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Original Research Article

The Effect of Calcium Alginate Entrapment on the Stability of Novel Lipases from *P. Reinekei* and *P. brenneri*

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HIGHLIGHTS

- Two novel lipases have been entrapped in calcium alginate for the first time.
- A statistically enhanced stability in 1M EDTA was observed following entrapment.
- The novel entrapped lipases display excellent storage stability and reusability.

ABSTRACT

The high cost of soluble enzymes can limit their use for commercial and industrial purposes. Immobilization can enhance enzyme reusability, thereby reducing product isolation costs and overcoming this economic barrier. In the current study, two novel, purified lipases from *Pseudomonas* sp. (*Pseudomonas reinekei* and *Pseudomonas brenneri*) were entrapped in a calcium alginate matrix, with the aim of simultaneously enhancing enzyme reusability and stability. Following entrapment, the retained activity of the enzyme-alginate composite was verified by an enzymatic hydrolysis reaction of a *p*-nitrophenol palmitate substrate. The effect of the enzyme-alginate entrapment against various physiochemical parameters such as pH, temperature, metal ions, and solvents were subsequently examined. The entrapment was found to have minimal beneficial stability gains. However, enhanced enzyme reusability (up to 3 cycles) and storage stability (up to 18 days at 4°C) of the calcium alginate entrapped lipase, as indicated by residual hydrolysis of *p*-nitrophenol palmitate, was observed, suggesting potential roles for calcium alginate entrapped lipases in cost efficient enzyme catalysis.

Introduction

With the advance in recombinant DNA technology and high-throughput screening, the cost of finding and employing a reaction-specific enzyme has been significantly reduced. However, enzymes can lose their activity during long-term reaction(s) and/or storage due to denaturation of their tertiary structure and/or active site (Homaei et al., 2013). Furthermore, the recovery of enzymes from the reaction medium for repeated use, and to enhance product purification, is a significant challenge for industrial applications of enzymes. Conversely, once immobilized or entrapped, the stability of an enzyme is typically enhanced and the enzyme composite can be easily recovered for reuse. Enhanced enzyme stability following immobilization or entrapment has been linked to a more rigid tertiary structure based (Bagi et al., 1997; Anwar et al., 2009; Shikha et al., 2017) and the inability of denaturants to interact with the enzyme (Kanmani et al., 2015). Enzyme immobilization has been a popular research topic since the 1960s and various enzyme immobilization methods; such as adsorption, covalent bonding, entrapment, and cross-linking, have been developed (Mohamad et al., 2015). An encapsulation

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or entrapment method for enzyme immobilization keeps the enzyme trapped inside an inert, porous material without affecting its structure. Several natural polymer materials like cellulose, alginate, collagen, chitosan and starch, and some conductive polymers such as polyaniline and Nafion have been used for encapsulating/entrapping enzymes (Chang, 2018). Enzymes entrapped inside these solid particles are protected from the external harsh reaction environment and, as a result, the stability of the enzyme tends to be unaffected, or sometimes enhanced. The current study involves investigating the entrapment of two novel lipases from novel bacterial strains (*P. reinekei* and *P. brenneri*) in calcium alginate beads and quantifying the effect of entrapment on stability and reusability of these lipases.

Materials and Methods

Chemicals

All chemicals were of analytical grade and were purchased from Sigma-Aldrich. Lipases for entrapment were from *P. reinekei* (termed H1; Priyanka *et al.*, 2019) and *P. brenneri* (termed H3; Priyanka, 2019b).

Standard spectrophotometric assay

p-NPP (*p*-nitrophenyl palmitate) was used as the substrate for the estimation of lipase activity as per Glogauer and colleagues (Glogauer et al., 2011); lipase activity was measured after a defined period of static incubation at 28°C (Priyanka et al., 2019).

