Catalytic Potential of a Poly(AAc-*co*-HPMA-*cl* MBAm)-Matrix-Immobilized Lipase from a Thermotolerant *Pseudomonas aeruginosa* MTCC-4713

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ABSTRACT: A purified alkaline thermo-tolerant lipase from *Pseudomonas aeruginosa* MTCC-4713 was immobilized on a series of five noble weakly hydrophilic poly(AAc-*co*-HPMA-*cl* MBAm) hydrogels. The hydrogel synthesized by copolymerizing acrylic acid and 2-hydroxy propyl methacrylate in a ratio of 5 : 1 ($HG_{5:1}$ matrix) showed maximum binding efficiency for lipase (95.3%, specific activity 1.96 IU mg^{-1} of protein). The $HG_{5:1}$ immobilized lipase was evaluated for its hydrolytic potential towards *p*-NPP by studying the effect of various physical parameters and salt-ions. The immobilized lipase was highly stable and retained ~92% of its original hydrolytic activity after fifth cycle of reuse for hydrolysis of *p*-nitrophenyl palmitate at pH 7.5 and temperature 55°C. However, when the effect of pH and temperature was studied on free and bound lipase, the $HG_{5:1}$ immobilized lipase exhibited a shift in optima for pH and temperature from pH 7.5 and 55°C to 8.5 and 65°C in free and immobilized lipase, respectively. At 1 m*M* concentration, Fe^{3+}, Hg^{2+}, NH_4^+, and Al^{3+} ions promoted and Co^{2+} ions inhibited the hydrolytic activities of free as well as immobilized lipase. However, exposure of either free or immobilized lipase to any of these ions at 5 m*M* concentration strongly increased the hydrolysis of *p*-NPP (by ~3–4 times) in comparison to the biocatalysts not exposed to any of the salt ions. The study concluded that $HG_{5:1}$ matrix efficiently immobilized lipase of *P. aeruginosa* MTCC-4713, improved the stability of the immobilized biocatalyst towards a higher pH and temperature than the free enzyme and interacted with Fe^{3+}, Hg^{2+}, NH_4^+, and Al^{3+} ions to promote rapid hydrolysis of the substrate (*p*-NPP). © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 4252–4259, 2006

Key words: lipase; *P. aeruginosa* MTCC-4713; immobilization; synthetic hydrogels

INTRODUCTION

Immobilized enzymes are used in many commercialized products for higher yields. The lipases that constitute a most versatile group of enzymes have been successfully immobilized on a variety of matrices/supports for performing esterification and trans-esterification reactions in organic solvents. Besides the nature/characteristics of the immobilization matrices/supports, the effect of solvents on enzyme activity, specificity and stability have been extensively studied and many solvent-parameters have been compared to develop a simple strategy for choosing the appropriate solvent for each enzymatic reaction.[1–6] A solvent is necessary for solubilizing the substrates, and for partitioning the substrates and products in different phases.[7] It can also be used for increasing the enzyme's thermal stability or for avoiding hydrolysis.[8] Moreover, the solvent might be necessary in case of cosoluble substrates (liquid or solids and liquids) for ester synthesis or trans-esterification.[9–11]

The polarity of the liquid exerts a great influence on water solubilization and distortion of H-bonds.[2] This polarity is characterized by the logarithm of partition coefficient in an octanol water two-phase system (Log *P* value). Water plays several roles in enzyme structure and functions[12,13] that included action on enzyme structure by contribution to all noncovalent bonding, alteration of protein structure by disruption of H-bonds, facilitation of reagent diffusion and participation in the equilibrium control (water can be substrate or product). The enzyme sometimes may be inactive in a completely dehydrated system.[14] The metal-ions are also effective antagonists and modulators of lipases. Chelating agents like EDTA inhibit the metallo-lipases while others do not.[15,16]

Thus it is essential to study the effect of various physical parameters besides metal-ions to perform the

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desired hydrolysis of a chosen water-compatible *p*-nitrophenyl ester such as *p*-nitrophenyl palmitate (*p*-NPP). In the present study an attempt has been made to evaluate the effect of reaction/incubation time, incubation temperature, thermal tolerance, reaction buffer pH and hydrolysis of *p*-nitrophenyl esters of varying C-chain length esters using acrylic acid (AAc)–hydroxy propyl methacrylate (HPMA)-based hydrogel-immobilized purified lipase of a thermotolerant *Pseudomonas aeruginosa* (*P. aeruginosa*) MTCC-4713.

