Hyper-activation of lipase in *poly* (*N*-AEAAm-*co*- AAc)-*cl*-MBAm hydrogel(s)

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A lipase of *Bacillus coagulans* MTCC 6375 was purified to homogeneity by DEAE-cellulose and Octyl-sepharose chromatography. The lipase possessed a *M*r of 103 kDa. The pure enzyme was immobilized onto a series of grafted hydrogels of weakly hydrophilic character by method of interfacial activation. Both immobilized preparations were much more stable than soluble enzyme. However, hydrogel *poly*(*N*-AEAAm-*co*-AAc)-*cl*-MBAm-bound lipase expressed hyper-activity and greater thermal stability in the presence of organic solvents. The bound-lipase maintaining 100% of activity at 45°C and 65°C after several days of incubation. The hydrogel bound lipase was used to synthesize unusual esters of methacrylates.

Key words: *Bacillus coagulans* MTCC 6375, hyper-activity, thermostability, methacrylate esters and hydrogels

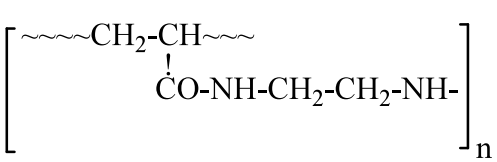
Introduction

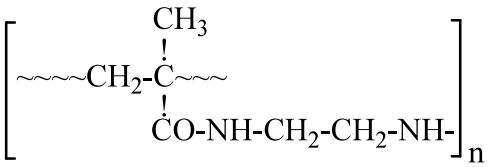
Use of immobilized lipase (*EC 3.1.1.3*) as biocatalyst is attractive tool to synthesize myriad of organic molecules *via* non-conventional routes with high precision, yield and purity. However, improved immobilization system owed to specific (grafted) hydrogel *i.e.* immobilized lipase in such system possesses good performance in the organic solvents [Kanwar *et al.*, 2005]. Based on our prior knowledge during purification of lipase on an anion exchanger, DEAE (diethyl amino ethane) showed high affinity towards *B. coagulans* lipase [Kanwar *et al.,* 2006]. Therefore such analogous anchoring designed hydrogel of *N*-aminoethyl acrylamide) [*N*-AEAAm] and *N*-aminoethyl methacrylamide [*N*-AEMAAm] could provide stable immobilization to this biocatalyst(s). The resulting hydrogels acquire diverse properties as compared to polymeric acrylic acid (AAc) and methacrylic acid (MAAc) and are thus expected to be of specific uses in organic synthesis. These hydrogels could be molded in any shape or size and are also biocompatible and biodegradable [Wang *et al.,* 2004].

Materials and methods

Microorganism and lipase immobilization: The *Bacillus coagulans* MTCC-6375 was used to obtain purified lipase as previously described [Kanwar *et al.,* 2005]. The homogenous purified soluble lipase was used for immobilization onto *poly* (*N*-aminoethyl acrylamide) *poly*[*N*-AEAAm] and *poly* (*N*-aminoethyl methacrylamide) *poly*[*N*-AEMAAm]. Hydrolytic activity of lipase was expressed as micro-mole(s) of *p*-nitrophenol (A_{410} nm) produced per minute by hydrolysis of *p*-NPP at 45°C by one ml of free (soluble) enzyme or at 55°C by one gram of hydrogel-immobilized enzyme under assay conditions

Synthesis of hydrogels network: The hydrophilic hydrogel(s) containing a domain of amino ethyl moiety were chosen from a series of hydrophilic support(s) possessing affinity to *B. coagulans* lipase. The hydrogels were prepared by co-polymerizing AAc (0.138 M) and AEAAm or AEMAAm (0.138 M each) separately and subsequently cross-linked (employing MBAm (*cl*); 400 mg and APS (*initiator*); 200 mg) to obtain a polymer network. The reactants were vigorously stirred and allowed to stand for 30 min at 25°C. The mixture was warmed at 60°C for 2 h in water bath. The polymer(s) so synthesized were washed thrice with excess of warm distilled water (60°C) to remove unreacted compounds. The polymer were completely dried in a vacuum oven at 30°C to get a constant weight to obtain a *xeroge*l. The *xerogel* was used subsequently for immobilization of the lipase of *B. coagulans* MTCC 6375. The *xerogel(s)* [*poly* (AEAAm-*co*-AAc)-*cl*-MBAAm] and [*poly* (AEAAm-*co*-AAc)-*cl*-MBAAm] had S_w value (swelling capacity in water) of approximately 25 times with tris-buffer (pH 8.5). The representative structures of these polyamides are proposed as:





