilization on solid support by covalent attachment offers several advantages, such as high concentration and even distribution of the enzyme, enhancing operational stability, continuous operation, and repeated usage of derivative and retention of the biocatalyst [15]. Multibinding of enzymes has been conducted mostly with organic synthetic polymers because of the ease of fabrication of desirable structures and the availability of the reactive functional groups [16-22].

In the present paper an attempt has been made to immobilize lipase on tri (4-formyl phenoxy) cyanurate (Tris support) via multipoint attachment. This is a simple protocol for the immobilization of lipase on Tris organic supports that permits a dramatic increase in enzyme stability. Immobilization on this support seems to be one of the most promising possibilities to improve enzyme stability, since the relative positions of all the groups implied are maintained during enzyme conformational change. Though different supports have been used for enzyme immobilization, Tris organic support is exceptionally efficient for immobilization of protein. Some of the properties that contribute for its successful utilization similar to glyoxyl support [23] are as follows:

1. Very high reactivity of carboxaldehyde group with non-ionized amino group of enzyme.

2. The Tris support is symmetric and has no steric hindrance in reaction with amino groups in the enzyme.

3. High stability of Tris support permits efficient enzyme support

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multi interaction and long storage times.

4. Multipoint covalent attachment was carried out by most reactive amine (in most cases the terminal amino group) and not by the area richest in Lys groups. Under this condition the reaction of the enzyme and the support directly yield stable bond.

The catalytic efficiency of the immobilized lipase was examined in terms of hydrolytic activity, stability and reuse in an aqueous medium at variable pH and temperatures. The kinetic studies were performed to determine the K_m and V_{max} values from the Lineweaver-Burk plots. The stability of native and immobilized enzyme was also studied by incubating enzymes in different organic solvents.

EXPERIMENTAL

1. Materials and Methods

1-1. Microbial Strain

The bacterial strain *Pseudomonas aeruginosa* SRT9, a high lipase producing strain. was isolated from petroleum-spilled soil and identified by 16S rDNA technology. All chemicals were procured from S.D. Fine Chemicals and Qualigens Fine Chemicals, Mumbai, India. The chemicals were of A.R grade (98%) and used without further purification unless stated otherwise. 4-hydroxybenzaldehyde, Cyanuric chloride was purified by recrystallization from petroleum ether (80-100 ºC). The partially purified lipase stored at 4 ºC was dissolved in 20 mM Tris HCl buffer pH 7.0 and used for the experimental work.

2. Enzyme Production and Purification

Pseudomonas aeruginosa cells were cultured aerobically in 500 ml production medium containing per liter: peptone 5 g, yeast extract 2 g, beef extract 1 g, NaCl 5 g, $CaCl_2$ 0.05 g, 10 ml Tween 20 for 20 h at 37 ºC with agitation at 150 rpm. The culture broth was then centrifuged at 8,000 rpm for 20 min at 4 ºC using Remi's cooling centrifuge. Solid ammonium sulfate was then added slowly to the culture filtrate to attain 30% and 70% saturation respectively with gentle stirring on ice bath [24]. The mixture was then centrifuged at 1,200 rpm for 30 min at 4 ºC. Pellets of fractions 30% and 70% were dissolved in 10 ml of 20 mM Tris HCL buffer, pH 7.0 and dialyzed overnight against 2 L of the same buffer. 30% active fraction showing maximum lipase activity was applied to 15 ml of activated Phenyl Sepharose CL-4B (1.5×24 cm) previously equilibrated with 20 mM Tris HCL buffer, pH 6.8. The enzyme was eluted with linear gradient of 0-1% (w/v) cholate in 20 mM Tris HCL buffer, pH 6.8 with flow rate of 1 ml/min. All the fractions were checked for enzyme activity. The active fractions were pooled and applied on pre equilibrated Mono Q HR5/5 column (1×6 cm). The enzyme was eluted by NaCl (0-1.0 M) gradient in the same buffer at a flow rate of 1 ml/min. Fractions containing active enzyme were pooled and assessed for protein content. The resulting partially purified lipase enzyme was used for further experiment.