EXPERIMEMTAL

Chemicals

Acrylic acid (AAc), 2-hydroxy propyl methacrylate (HPMA), and *N*,*N*-methylene bisacrylamide (MBAm) were from Merck, Germany; *p*-nitrophenyl palmitate (*p*-NPP), *p*-nitrophenyl formate (*p*-NPF), *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl caprylate (*p*-NPC), and *p*-nitrophenyl laurate (*p*-NPL) were from Lancaster Synthesis, England; 2-propanol; ammonium persulphate (APS) and acetone were from Qualigens Fine Chemicals, India. All these chemicals were used as received.

Microorganism and lipase

The *P. aeruginosa* MTCC-4713 was originally isolated from kitchen waste of a sweet shop by enrichment at 56°C at Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla (India). The purified lipase was used for immobilization on a polymer matrix prepared by polymerizing AAc and HPMA. One unit (IU) of hydrolytic activity of immobilized lipase was expressed as micromoles of *p*-nitrophenol produced per minute by hydrolysis of *p*-NPP (prepared as 20 m*M* stock in 2-propanol) by 1 mL of free enzyme or 1 g of matrix-bound enzyme under assay conditions. The lipase from *P. aeruginosa* MTCC-4713 was purified by successive salting out at 80% (w/v) ammonium sulfate saturation and DEAE cellulose anion exchange chromatography as previously described.[4,5]

Preparation of crude lipase

P. aeruginosa MTCC-4713 was maintained in the Department of Biotechnology at Himachal Pradesh University, Shimla. The culture was repeatedly subcultured at 56°C on a mineral-based (MB) broth supplemented with 1% (v/v) cottonseed oil as a sole carbon source. The MB broth contained (gL^{-1}) $NaNO_3$, 3.00; K_2HPO_4, 0.10; $MgSO_4{\cdot}7H_2O$, 0.50; KCl, 0.50; $FeSO_4{\cdot}7H_2O$, 0.01; yeast extract, 40 and sucrose 5.0. The cottonseed oil (100 mL) warmed to 70°C was initially emulsified with gum acacia (0.5 g) by continuous stirring (Magnetic Stirrer, Remi Equipments, Bombay, India). The pH of medium was adjusted to 7.5. For lipase production, 7.5% (v/v) of 48 h old inoculum was used to inoculate production medium (50 mL each in 250 mL capacity Erlenmeyer flasks). The lipase producing broth was rendered free of cells by centrifuging at 10,000 × *g* for 10 min at 4°C (SIGMA 3K30, Germany). The supernatant filtered through Whatman paper no. 1 was subjected to precipitation with 80% (w/v) ammonium sulfate at 4°C. The precipitates sedimented by centrifugation were reconstituted in 40 mL of tris buffer (0.05*M*, pH 7.5) and extensively dialyzed against same buffer to remove ammonium sulfate. Finally, the lipase activity was assayed and the concentrated lipase preparation was stored at −20°C till further use. The protein content was measured.[17] This filtrate/broth preparation was henceforth referred as crude lipase.

Purification of the enzyme

The purification of the lipase enzyme was performed on a DEAE-cellulose anion exchange column (Pharmacia Amersham, Sweden). A column (12 × 2 cm^2, Vt = 50.2 cm^3) was packed with preswollen DEAE-cellulose. The matrix was activated sequentially with 0.1*N* HCl and 0.1*M* NaOH. The column was washed thoroughly with 0.05*M* phosphate–buffer (pH 7.5). The crude enzyme (1 mL = 9.8 mg protein; 3.9 IU mL^{-1}) was loaded on the column. Initially, 12 fractions were eluted using 0.05*M* phosphate–buffer (pH 7.5) followed by another 13 fractions with 0.05*M* phosphate–buffer containing 1.0*M* KCl (fraction volume = 2 mL each). The fractions were analyzed for lipase activity and protein content. The fractions possessing lipase activity were pooled and lyophilized. The specific activity of the purified enzyme was compared with that of the crude enzyme and fold purification was calculated.