Immobilization of lipase on hydrogel(s): Each matrix weighed 100 mg was pre-equilibrated with appropriate amount of tris-buffer (0.05M; pH 8.5) and subsequently contacted/coupled with purified lipase (200 µl = 0.325 mg of protein) at 8^0C upto 20 h in separate glass vials. The residual supernatant was traced for volume, activity and protein (unbound) in each matrix. The mass of enzyme-incubated matrices was measured and activity was assayed using 100 mg of immobilized-matrix. The immobilization efficiency of matrices was determined by subtracting unbound protein in the supernatant from the total protein used for immobilization by dividing with total protein content. Adsorption coefficient (A_{coeff}) was expressed as loading capacity of milligram(s) protein to one gram of matrix. Since immobilized *poly* (*N*-AEAAm-*co*-AAc)-*cl*-MBAAm) showed the maximum activity alongwith protein binding and optimum water up take, surface activity, modulators effect *etc*. hence taken as the model support. Subsequently other parameters like pH, temperature, organic solvent stability, specificity and reusability *etc*. were studied systematically with this support. All the experiments were in triplicate unless otherwise stated.

Esterification reactions using immobilized lipase: The esterification of methacrylic acid with hexan-1-ol (equimolar amounts *i.e.* 0.625 moles each) was carried out using immobilized lipase (200 mg) as model reaction. The reactions was carried out an optimal temperature (55^oC) at continuous shaking (200 rpm). The ester synthesized was quantified by estimating residual acid (at an interval 30 minutes; 0-280 min) by simple acid-base titrimetry assay using phenolphthalein as an indicator. The immobilized-matrix was filtered off. The reaction system formed two layers; ester formed was separated from the upper layer and lower layer was again treated with alkali to neutralize the unreacted acid. The total. The determination of ester formed was also confirmed by polymerizing in presence of APS (1%) at 60^oC for half an hour. The resultant monomers and polymers were characterized by FT-IR. The control esterification reaction (without immobilized lipase) was also maintained under similar reaction conditions.

Results

Immobilization of enzyme on hydrophilic-hydrogel(s)*:* Immobilization of purified lipase on to a weakly hydrophilic hydrogel was studied as a function of structural aspects of hydrogel besides the role of environmental factors like pH, temperature and incubation time. The purified lipase of *B. coagulans* MTCC 6375 was optimally bound to both hydrophilic-hydrogel however, with varing A_{coeff} *i.e.* 525 mg protein g^{-1} matrix (approx. 95% binding; possessed 145 IU g^{-1} matrix) of *poly* (AEAAm-*co*-AAc)-*cl*-MBAAm-bound lipase whereas *poly* (AEMAAm-*co*-AAc)-*cl*-MBAAm had low A_{coefft} (Table 1). The immobilization though a surface phenomenon, yet strong enough interactions take place between with lipase and the support, as is evident from the loss of only 50% activity even after the tenth cycle.

Effect of pH on activity of immobilized lipase*:* Hydrophilic hydrogel AEAAm-*co*-AAc-*cl*-MBAm characteristically displayed broad pH optima from 5.7 to 8.0 pH for the hydrolysis of *p*-NPC (Fig 1). These results suggested that the hydrogel immobilized lipase efficient in attaining hydrolytic potential on both acidic (pH 5.7) as well as in slightly alkaline buffers (8.0 pH).