3. Synthesis and Characterization of Tri (4-formyl phenoxy) Cyanurate

In a 500 ml three-neck round bottom flask (arranged with a dropping funnel, a thermal and stirring arrangement) a solution of sodium hydroxide (6.2 g, 0.155 mol) and 4-hydroxy benzaldehyde (18.929 g, 0.155 mol) prepared in 100 ml distilled water was taken. The solution was cooled to 5 ºC by using ice; then a solution of cyanuric chloride (9.22 g, 0.05 mol) in 100 ml acetone was added dropwise in about 30 min with constant stirring. The reaction was continued further at the same temperature for 3 h. At the end of the reaction, the solution in the flask was poured on the crushed ice. The white product so formed was separated by filtering through Whatman paper No I and washed several times with distilled water and dried under reduced pressure at 100 ºC for 5-6 h [25]. The synthesized product tri (4-formyl phenoxy) cyanurate was designated as A and was monitored by thin layer chromatography [26].

The textural parameters (surface area, *S*BET; pore volume, *V*p and pore diameter *d*p) of Tris support were obtained from N2 adsorption data measured at 77.4 K using a volumetric adsorption setup (Micromeritics ASAP 2010, USA). All the samples were degassed at 50 ºC for 3 h prior to N2 adsorption. The specific surface area of the sample was calculated by using the multiple-point Brunauer-Emmett-Teller (BET) method in the relative pressure range (*P*/*P*0) of 0.05-0.3. The pore size distribution was determined using the Barrett-Joyner-Halenda (BJH) method, and pore sizes were obtained from the peak positions of the distribution curves. Fourier transform infrared (FTIR) spectra were collected on Bomem Canada, model MB-104 spectra in the range of 400-4,000 cm^{-1}. Molecular weight of the immobilized enzyme was determined by MALDI TOF-TOF 4800 mass spectrometer from Applied Bio systems utilizing a nitrogen laser emitting at 337 nm and an accelerating voltage of 25 Kv. Measurements were performed in the delayer-extraction mode using a low mass gate of 2,000. The mass spectrometer was used in the positive ion detection and linear mode. Samples of the digestion mixture were placed directly on a 96 well plate and allowed to air dry after the addition of an equal volume of saturated solution of 3,5-dimethoxy-4-hydorxycinnaminic acid (sinapinic acid) in 50% acetonitrile and 0.3% trifluoroacetic acid.

4. Immobilization of Enzyme

The enzymes were immobilized by carrier binding method, in which enzymes are made to bind to the support material by covalently. For immobilization, 30 mg of tri (4-formyl phenoxy) cyanurate and 90 mg of lipase in 10 ml of 20 mM Tris HCl buffer pH 7.0 was taken and stirred continuously for 30 min at room temperature. The amino group of enzyme was made to bind reversibly to the aromatic aldehyde group of tri (4-formyl phenoxy) cyanurate to form Schiff base [27,28].

After immobilization the amount of unbound and bound (immobilized) enzymes were subjected to protein estimation and the amount of lipase immobilized (mg/mg support) was calculated by the formula.

$$\text{The amount of enzyme immobilized } P_b=(C_oV_o-C_fV_f)\,W$$

Where C_o is the initial protein concentration (mg/ml)
C_f is the protein concentration of the filtrate (mg/ml)
V_o is the initial volume of lipase solution (ml)
V_f is the volume of lipase of the filtrate (ml)
W is the weight of the support.

The percentage of enzyme immobilized to support was determined by the difference between the initial activity of the native enzyme and the activity of the filtrate after the immobilization process using pNPP as a substrate.

$$\text{Immobilization efficiency } (\%)=[(E_oV_o-E_fV_f)/E_oV_o]\times 100$$

Where E_o is the initial lipase activity (U/ml)
E_f is the lipase activity in the filtrate (U/ml)

The catalytic efficiency of immobilization enzyme was determined as a ratio between the specific enzyme activities of 1 mg native lipase and the amount of immobilized enzyme which contains 1 mg enzyme by taking into account the % of covalently immobilized enzyme. It is calculated by the formula:

$$\text{Catalytic efficiency } (\%) = (E_{imm}/E_{nat}) \times 100$$

Where E_{imm} is specific activity of the immobilized lipase
E_{nat} is specific activity of the native lipase

5. Enzyme Loading Capacity of Tri (4-formyl phenoxy) Cyanurate

Enzyme was loaded to the supporting material giving a stoichiometry of 3 : 1 ratio [enzyme 90 mg (0.477 mmol) and 30 mg (0.159 mmol) support]. The three enzyme molecules are bound to the three binding sites present on the single molecule of support. After immobilization the amounts of unbound (filtrate) and bound (immobilized) enzymes were subjected for protein estimation.