Synthesis of hydrogel networks

A series of five networks were prepared in acetone: water system (1 : 1, v/v) by polymerizing AAc with varying amount of HPMA (5 : 1, 5 : 2, 5 : 3, 5 : 4, and 5 : 5, v/v) in the presence of a crosslinker (MBAm 5%, w/w) and initiator (APS 1%, w/w). The reactants were vigorously stirred and transferred inside an airtight glass vial under vacuum. The vial was sealed and heated at 80°C for 30 min. Each of the networks synthesized was washed with different solvents by polarity gradient method to remove unreacted compounds to obtain a xerogel. The swelling capacity (S_w) of each of the xerogels in distilled water was determined as given below. The excess water after swelling of matrix was removed by decantation.

$$S_w = \frac{W_2 - W_1}{W_1}$$

where W_1 is the weight of xerogel in milligrams; W_2 is the weight of hydrogel in milligrams, i.e., net weight of polymeric matrix (xerogel) after suspending it in excess volume of water for 1 h at 55°C.

Assay of hydrolytic activity

The hydrolytic activity of free or immobilized lipase was assayed in reaction buffer (0.05*M* tris pH 7.5 for assay of free or pH 8.5 for immobilized enzyme) by a standard colorimetric method using *p*-NPP as a substrate.[18] The purified lipase had an activity of 5.4 IU mL^{-1} under assay conditions with a protein content of 5.4 mg mL^{-1} of soluble enzyme. In all the assays the reaction mixture before addition of biocatalyst was preincubated at 55 or 65°C for determining free- and bound-lipase activity, respectively. Chilling the reaction mixture at −20°C for 7 min stopped further enzymatic reaction.

Immobilization of the lipase on hydrogel network

The matrix (50 mg) taken in a glass vial was suspended in tris buffer (0.05*M*, pH 8.5) containing 200 μL (lipase activity, ~1.08 μmol mL^{-1} min^{-1}; specific activity 1.08 IU mg^{-1} protein) of purified lipase. The suspension was kept at 8°C for 20 h. The hydrogel was separated by centrifugation. The supernatant was decanted, its volume and protein (unbound) was estimated. The immobilized protein was determined by subtracting unbound protein in the supernatant from the total protein used for immobilization.

Reusability of immobilized lipase for hydrolytic activity towards *p*-NPP

To determine leaching of bound lipase, 10 mg of the matrix was added to 2.92 mL of reaction buffer containing 75 μL of *p*-NPP stock (20 m*M* prepared in 2-propanol). The incubation was performed at 55°C for 10 min and thereafter lipase activity was determined. The matrix was recovered after decantation of the reaction buffer, subjected to two washings with 0.05*M* tris buffer (3 mL each wash) and reused for another cycle of hydrolysis in fresh reaction buffer. The reusability of the immobilized lipase was determined for up to 5 cycles of hydrolysis of *p*-NPP. The residual activity after each cycle of hydrolysis was determined in comparison to the activity recorded in the first cycle.

Effect of pH of reaction buffer on activity of immobilized and free lipase

Effect of pH of the reaction buffer (0.05*M* tris) on catalytic potential of bound and free lipase was assayed by incubating 10 mg of hydrogel-immobilized lipase or 5 μL of free lipase in reaction buffer adjusted at pH 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, and 10. The lipase activity was assayed after 10 min of incubation.

Effect of incubation temperature on free and immobilized lipase

The activity of free and bound lipase was assayed separately by incubating the reaction mixture taken in glass-tubes at temperature ranging from 40 to 100°C in water-bath-incubator-shaker set at 160 rpm. The lipase activity was assayed after 10 min.

Thermostability of free and immobilized lipase

Thermostability of the immobilized and free lipase was examined at 65 and 55°C, respectively. Immobilized and free lipases were incubated separately in 0.05*M* tris buffer (pH 8.5 for bound and 7.5 for free lipase) up to 5 h with continuous shaking. Periodically, residual activities of the free and immobilized lipase were determined.