Entrapment of lipase(s) in calcium alginate beads

A 5% (w/v) stock solution of sodium alginate was prepared in double distilled water under continuous stirring. Lyophilized purified lipase (from both H1 and H3; Priyanka, 2019b) and porcine pancreas lipase (as a commercial standard) were reconstituted separately in 10mM Tris-HCl buffer (pH 7.0±0.2) to a final concentration of 1IU/ml. Reconstituted lipase solutions were used to dilute the stock sodium alginate solution to achieve a final concentration of 2% (w/v) sodium alginate. Under continuous stirring, the alginate enzyme solution was mixed for 10 minutes following reconstituted lipase solution addition. Each alginate-enzyme solution was subsequently added, dropwise, to a beaker containing 0.2M $CaCl_2$.

Hard calcium alginate beads were formed after the sodium alginate drops remained in the $CaCl_2$ solution for 60mins at room temperature. After 60mins, the beads were separated from the $CaCl_2$ solution by using a 1.2µm filter and were washed twice in double distilled water, following filtration after every wash. The beads were stored overnight (10h) at room temperature in a vacuum desiccator to remove any water present on the bead surface. The beads were then stored at 4°C, for no more than two weeks, in glass air-tight container until further use.

Characterization of lipases entrapped in calcium alginate beads

Estimation of reaction time

Calcium alginate beads, with and without entrapped lipase, were checked for lipase activity as per the standard *p*-NPP-based spectrophotometer assay. In brief, the catalysis of *p*-NPP substrate was verified by measuring absorbance at 410nm every 15 min, for 90mins, at 28°C. Simultaneously, the rate of hydrolysis of the free enzyme was also monitored under the same conditions. The amount of enzyme activity obtained with free and entrapped enzyme over a 90min incubation period was calculated and compared.

Calcium alginate beads characterization

For all the free lipase versus calcium alginate entrapped lipase trials; the ratio of enzyme and substrate (*p*-NPP) was maintained constant as per the standard *p*-NPP spectrophotometer assay. Calcium alginate beads generated were weighed and the concentration of enzyme entrapped in the beads was calculated by comparing with initial concentration of lipase used for bead generation as follows: If Xg of beads were generated from Yg of lipase, then the amount of lipase in 1 bead = X / Y. The volume of *p*-NPP required for Yg of beads was calculated by using the same enzyme:*p*-NPP ratio as that for free lipase.

Stability studies

The effect of pH, temperature, solvent and other additives on the stability and activity of calcium entrapped lipase from *P. reinekei* (H1) and *P. brenneri* (H3) was calculated using the standard *p*-NPP spectrophotometric assay following timed exposure to the variable of choice. To ascertain the effect of pH on the stability of the lipases, purified lipase solution and calcium alginate entrapped lipases were incubated in 50mM of the appropriate buffer, depending on the required pH of incubation. In this study, 50mM of Glycine-HCl (pH 3, 4), 50mM of Tris-Acetate (pH 5, 6), 50mM of Tris-HCl (pH 7, 8, 9) and 50mM of Borate Buffer (pH 10) were used to examine the effect of pH at 28°C following 24 hr of incubation. To examine the thermostability characteristics, both free purified lipase and calcium alginate entrapped lipase were prepared in 50mM Tris-HCl buffer (pH 7.0) and were incubated at 40, 50, 60°C for 45mins. The effect of various metal ions and chemical reagents on purified free and entrapped lipase activity was examined at 28°C and 40°C. To achieve

this purified free lipase solutions and calcium alginate entrapped lipase beads were incubated in 50mM Tris-HCl (pH 7.0) independently with the additive. To understand the effect of organic solvents, purified lipase solutions and calcium alginate entrapped lipase were incubated in airtight vials with organic solvents at 28°C and 40°C for 1 hr and 24 hr under continuous, gentle, stirring. Buffer and solvent blanks were used to identify and verify any interference on the assay caused by each solvent. To characterise the effect of selected additives the purified free lipase and the entrapped lipase were incubated with the selected additives for 24 hr. After incubation, the various additives were removed, the beads were washed twice with Tris-HCl buffer at pH 7.0 ± 0.2. The substrate, *p*-NPP, was added to the beads and incubated for 60mins and the residual activity of the free and entrapped lipases was calculated based on the standard spectrophotometric assay.