6. Protein Measurement

The protein content of the enzymes was determined from the calibration curve established with bovine serum albumin (fraction V) by the standard method of Lowry [29].

7. Enzyme Assay

The hydrolytic activities of free and immobilized lipase were assayed by the olive oil emulsion method according to the modification proposed by Soares et al. [30]. The substrate was prepared by mixing 50 ml of olive oil with 50 ml of gum arabic solution (7% w/v). The reaction mixture containing 5 ml of the emulsion, 2 ml of 100 mM sodium phosphate buffer (pH 7.0) and either free (1 ml) or immobilized (1.2mg/mg support) lipase was incubated for 5 min at 37 °C. The reaction was stopped by the addition of 10 ml of acetone-ethanol solution (1 : 1). The liberated fatty acids were titrated with 25 mM sodium hydroxide solution using phenolphthalein as an indicator. One unit (U) of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of free fatty acid /min under the assay conditions.

The spectrophotometric assay with p-nitrophenyl palmitate (p-NPP), as the substrate was determined according to Kordel et al. [31]. The liberated p-nitrophenol was measured at 410 nm with UV-vis Shimadzu spectrophotometer. One unit (U) of enzyme was defined as the amount of enzyme that releases 1 μmol of p-nitrophenol from the substrate under the assay conditions.

8. Kinetic Assay

Assays were performed by using better physicochemical conditions for optimization of enzyme activity (free and immobilized) at various pH ranging from 4.0 to 10.0 and temperature ranging from 30 to 90 °C. The optimum pH and temperature for both native and immobilized enzymes were determined by spectrophotometric assay using pNPP as substrate. Thermostability of both native and immobilized enzymes was examined at a temperature ranging from 50-70 °C for different time intervals (0.5-3 h) in 20 mM Tris HCl buffer pH 7.0.The pH was adjusted at the start of incubation of enzyme at respective temperature by adding 0.01 N HCL or 0.01 N NaOH.

The residual activity was determined by taking 1 ml of the enzyme solution after specified time using pNPP as substrate. Kinetic studies were performed to determine the Michaelis constant, K_m, and the maximum velocity, V_{max}, of the native and immobilized lipase. The values of these parameters were determined from the Lineweaver-Burk plots using pNPP as substrate. The effect of organic solvents on lipase activity was analyzed by incubating the enzyme mixture with 30% organic solvents for 30 min at 30 °C. The control contained no organic solvent. The residual activity was also measured using pNPP as substrate.

RESULTS AND DISCUSSION

1. Microbial Strain

The highest lipase producing strain of *Pseudomonas aeruginosa* was confirmed by 16S rDNA technique. Amplification of gene was carried out by polymerase chain reaction using forward primer, was 5′-AGAGTTTGATCATGGCTCAG-3′ and the reverse primer, 5′-TACGGTTACCTTGTTACGACTT-3′ were used to amplify a 500-700 bp fragment of this gene. The PCR conditions used were initial denaturation at 94 °C, for 3 min followed by 30 cycles of 94 °C for 15 s; 60 °C for 15 s; 72 °C for 30 s and a final extension of 72 °C for 5 min. the 50 μl reaction mixture contained 1×PCR buffer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 10 pmoles of each primer, 1-10 ng of DNA and 2.5 U of Taq DNA polymerase. 5 μl of the amplified product was run on the agarose gel for purity check. In the sequencing PCR reaction either the forward or reverse primer was used. The sequence was edited and aligned with the sequence in the public domain Gen bank (http://www.ncbi.nih.gov) by BLAST program [32]. Based on the sequence similarity with the 16 S ribosomal genes of known organisms, the organism was granted a genus and a species.

2. Purified Enzyme Characterization

The purified enzyme showed total activity of 111,024 U in the culture filtrate with specific activity 126 U/mg. Partially purified enzyme obtained on Mono Q column showed total activity 8,369 U with specific activity of 12,308 U/mg, with purification of 98 fold. The yield obtained was 7.5% (Table 1).