Effect of various salt-ions on free and immobilized lipase

The effect of salt-ions on the hydrolytic potential of free lipase was determined by adding each of the selected salts at 1 m*M* concentration separately in the reaction mixture containing substrate (*p*-NPP). The lipase-bound matrix was incubated with each of the salts separately for 10 min at 8°C. The matrix was removed, soaked (on Whatman paper no. 1) to remove excess liquid and transferred into the reaction mixture (0.05*M* tris, pH 8.5). The hydrolytic activity of free and immobilized lipase was assayed.

Effect of acyl chain length on lipase activity

The immobilized or free lipase was reacted with any of the five *p*-nitrophenyl esters (20 m*M* stock prepared in 2-propanol). The *p*-nitrophenyl esters were *p*-NPF (1 : 0), *p*-NPA (2 : 0), *p*-NPC (8 : 0), *p*-NPL (12 : 0), and *p*-NPP (16 : 0). The hydrolytic activity of free and bound lipase was determined at 55°C and pH 7.5, and 65°C and pH 8.5 respectively, after 10 min of incubation in shaking water-bath.

RESULTS

Purification of bacterial lipase

The cell-free broth had a lipase activity of 0.86 IU mL^{-1} (protein 20.8 mg mL^{-1}). The protein was optimally precipitated at 80% (w/v) ammonium sulfate saturation. The precipitate reconstituted in 40 mL of

TABLE I
Immobilization of Lipase on Different Hydrogel Matrices

Hydrogel network	AAc:HPMAc ratio	S_w	Code	Protein binding efficiency (%)	Bound protein (mg)	Specific activity (IU mg^{-1}) with *p*-NPP
AAc-*co*-HPMA-*cl*-MBAm	5 : 1	2.6	$HG_{5:1}$	95.3	1.03	1.96
	5 : 2	2.2	$HG_{5:2}$	90.7	0.98	1.44
	5 : 3	2.4	$HG_{5:3}$	88.8	0.96	1.40
	5 : 4	2.2	$HG_{5:4}$	81.4	0.88	1.38
	5 : 5	2.2	$HG_{5:5}$	80.5	0.87	1.36

tris buffer, pH 7.5 and was extensively dialyzed against the same buffer. The dialyzed fraction showed lipase activity of (3.9 IU mL^{-1}, 9.8 mg protein mL^{-1} and specific activity 0.39 IU mg^{-1}). The DEAE-anion exchange chromatography resulted in a single peak. The fractions showing lipase activity were pooled (21 mL, 5.45 IU mL^{-1}, 5.4 mg of protein per milliliter, specific activity 1.01 IU mg^{-1}) and were subjected to concentration by lyophilization.

Protein binding efficiency of hydrogels and their characteristics

A series of five hydrogels were evaluated for their ability to bind protein (lipase activity, ~1.09 IU mL^{-1}, specific activity 1.01 IU mg^{-1} protein). The efficiency of hydrogels decreased with an increase in the concentration of the copolymer HPMA (Table I). The lipase was optimally immobilized/bound to the hydrogel $HG_{5:1}$ that retained 95.3% of the total protein.

The use of lipase bound to $HG_{5:1}$ matrix for hydrolysis of *p*-NPP in repeat cycles showed marked retention of the catalytic activity upto five cycles of hydrolysis. The matrix after five cycles of reuse retained ~92% of its originally bound hydrolytic lipase activity (Fig. 1).

Effect of pH of reaction mixture on hydrolytic activity of bound as well as free lipase was evaluated by adjusting the pH in the range of 5–10 using tris buffer. pH of 7.5 for free lipase and 8.5 for $HG_{5:1}$ matrix-immobilized lipase was found to be optimum for hydrolysis of *p*-NPP (Fig. 2). Immobilized lipase was quite stable and retained activity over a wide range of pH.