Reusability and stability of calcium alginate entrapped lipases

To investigate the reusability of the enzyme entrapped calcium alginate beads, the beads were incubated in *p*-NPP, as per the standard spectrophotometric assay. After 60mins of incubation, the product generated was removed and measured at 410 nm. The calcium alginate beads were subsequently washed twice with 50 mM Tris-HCl buffer at pH 7.0±0.2, before fresh *p*-NPP substrate was added to measure the remaining enzyme activity entrapped in calcium alginate beads. This washing process was repeated for 7 cycles to calculate the reusability of calcium alginate entrapped lipases.

To explore the stability of enzyme entrapped calcium alginate beads, the beads were stored at 4°C. A fixed weight of beads was removed at respective time-points and was checked for lipase activity by incubation in *p*-NPP substrate for 60mins; stability was compared against the activity of lipase obtained at day 0 (freshly entrapped lipase in calcium alginate beads).

Results and Discussion

For enzyme immobilization and entrapment, it is preferable that the method employed causes as little disturbance to the enzyme as possible. Enzyme entrapment in porous matrices (alginate and acrylamide beads) is a rapid, nontoxic, inexpensive and versatile technique (Datta et al., 2013). This technique offers the benefits of good mechanical strength as well as high porosity for substrate and product diffusion; with alginate being by far the most widely used polymer for enzyme entrapment (Zhang et al., 2013). Alginate is an anionic linear copolymer composed of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid in different proportions and sequential arrangements. Porous alginate beads are synthesized by crosslinking the carboxyl group of the α-L-guluronic acid with a cationic crosslinker ($CaCl_2$ or $BaCl_2$). Calcium alginate is the most commonly used due to its natural biocompatibility, ease of formation, and mild physiological gelation conditions. Enzymes are entrapped by drop-wise addition of sodium alginate solution containing the enzyme to a solution of $CaCl_2$. The cation acts as a cross-linking agent for alginate biopolymer and the droplets precipitate as beads with the enzyme entrapped within (Won *et al.*, 2005). The results from the use of a calcium alginate matrix to entrap two novel lipases is explored subsequently.

Calcium alginate matrix composition

In calcium alginate matrices, the pore size of the beads and the amount of entrapped enzyme can be controlled by changing the concentration of sodium alginate. The capacity to retain enzyme inside the beads (i.e. to reduce leaching) increases with increasing concentration of sodium alginate but, conversely, the activity of immobilized enzyme decreases. An increase in sodium alginate concentrations causes extensive cross networking of the matrix, resulting in reduced pore size of beads and causing reduced mass transfer or diffusion of substrate to the enzyme active site. Talekar and Chavare (2012) reported that appropriate and active enzyme entrapment inside calcium alginate beads also depended on the concentration and contact time between alginate and $CaCl_2$. Bhushan and co-workers (2008) noted that the maximum activity of entrapped lipase was achieved at 1.5% (w/v) alginate concentration and decreased with an increase in the amount of alginate used. In proteases, for comparison, the maximum percent entrapped activity was observed at 2% (w/v) sodium alginate; maximum entrapped enzyme leakage occurred at 1% (w/v) sodium alginate concentration while at 3% and 4% (w/v) sodium alginate concentration the entrapped activity of the enzyme was low (Anwar et al., 2009). Enzyme entrapment efficiency is unaltered by variations in $CaCl_2$ concentration within the working range (0.05–0.3M; Won et al., 2005). Previous studies have shown 0.2M $CaCl_2$ to retain highest activity of entrapped enzyme; however, above 0.3M $CaCl_2$, enzyme activity decreases, as the pH of $CaCl_2$ concentration changes it affects the activity of entrapped enzyme (Anwar et al., 2009). Therefore, for the entrapment of lipases from *P. reinekei* (H1) and *P. brenneri* (H3) the final concentration of sodium alginate was 2% (w/v), with 0.2M $CaCl_2$ as a cross-linking agent.