The protein content of native (free) enzyme estimated for crude enzyme was 2.21 mg/ml and for unbound enzyme was found be 15.3 mg/ml, while the protein concentration in bound (immobilized)

Table 1. Purification of lipase from *Pseudomonas aeruginosa* SRT9

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture filtrate	884	111024	126	1	100
Ammonium sulfate	5.59	14635	2618	20	13
Phenyl sepharose CL-4B	2.94	13353	4542	36	12
Mono Q HR 5/5	0.68	8369	12307	98	8

enzyme for 1 mg of support was 0.155 mg/ml.

3. Tris Support: Spectral Analysis and MALDI TOF

Tris support is solid monomers (MP 174 °C), which have hydrophobic nature, as it possesses three aromatic rings linked to central triazine moiety through three oxygen atoms. This support contains 3-aldehyde groups at the peripheral position and forms Schiff's base with amino group of lipase. After immobilization the free amino groups of the immobilized enzyme forms Schiff's base with other support monomers to form a cross-linked enzyme support complex. Tris support is having specific area S (m^2/g), average pore diameter d_m (A°), pore volume v_p (cm^3/g) were 82, 95 and 0.93, respectively.

The immobilization of lipase by Schiff's base formation was confirmed by IR spectral studies and MALDI TOF. The prominent peaks obtained in IR spectra of compound A are 1,102, 1,602, 1,702, 2,741, and 2,833 cm^{-1} can be assigned to C-O, C=C, C-H of aldehyde, C=O and C-H (aromatic) stretching frequencies. However, IR spectra of the compound B indicated the presence of peaks at 1,100, 1,569, 1,606, 1,702 and 2,851 cm^{-1}. These can be assigned to C-O, C=C, CH=N (imine), C=O and C-H (aromatic) stretching frequencies. The appearance of a peak at 1,569 cm^{-1} for imine, disappearance of a peak at 2,741 and reduction of peak height at 1,702 cm^{-1} clearly indicated lipase has immobilized on Tris support.

The molecular weight 441 m/z for Tris support was confirmed by EI mass spectra. After immobilization the molecular weight increased to 46,100 m/z as determined by MALDI-TOF is an evidence for lipase immobilization on Tris support (Fig. 1).

4. Kinetic Studies of Immobilized Lipase

The pH of an enzyme solution can affect overall enzymatic activity in a number of ways. Like all proteins, enzymes have native tertiary structure sensitive to pH, and in general denaturation of enzymes

Table 2. Effect of pH, temperature and thermal stability on lipase activity

Enzyme	Optimum pH	Optimum temp. (°C)	Thermal stability* (h)	Enhancement	Half-life (min)	Stabilization factor
Free lipase	6.9	55	<2	-	60	-
Immobilized lipase	7.5	70	>5	>2.5	330	5.5