Effect of varying incubation temperature (40–100°C) for the hydrolytic reaction was studied by maintaining the reaction buffer pH at 7.5 and 8.5, respectively, for free and immobilized lipase. A temperature of 55°C for free and 65°C for immobilized enzyme was found to give maximum hydrolytic activities for the *p*-NPP (Fig. 3). The free and immobilized biocatalysts were subjected to prolonged exposure at their optimum temperature of 55 and 65°C, respectively. The free and immobilized enzyme when preincubated at 55 and 65°C retained 56 and 75% of their original activity after 1 h, and 40 and 75% of their original activity after 2 h, respectively, (Fig. 4).

Effect of salt ions on hydrolytic potential of free and immobilized lipase

The Fe^{3+}, NH_4^+, Hg^{2+}, and Al^{3+} at 1 m*M* concentration modulated the lipase activity of free lipase (Fig. 5). The presence of Co^{2+} ions drastically decreased the lipase activity of both free (50.5% decline) and immobilized (37.9% decline) biocatalyst in compassion to the control. Fe^{3+}, Hg^{2+}, NH_4^+, and Al^{3+} mildly promoted the activity of free and bound lipase. Hg^{2+} ions enhanced the activity of the free and bound lipase by 15.8 and 15.5%, respectively, in comparison to the control. Four cations, i.e., Fe^{3+}, Hg^{2+}, NH_4^+, and Al^{3+} were further evaluated at 3–7 m*M* for their effect on

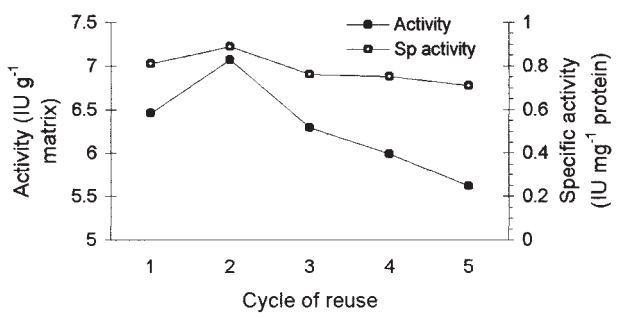

Figure 1 Reusability of immobilized lipase in hydrolysis of *p*-NPP.

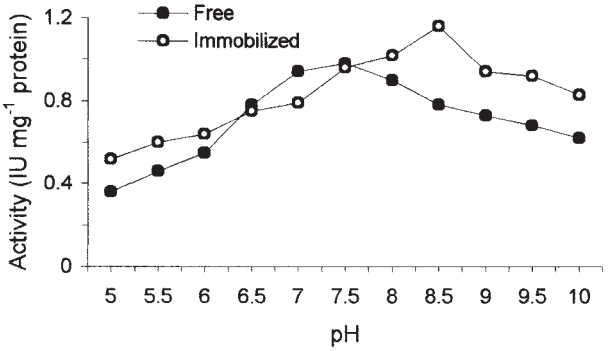

Figure 2 Effect of pH on activity of free and immobilized lipase.

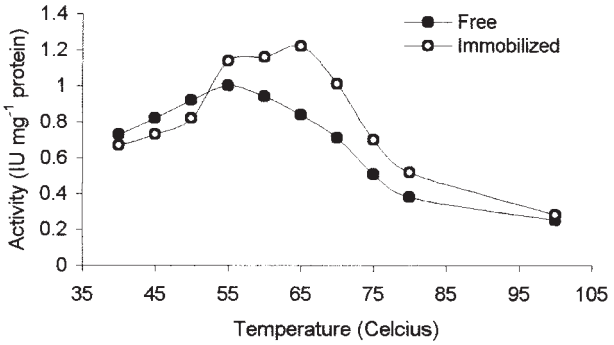

Figure 3 Effect of incubation temperature on hydrolytic activity of free and immobilized lipase.