Catalysis reaction time optimisation

The higher the concentration of alginate, the lower the bead porosity and, hence, the lower the enzyme activity will be due to limited mass transfer of substrate and

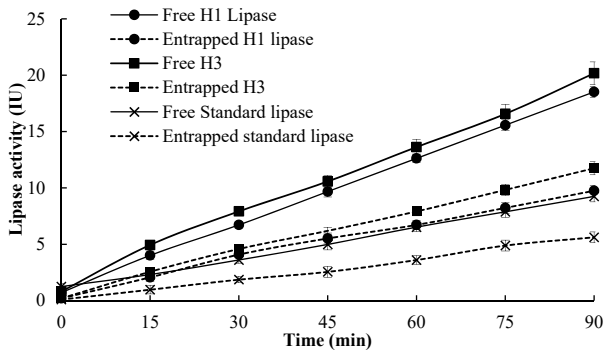

Figure 1. *p*-NPP spectrophotometric determination of the optimal time required to obtain equal lipase activity between free lipase and lipase entrapped in calcium alginate beads. 60 min of incubation time for enzyme entrapped in calcium alginate beads resulted in the same activity as 30 min of free enzyme. Standard lipase is the positive control while *p*-NPP substrate was used as negative control. No spectrophotometric absorbance was obtained in negative control.

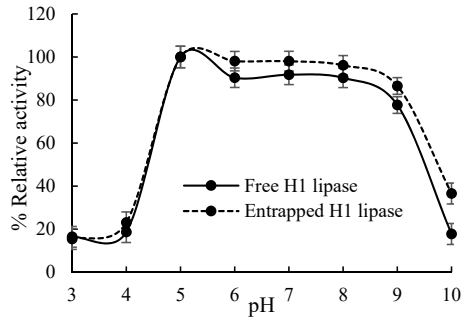

Figure 2. For pH stability studies, residual activities of entrapped H1 lipase (*P. reinekei*) was measured after 24 hr of incubation at 28°C in the presence of different pH buffers (pH 3.0±0.2 to pH10±0.2). Data represented here is the comparison of free and entrapped lipase, while values are the mean of three independent assays and error bars represent standard deviation.

product. Important parameters influencing internal mass transfer are particle size, pore size and the effective diffusion coefficient of the substrate within the pores of the enzyme (Won et al., 2005; Talekar and Chavare, 2012; Kumar et al., 2014). Entrapment based immobilization can also affect the flexibility of the enzyme. In this study, a 60min incubation of calcium alginate beads entrapping lipase from *P. reinekei* (H1) and *P. brenneri* (H3) generated equivalent lipolytic activity comparable to that of free lipase from the respective source, however, a two-fold difference in the reaction time was noted (see Fig. 1). For all calcium alginate immobilisation characterisation studies, a 60min incubation with the substrate was used in order to compare it with free lipase enzymatic activity.

Effect of pH

Due to changes in the degree of ionization of amino acid residues at the active site, leading to changes in electrostatic potential (Ψ), the optimum pH of many enzymes shift to higher pH if it they are entrapped in a carrier that is anionic and towards low pH if it is cationic (Palmer and Bonner, 2008). It has been suggested that this shift in optimum pH may be due to changes in acidic and basic amino acid side chain ionization in the microenvironment around the active site (Talekar and Chavare, 2012). This property of the calcium alginate carrier also has an effect on the optimal pH of enzymatic activity (Zhang et al., 2013). The carboxyl is the acidic group present in alginate. The pK_a of guluronic acid is 3.65, and the pK_a of mannuronic acid is 3.38. Therefore, the alginate gels are usually negatively charged at neutral pH. If the enzyme solution is positively charged (pH of the solution < pI of the enzyme); the enzyme could be easily adsorbed on the alginate network (facilitated by the overall negative charge on the enzyme), thus reducing enzyme leakage (Zhang et al., 2013). This charged surface of alginate beads and entrapped enzyme produces a charged microenvironment, which can affect the nature of the enzyme and alters the pH and stability of the entrapped enzyme (Anwar et al., 2009). However; in this study, no statistically significant change in lipase stability/activity was observed in the different pH solutions tested compared to free lipase (see Fig. 2 and Fig. 3). The optimum pH of lipase before and after entrapment was similar for both lipases (pH 5.0±0.2 for *P. reinekei,* H1; pH 8.0±0.2 *P. brenneri,* H3). Similar observations have been made for immobilized proteases where the optimum pH of free and immobilized enzyme remained same (pH 7.5; Anwar et al., 2009). Bhushan and colleagues (2008) have also reported no loss in enzyme activity for immobilized lipase at pH 7; however, a loss in activity was observed at the lower pH values after prolonged incubation (96h); indicating a shift in optimum pH of the enzyme after entrapment.