*Thermo stability for native immobilized lipase was examined at 70 °C

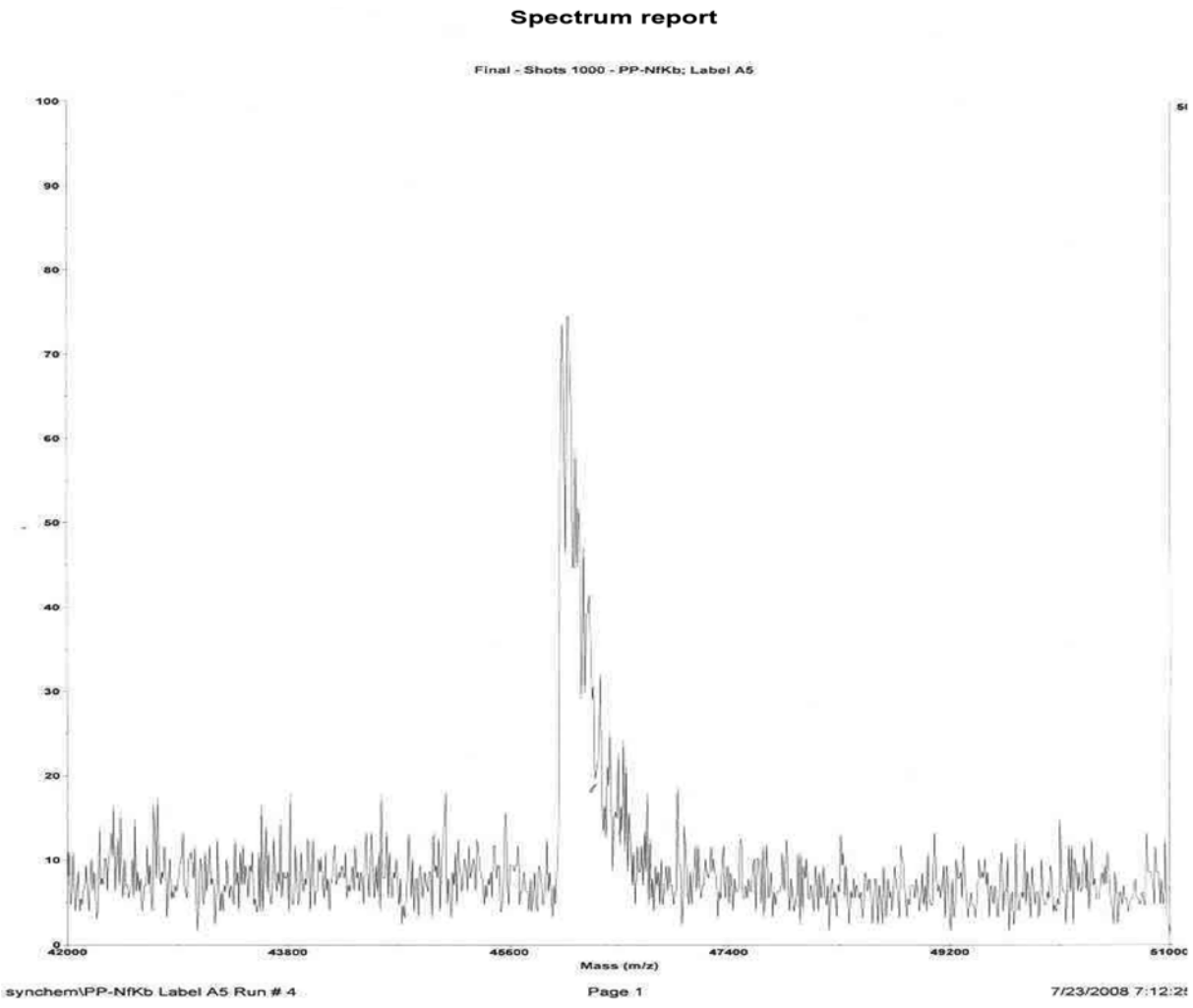
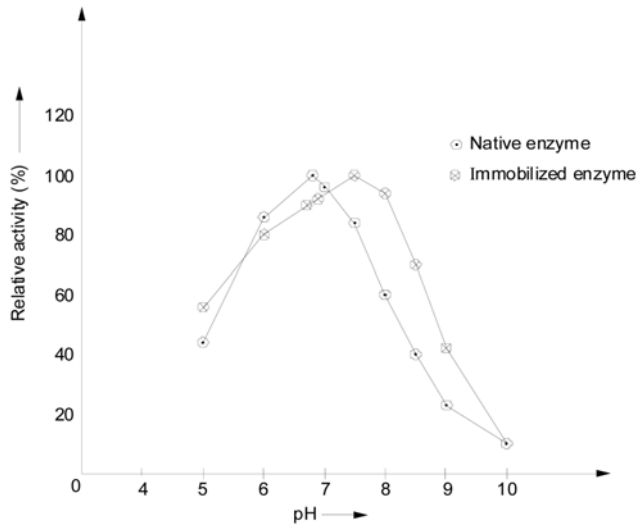


Fig. 1. Maldi TOF analysis.



Reaction conditions: Activity vs pH was determined at temp. 55 ºC for native and 70 ºC for immobilized lipase under standard conditions. Activity is expressed as percentage of the activity determined at pH 6.9 for native and 7.5 for immobilized lipase using pNPP as substrate.

Fig. 2. Effect of pH on lipase activity.