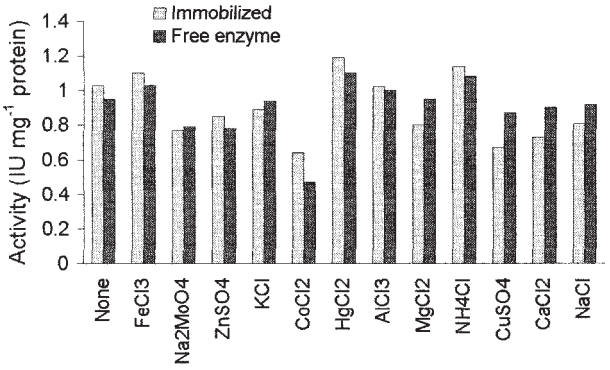

Figure 5 Effect of various salt ions (1 m*M*) on hydrolytic activity of free and immobilized lipase.

activities of free and bound lipase (Figs. 6 and 7). All the chosen cations at a concentration of 3–5 m*M* promoted lipase activity of both free as well as bound lipase. At 5 m*M*, Fe^{3+}, Al^{3+}, NH_4^+, and Hg^{2+} ions enhanced the activity of free lipase by 269, 387, 455, and 217%, and for immobilized lipase by 297, 397, 478, and 170%, respectively, of their original activities. However, at 7 m*M*, all these ions prompted a decline in the hydrolytic activity of free as well as immobilized lipase.

Specificity of free and immobilized lipase towards *p*-nitrophenyl esters of varying C-chain length and role of different metal-ions

The free lipase showed maximum hydrolytic activity for *p*-NPP (Table II). The $HG_{5:1}$ matrix-bound lipase was highly hydrolytic towards *p*-NPC. Interestingly, a marked enhancement in the hydrolytic activity of free as well as immobilized lipase was observed following the exposure to Hg^{2+} ions at 5 m*M* concentration. The Hg^{2+} ions-exposed free lipase showed 331% increase in the hydrolytic activity (specific activity) towards *p*-NPP. However, $HG_{5:1}$ matrix-bound lipase was preferentially more active towards *p*-NPL than *p*-NPP (Table III).

DISCUSSION

Over the last decade, the interest in industrial processes employing lipase biocatalysts is still growing because of their important and multiple applications.[19–23] In the present study, five network polymers with weak-hydrophilic nature but differing in concentration of copolymer (HPMA) were synthesized and employed for immobilization of an alkaline lipase from a thermotolerant *P. aeruginosa* MTCC-4713. The polymer $HG_{5:1}$ that was obtained using lowest concentration of weakly hydrophilic copolymer HPMA showed highest binding efficiency towards lipase of *P. aeruginosa* MTCC-4713. In the present study, it was established that *P. aeruginosa* MTCC-4713 lipase when immobilized on $HG_{5:1}$ exhibited a relatively higher hydrolytic activity, as evident by its repetitive use for hydrolysis of *p*-NPP than the free form. Another observation that was of great significance was the ability of the $HG_{5:1}$ hydrogel-bound lipase to retain a higher efficiency for hydrolysis both at an acidic as well as an alkaline pH tested in the present study. Such an observation gave an additional advantage to the *P. aeruginosa* MTCC-4713 lipase to achieve catalysis at a low as well as a high pH unlike other alkaline lipases sourced from various microorganisms. Lipases that

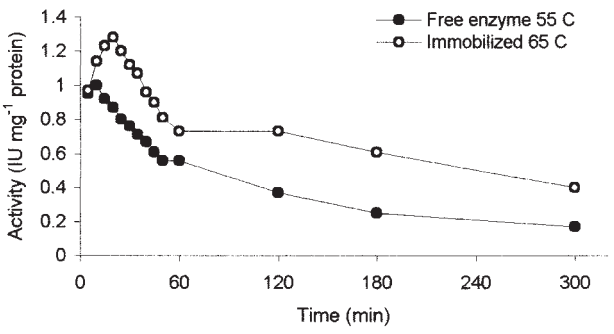

Figure 4 Thermostability of free and immobilized lipase.