Thermostability

Entrapment restricts the overall flexibility of the enzyme, thereby typically increasing enzyme thermal stability through enhanced enzyme rigidity. It has been suggested that immobilization creates a microenvironment that protects the hydrogen bonding patterns in lipase at

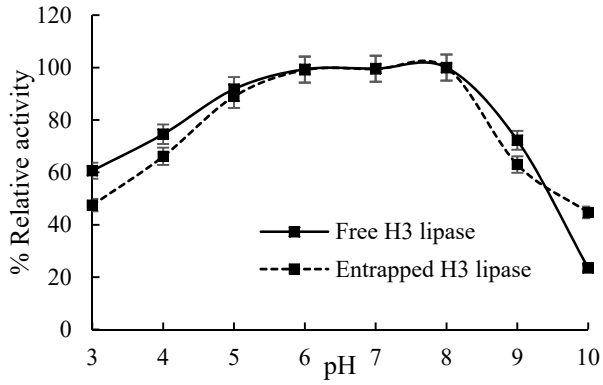

Figure 3. For pH stability studies, residual activities of entrapped H3 lipase (*P. brenneri*) were measured after 24 h of incubation at 28°C in the presence of different pH buffers (pH 3.0±0.2 to pH10±0.2). Data represented here is the comparison of free and entrapped lipase, while values are the mean of three independent assays and error bars represent standard deviation.

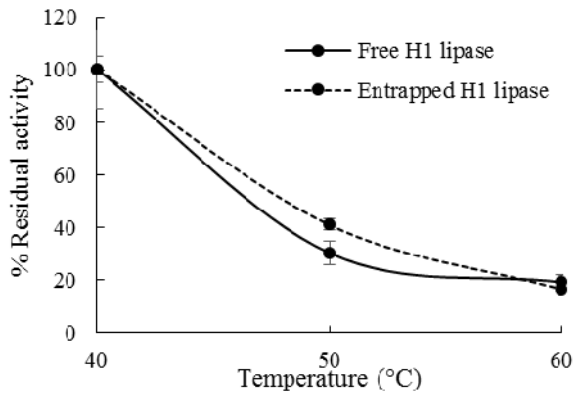

Figure 4. Thermal stability of entrapped purified lipase from H1 (*P. reinekei*) was studied by incubating the entrapped enzymes at 40, 50, 60°C for 1h in Tris-HCl pH 7.0±0.2. Residual activity (%) at each temperature was calculated compared to that at 0hr. Data represented here are the mean of three independent assay values and error bars represent standard deviation.

higher temperatures (Knezevic et al., 2002). Thus, at higher temperatures enzyme unfolding can be reduced, to some extent, through immobilization. In most cases, immobilization results in a broadening of the functional temperature range of enzyme activity compared to the free enzyme (Zhang et al., 2013). However, in the current study, calcium alginate beads entrapping lipases from *P. reinekei* (H1) and *P. brenneri* (H3) showed no statistically significant increase in thermostability compared to their free forms (see Fig. 4 and Fig. 5). Since the free form of lipase from H1 (*P. reinekei*) was unstable above 50°C (Priyanka et al., 2019) and the free form of lipase from H3 (*P. brenneri*) was unstable above 60°C (Priyanka, 2019b); thermostability of their calcium alginate entrapped forms was not documented at higher temperatures and prolonged durations. To fully understand the significance of lipase entrapment in calcium alginate beads in terms of thermostability; incubation at higher temperatures (>60°C) and prolonged times (>45mins) would have been required.