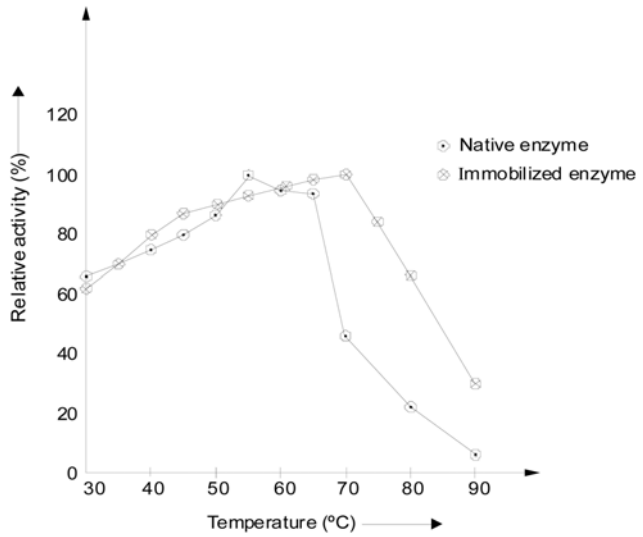
occurs at extreme low and high pH values [33]. The enzyme activities of both native (free) and immobilized lipase on tri (4-formyl phenoxy) cyanurate were studied at variable pH and temperatures. The optimum pH was 6.9. After immobilization the optimum pH was increased from 6.9 to 7.5 (Fig. 2). The shift in pH optimum may be due to the change in the H^+ ion concentration of the microenvironment of the immobilized lipase on tri (4-formyl phenoxy) cyanurate. The H+ ion concentration in the immobilized enzyme and support medium may be high as compared with the H+ ion concentration around the immobilized enzyme. Hence, to balance the identical H+ ion concentration in both the environment, H+ ion may be released from inside towards the outer environment, thereby shifting optimal pH towards the alkaline side.

Another reason for increase in activity at pH 7.5 seems to be related mainly to the rigidification of the enzyme and formation of stable enzyme support complex with a very suitable open conformation with little restriction to access of substrates [23,34,35].

The optimum temperature recorded for native form of lipase was 55 ºC. The lipase enzyme undergoes thermal denaturation at elevated temperatures. After immobilization, a shift in optimum temperature from 55 ºC to 70 ºC was revealed (Fig. 3). The shift in optimum temperature towards higher temperature may be due to the immobilization of the enzyme to the support providing rigidity and stability resulting in formation of the enzyme substrate complex. The rise in temperature above 70 ºC might have denatured the enzyme, and decrease in activity was therefore obtained at higher temperatures [36].

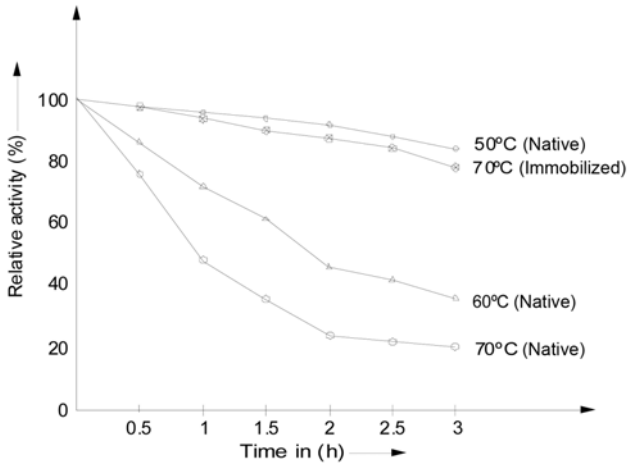
The thermal stability of native and immobilized lipase was examined by heating at 50-70 ºC, respectively (Fig. 4). It appears that the immobilized enzyme is remarkably more stable than the native form. The immobilized enzyme retained 75% of its original activity after 2 h incubation at 70 ºC, while in the native form it lost >70% of its original activity within 30 min. The half-life of the immobilized lipase estimated was >330 min at 70 ºC. The higher stability of the immobilized lipase could be due to the diminished denaturation of enzyme fixed to the support. Also, thermal inactivation involves considerable conformational changes in the protein molecules, i.e., partial unfolding, resulting in enzyme inactivation, whereas an enzyme molecule linked to the solid support is much more rigid than that of its free predecessor and is much less readily inactivated [37].

The kinetic studies showed an increase in the values of K_m of the native and immobilized enzymes from 0.043 mM to 0.10 mM, respectively, indicating that the affinity the substrate is significantly decreased. The immobilized enzyme has lower affinity towards the



Reaction conditions: Activity vs temperature was determined at pH 6.9 for native and 7.5 for immobilized lipase under standard conditions. Activity is expressed as percentage of the activity of the initial enzyme determined at 55 ºC for native and 70 ºC for immobilized lipase using pNPP as substrate.