Table 1: Immobilization characteristics of hydrogel(s)

Hydrogel series	Bound lipase activity (IU g^{-1})	[#]Percent change in activity	A_{coeff} (mg protein.g^{-1} matrix)
Poly (*N*-AEAAm-*co*-AAc)*	152	+9.5	525
Poly (*N*-AEMAAm-*co*-AAc)*	147	+2.9	500

*-*cl-N,N*-MBAAm

[#]*A decrease or an increase in the in lipase activity was calculated on the basis of amount of lipase (200 µl ~ 0.352 IU) used during immobilization/ binding onto the hydrogel. The values in the square parentheses indicate Mean Standard Error (MSE).*

Effect of temperature on activity of immobilized lipase*:* The effect of various incubation temperatures (35 to 65^oC) on the activity showed good lipase activities over wide range of temperatures. However, the optimal activity of 1.38 IU g^{-1} was recorded at a temperature of 55^oC (Fig. 2).

Substrate (C-chain length) specificity of silica-immobilized lipase: A marked increase in the hydrolytic activity towards medium C-chain length ester (*p*-NPC) was noticed with hydrogel-immobilized lipase preparations (Table 2).

Table 2: Effect of acyl chain length on *poly* (AEAAm-*co*-AAc-*cl*-MBAm)-immobilized lipase of *B. coagulans.*

***p*-nitrophenyl acyl substrate**	**C-length**	**Lipase activity (IU g^{-1})**
p -nitrophenyl formate	1: 0	166
p -nitrophenyl acetate	2: 0	171
***p* -nitrophenyl caprylate**	**8: 0**	**251**
p -nitrophenyl laurate	12: 0	170
p -nitrophenyl palmitate	16: 0	171

Effect of organic solvents on immobilized lipase: When the effect of various organic solvents on the activity of the immobilized lipase was studied, amongst various alcohols, exposure of bound enzyme to *iso*-propanol, amongst alkanes exposure to *n*-hexane showed a maximum residual activity of 1.0 and 1.50 IU g^{-1}, respectively. (Fig. 3-4).

Direct esterification using immobilized lipase: The direct esterification of methacrylic acid with hexan-1-ol resulted in high conversion (≅ 98%) into *n*-hexyl methacrylate in solvent free system under optimal conditions. Evidence of ester formation was validated by FT-IR spectra; showed peaks at 1725.6 cm^{-1} (C=O stretching of ester), 1172.2 cm^{-1} (symmetric stretching of O-C-O grouping of ester), 1637.6 cm^{-1}(C = C) stretching of methacrylic group). and (spectra are not included) peaks at 1729.9 cm^{-1} (C=O stretching of ester), 1151.3 cm^{-1} (symmetric stretching of O-C-O grouping of ester), while the peak near 1640 cm^{-1} (C = C stretching of methacrylic group) is absent after polymerization.

Discussion

Lipases (*EC*: *3.1.1.3*) that were capable o catalyzing hydrolysis, esterification and transesterification reactions both in aqueous and organic phases predict multifold advantages when employed in immobilized form(s) for syntheses pursuit. In present study, a novel approach to immobilize lipase into microporous polymeric-hydrogel *via* embedding enzyme in surface-active matrix has been explored. The biocatalytic properties of bound-lipase was greatly enhanced (hyper-activation) in hydrolytic reaction as well as under influence of organic media exposed hydrogel. This is first report to our knowledge whereby hydrophilic-hydrogel had hyper-activation of lipase activity in contrary to reports with hydrophobic polymers (Aucoin *et al.,* 2004). The p*oly* (AEAAm-*co*-AAc-*cl*-MBAm)-hydrogel possessed a copolymer moiety aminoethyl-acrylamide (AEAAm) that contained two highly basic amino groups, which might have interacted with the amino acid residues at the active site of the enzyme to yield enhanced catalytic activity. Besides, the presence of hydrophilic AEAAm might have influenced the availability of the reactants at the catalytic site of poly (AEAAm-*co*-AAc-*cl*-MBAm)-hydrogel immobilized lipase at a relatively low pH (acidic) microenvironment created by presence of excess of acid. In this study, higher conversion rate (> 98%) in esterification of *n*-hexyl methacrylate indicated improved efficiency of this hydrogel bound-lipase in comparison to previously attained esterification rate.