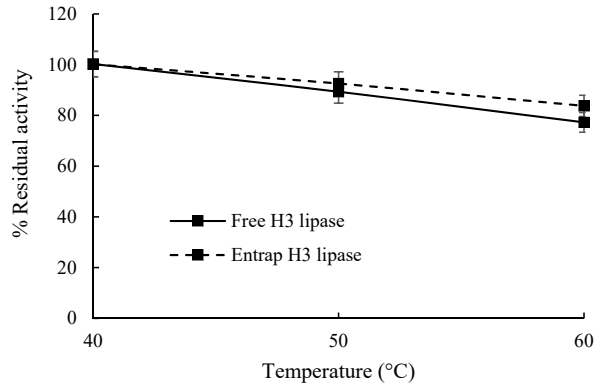

Figure 5. Thermal stability of entrapped purified lipase from H3 (*P. brenneri*) was studied by incubating the entrapped enzymes at 40, 50, 60°C for 1h in Tris-HCl pH 7.0±0.2. Residual activity (%) at each temperature was calculated compared to that at 0hr. Data represented here are the mean of three independent assay values and error bars represent standard deviation.

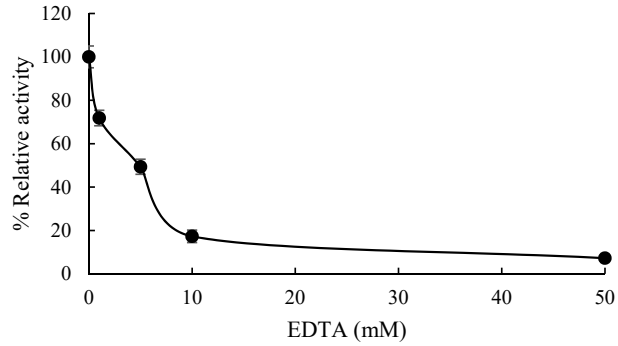

Figure 6. The effect of various concentration of EDTA on the stability of entrapped H1 lipase. All samples were prepared in 50 mM Tris-HCl buffer pH 7.0±0.2 with different concentrations of EDTA and were incubated at 28°C for 24 hr. Relative activity (%) was calculated relative to that of entrapped enzyme with same conditions but no EDTA after 24 hr of incubation. Data represented here are the mean of three assay values and error bars represent standard deviation.

Effect of additives

In the presence of 1mM EDTA, there was a significant increase in stability for both entrapped lipases compared to their free forms (see Table 1). The enhanced stability could be due to the reduced accessibility of EDTA to entrapped lipase; or, more probably, due to the chelation of Ca^{2+} ions present in alginate beads instead of metal ions required for the stability/activity of the lipases. With increased concentrations of EDTA, the stability of entrapped lipases decreased (see Fig. 6) indicating that the chelation of divalent metal ions associated with the

Table 1. The effect of various metal ions and effector molecules/chemicals on the stability of entrapped and free purified lipase from H1 (*P. reinekei*) and H3 (*P. brenneri*).

Additive	Lipase from H1 (*P. reinekei*)		Lipase from H3 (*P. brenneri*)	
	Free	Entrapped	Free	Entrapped
Control (no additive)	100.00	100.00	100.00	100.00
$CaCl_2$	99.92 ± 2.65	97.78 ± 2.77	106.03 ± 2.76	103.03 ± 2.37
$MgCl_2$	94.67 ± 1.57	104.21 ± 2.06	93.52 ± 2.32	100.21 ± 2.49
KCl	95.30 ± 1.16	94.76 ± 2.19	103.12 ± 2.24	101.76 ± 2.19
NaCl	100.95 ± 2.23	104.76 ± 2.19	99.84 ± 2.18	104.76 ± 2.19
EDTA (1mM)	26.25 ± 1.39	75.29 2.65*	0.00	71.82 ± 2.09***
4-Mercaptaethanol	122.22 ± 2.22	123.17 ± 2.46	103.10 ± 2.13	123.17 ± 2.46
Polysorbate-80	62.25 ± 2.39	33.25 ± 2.39**	98.29 ± 2.11	38.25 ± 2.39****
Triton X-100	100.00 ± 1.50	45.16 ± 1.87****	89.94 ± 2.75	42.16 ± 2.87****
SDS	110.03 ± 2.17	4.60 ± 1.31****	59.70 ± 1.46	9.60 ± 1.32****
Urea (1M)	84.16 ± 2.87	99.31 ± 2.35*	61.39 ± 2.24	94.03 ± 2.95**
$CoCl_2$	95.84 ± 2.12	91.94 ± 2.42	91.14 ± 2.92	80.61 ± 2.34*
$MnCl_3$	103.078 ± 2.93	106.54 ± 2.14	94.55 ± 2.13	121.54 ± 2.06*
$FeCl_3$	102.95 ± 2.24	98.55 ± 2.63	6.68 ± 1.86	128.56 ± 2.47****
$ZnSO_4$	81.75 ±2.66	82.05 ± 2.13	0.00	37.06 ± 1.95*