Fig. 3. Effect of temperature on lipase activity.



Reaction conditions: Enzyme stability was studied by measuring residual activity after incubation at various temperatures (50-70 ºC) for 0-3 h using pNPP as substrate. The experiments were carried out in triplicate and the data represented is the mean value of three sets of experiments.

Fig. 4. Effect of temperature on lipase stability.

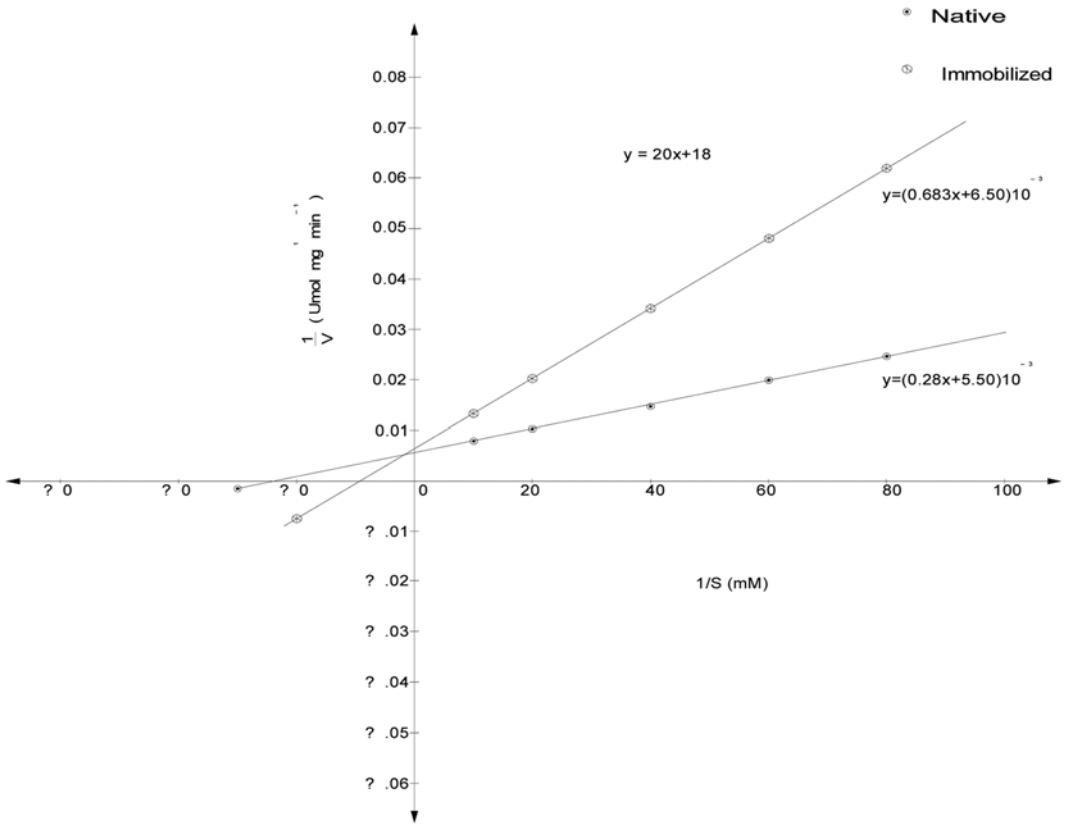
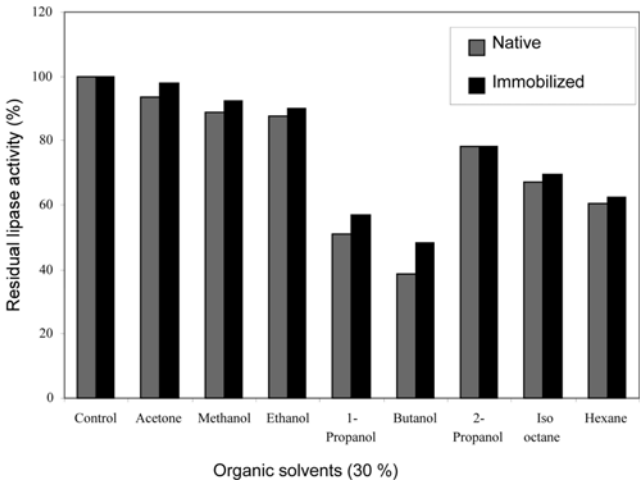