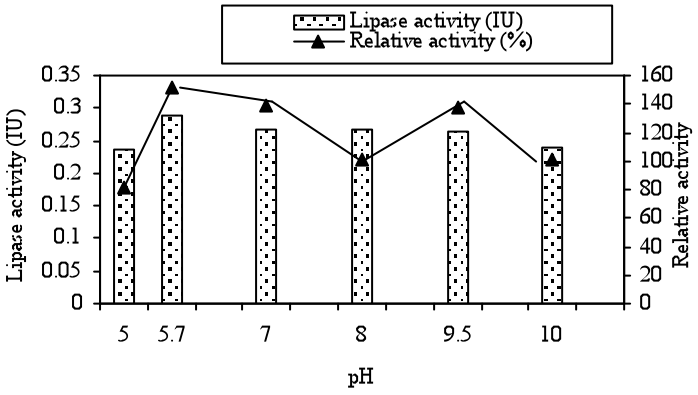


Fig. 1 : Effect of pH on poly (AEAAm*co*-AAc)-*cl*-MBAAm)-hydrogel immobilized lipase.

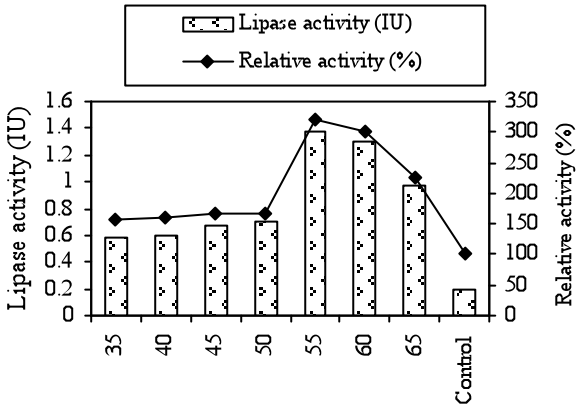


Fig 2: Effect of temperature on *poly* (AEAAm*co*-AAc)-*cl*-MBAAm)-hydrogel immobilized lipase.

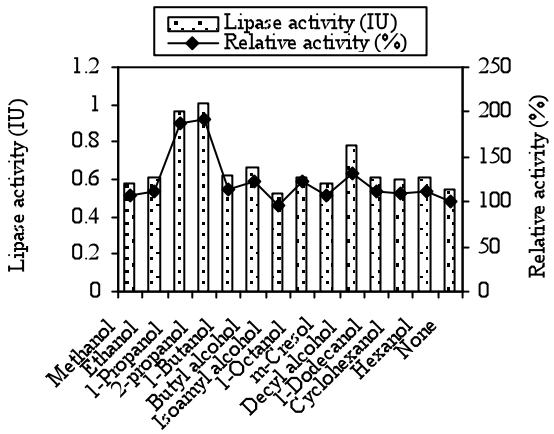


Fig. 3: Effect of various alcohols on *poly* (AEAAm*co*-AAc)-*cl*-MBAAm)-hydrogel immobilized lipase.

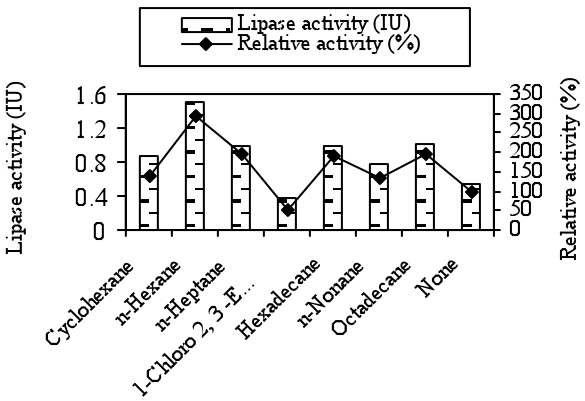


Fig. 4: Effect of various alkanes on *poly* (AEAAm-*co*-AAc)-*cl*-MBAAm)-hydrogel bound lipase.

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