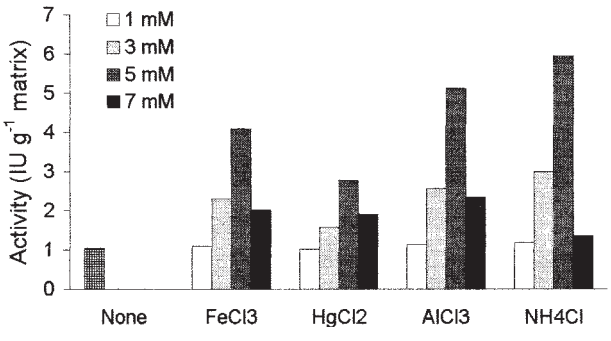

Figure 6 Effect of concentration of salt ions (1 m*M*) hydrolytic activity of immobilized lipase.

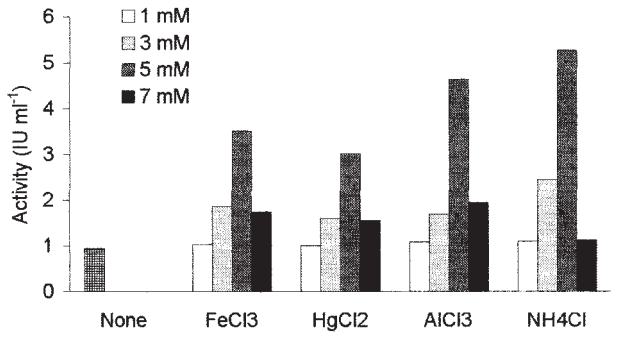

Figure 7 Effect of concentration of salt ions on hydrolytic activity of free lipase.

are hydrolytic at alkaline pH are conventionally used in detergents and laundry. However, if a lipase retains its activity in acidic as well as alkaline environment, its usefulness can be extended for achieving catalysis under mild acidic conditions also. Most lipases can act in a wide range of pH and temperature, although alkaline bacterial lipases are more common.[4,6,24] Lipase immobilized onto poly(HEMAc-*co*-MMAm) gave a higher yield for both hydrolysis and esterification activity compared to other polymers.[25]

Immobilized lipases generally offer economic incentives of enhanced thermal and chemical stability, ease of handling, recovery and reuse relative to nonimmobilized forms.[26,27] Various features of reaction selectivity of lipases are modulated by exogenous factors such as choice of cosubstrates/reactants, water activity, pH, temperature and immobilization.[28–31] Lipase of *P. aeruginosa* after immobilization on $HG_{5:1}$ hydrogel became relatively more thermotolerant than the free lipase. The temperature optimum for immobilized form was 10°C higher than the free form (55°C). Moreover, the bound lipase retained approximately 50% of its activity at its optimum temperature for 2 h than 1 h observed in case of free enzyme. It appeared that temperature also had an important effect on physical state of enzyme. Higher temperature and liquefaction could be assumed to make the substrate more acceptable to enzyme.[32] It was likely that the structure of lipase immobilized onto $HG_{1:5}$ matrix became more fluid and open at an elevated temperature. Specialized matrices with distinguishing chemical character such as the one used in the present study might have greater influence on lipase activity and selectivity, by virtue of support matrix effects directly on enzyme conformation or microenvironment, differences in substrate diffusion rates or physiochemical interaction directly with substrate and or products as previously postulated.[33,34] In a previous study, lipase immobilized onto poly(2-hydroxyethyl methacrylate-*co*-methyl methacrylate) matrix gave a higher yield for both hydrolysis and esterification activity compared to other polymers.[25] Moreover, immobilization also facilitated dispersal of enzyme on a solid surface to provide far greater interfacial area and accessibility of substrate relative to the use of enzyme powders in low water reaction media.[35] The emergence of commercial applications of lipases for preparing value-added speciality products from lipids or by esterification/trans-esterification would be dependent on understanding and controlling reaction selectivity, which is defined as comparative differences in rate of reaction towards different substrates.[36]

Structural elucidation of lipases has shown that catalytic site of most lipases resembles that of serine proteases.[37] The active site is a catalytic triad of serine, aspartic (or glutamic) acid, and histidine; serine is a highly conserved residue in various lipases. The presence of ionizable salts of metals as well as nonmetals is likely to cause structural perturbation(s) at the catalytic site of lipase. Interestingly, both divalent- (such as Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, and Hg^{2+}) as well as monovalent cations (Na^+ and K^+) are strong modulators of microbial lipase activity.[15,38–45] In the present study, cations of some of the metals (Hg^{2+}, Al^{3+}, and Fe^{3+}) as well as a nonmetal (NH_4^+) were found to modulate lipase activity of *P. aeruginosa*. Interestingly, an increase in concentration of Hg^{2+}, Al^{3+}, Fe^{3+}, and NH_4^+ enhanced lipase activity by 3- to 4-fold and maximum being noticed with $HG_{5:1}$ hydrogel-bound lipase in presence of Hg^{2+} ions at 5 m*M*. This