Fig. 5. Lineweaver-Burk plot of native and immobilized lipase.

substrate. It may be due to a slight change in conformation of the enzyme with some restriction to binding of substrate. The increase in K_m value seems to be related to the formation of an enzyme-support complex with a very suitable open conformation with some restrictions to the access of substrates (Fig. 5). On the other hand, the values of V_{max} showed decrease in values (181.8 mmol min^{-1} mg^{-1} and 142.8 μmolmin^{-1} mg^{-1} for native and immobilized, respectively) suggesting that the activity of immobilized lipase decreased in the course of covalent binding [38,39].



Reaction conditions: Residual activity was measured using pNPP as substrate by incubating enzyme mixture with organic solvents (30%) for 30 min at 30 °C. The experiments were carried out in triplicate and the data represented is the mean value of three sets of experiments.
Fig. 6. Effect of organic solvents on lipase activity.

The effect of organic solvents on enzyme activity revealed that enzyme was more stable in acetone, methanol and ethanol with residual activities of 93.47, 88.93 and 87.54%, respectively. However, significant loss of activity was observed in presence of butanol (30%) with residual activity of 38.74%. The lower enzyme activity may be attributed to the stronger ability of the polar solvent to strip the essential water off the enzyme molecules. However, when immobilized on to the support the activity increased to 48.32%. Increase in the enzyme activity may be due to the fact that the immobilized lipase is more resistant than its counterpart to the deleterious effects of butanol [40-43]. Enzyme stability in deferent organic solvents showed that Iipase enzyme is more stable when compared to its native form (Fig. 6).

5. Repetitive Use of Immobilized Lipase

Upon repeated use, the tri (4-formyl phenoxy) cyanurate immobilized lipase retained 75% of its initial activity after seven cycles. The decrease in activity after seven cycles can be correlated to slight inactivation of enzymes after every use, as evidenced by leaching in supernatant. The immobilized lipase could be stored at 4 °C in Tris HCl buffer, pH 7.0 for more than 30 days without any significant loss in its initial activity (Fig. 7).

CONCLUSION

Lipase when immobilized on Tris support showed 80% immo-

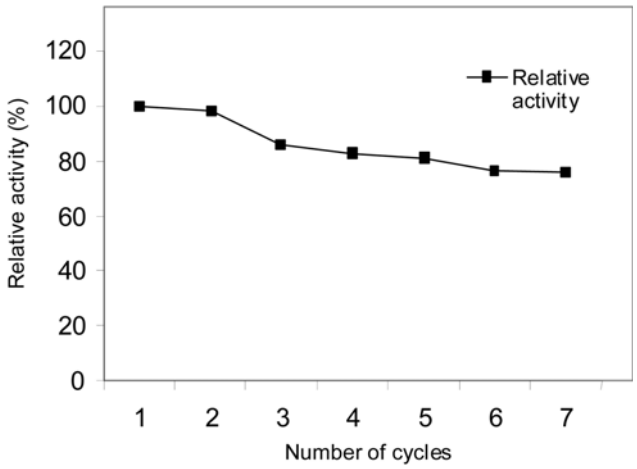


Fig. 7.Stability of immobilized lipase in repeated use.

bilization with catalytic efficiency of 75%; therefore, Tris support is found to be a better support for immobilization when compared to other supports. Moreno et al. reported the percentage immobilization of lipase iso enzymes A & B on agarose & silica via covalent coupling to be 33-40% with catalytic efficiency of 70% [20]. Lipase immobilized on Zeolite type Y showed 33% immobilization with 35% enzyme activity of the free enzyme (Knezevic et al.).

Tri(4-formyl phenoxy) cyanurate possesses a unique configuration providing some advantages such as enzyme loading and multipoint covalent attachment sites for reversible binding of the enzyme, and therefore could serve as a better carrier material for immobilization. In terms of solubility and thermostability, this supporting medium is highly insoluble in water, crystalline in nature and thermally stable. Therefore, it is expected that the present immobilization technology can be used as ideal system for long term chemical processing such as regio selective reactions; in the esterification of sugars, nucleosides, steroids and in enantio selective reaction, in the resolution of secondary alcohols via hydrolysis or esterification in organic solvents.

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