TABLE II
Hydrolytic Activity of Free and Bound Lipase Towards *p*-Nitrophenyl Esters of Varying C-Chain Length

	Free lipase		$HG_{5:1}$ matrix-bound lipase	
p-Nitrophenyl ester (C-chain length)	Relative activity (%)	Specific activity (IU mg^{-1})	Relative activity (%)	Specific activity (IU mg^{-1})
p-NPP (C 16)	100.0	1.00	80.0	1.09
p-NPL (C 12)	93.6	0.94	84.0	1.14
p-NPC (C 8)	74.6	0.75	100.0	1.36
p-NPA (C 2)	36.5	0.36	38.0	0.51
p-NPF (C 1)	17.3	0.17	9.9	0.13

TABLE III
Hydrolytic Activity of Hg^{2+} Ions-Exposed Free and Bound Lipase Towards *p*-Nitrophenyl Esters of Varying C-Chain Length

	Free lipase		$HG_{5:1}$ matrix-bound lipase	
p-Nitrophenyl ester (C-chain length)	Relative activity (%)	Specific activity (IU mg^{-1})	Relative activity (%)	Specific activity (IU mg^{-1})
p-NPP (C 16)	100.0	4.31	90.6	4.35
p-NPL (C 12)	88.9	3.83	100.0	4.80
p-NPC (C 8)	55.5	2.39	76.1	3.65
p-NPA (C 2)	25.9	1.11	24.4	1.17
p-NPF (C 1)	17.7	0.17	9.1	0.43

observation was highly noticeable, as previously Hg^{2+} ions, nowhere in literature were reported to modulate the lipase activity. Exposure of Ca^{2+} ions to an extracellular lipase of *Pseudomonas flourescens* 2D was reported to cause 360% increase in the lipase activity but the presence of Hg^{2+} and Co^{2+} strongly inhibited the activity.[44] Hg^{2+}, Al^{3+}, Mn^{2+}, and Co^{2+} ions exerted a drastic decline in lipase activity of *Rhizopus oryzae*.[46]

The *P. aeruginosa* lipase contains two cysteine residues that form an intramolecular disulfide bond essential for stabilizing the active conformation of lipase.[47] It appeared that disulfide bond might have interacted with the Hg^{2+} ions. The presence of metal-ions has been known to influence the hydrolytic activities of microbial lipases of bacterial as well as fungal origin. Na^{+} ions have been reported to be essential for lipase activity of *Pseudomonas pseudoalkaligenes*.[43]

The present study showed that immobilization of lipase on a noble weakly hydrophilic $HG_{5:1}$ hydrogel promoted the thermostability as well as pH tolerance at low and high pH. Unlike free lipase, that was preferentially more active towards a longer C-length ester (*p*-NPP), the immobilized lipase showed greater specificity towards *p*-NPC, a medium C-chain length ester. Interestingly, $HG_{5:1}$ bound-lipase pre-exposed to Hg^{2+} ions, was highly hydrolytic towards *p*-NPL and *p*-NPP than *p*-NPC. This altered preference for hydrolysis towards *p*-NPL indicated that Hg^{2+} ions appeared to interact with the catalytic site of the lipase thereby, somehow opening up the polypeptide structure that then accommodated a relatively longer C-length ester (C 12 or C 16). Thus, it may be concluded that immobilization provided stability to the lipase to tolerate a relatively higher temperature, prolonged exposure to heat, low acidic as well as high pH than the free lipase besides interactive-binding of Hg^{2+} ions at the catalytic site that promoted hydrolysis of the longer C-chain length esters by changing the specificity of bound enzyme.

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