All samples were prepared in 50 mM Tris-HCl buffer pH 7.0 ± 0.2 and were incubated with 10 mM concentration of respective additive (except EDTA and Urea) at 28°C for 24 hr. Relative activity (%) was calculated relative to that of entrapped enzyme at same temperature but no additive after 24 hr of incubation. Data represented here are the mean of three independent assay values, with standard deviations noted. *p ≤ 0.05, **p ≤ 0.01, ***p < 0.001, ****p < 0.0001 represents significant, very significant and extremely significant change in lipase stability (after entrapment) based on t-test.

Table 2. The effect of various detergents on the leaching of lipase from calcium alginate beads containing entrapped purified lipase from H1 (*P. reinekei*) and H3 (*P. brenneri*).

Lipase source	Detergent	Initial activity (%)	% Activity retained in beads	% Activity in buffer
H1 (*P. reinekei*)	Triton X-100	100.00	12.42 ± 1.22	37.82 ± 1.44
	Poly-80	100.00	37.18 ± 1.17	62.16 ± 1.05
	SDS	100.00	18.26 ± 1.38	62.59 ± 1.55
H3 (*P. brenneri*)	Triton X-100	100.00	18.59 ± 1.32	30.71 ± 1.09
	Poly-80	100.00	38.43 ± 1.05	61.41 ± 1.21
	SDS	100.00	15.68 ± 1.28	54.87 ± 1.98

All samples were prepared, in independent triplicates, in 50 mM Tris-HCl buffer pH 7.0±0.2 and were incubated with 10mM concentration of respective detergent at 28°C for 24 hr. Data represented here are the mean of three independent assay values, with standard deviations noted.

enzyme, along with the Ca^{2+} ions of calcium alginate beads, eventually becomes critical for enzyme stability. Metal ions at lower concentration are known to inhibit the activity of immobilized enzymes; 3.0 mM of various metal ions (Ca, Mg, K, Na, Cu) decreased the lipolytic activity of entrapped rice bran lipase (Kanmani et al., 2015). In this current study, no statistically significant changes in lipolytic activity were observed in calcium alginate entrapped lipase from *P. reinekei* (H1) or *P. brenneri* (H3) respectively in presence of 10mM of a variety of metal ions. Since the free form of these enzymes were also stable in 10mM of metal ions, no loss of lipolytic activity after entrapment suggests that entrapped forms of these lipases could be used in presence of these metal ions during lipase-based catalysis. Kanmani and co-workers (2015) have noted a decrease in calcium alginate entrapped rice bran lipase activity in presence of 1% (v/v) Triton X-100 and Tween 80; while activity increased in the presence of SDS. A loss of lipolytic activity was also observed in the present study when calcium alginate entrapped lipases from *P. reinekei* (H1) and *P. brenneri* (H3) were incubated with 10mM Poly 80, Triton X-100 and SDS respectively (see Table 1). An observed loss of lipolytic activity from calcium alginate entrapped lipase in presence of detergents was as a result of lipase leaching from calcium alginate beads; as lipolytic activity was observed in the calcium alginate bead free solution (see Table 2). Enzyme leaching could be due to the hydrolysis of the calcium alginate beads into calcium chloride and alginic acid in the presence of the detergents, causing them to lose integrity and permit the entrapped lipase to leach from the beads (Sharma et al., 2014).

Effect of solvents

A statistically significant loss of activity was observed in the presence of different solvent concentrations at 28°C for calcium alginate entrapped lipase from both sources (*P. reinekei*, H1 and *P. brenneri*, H3; see Table 3). Similar observations have been made for rice bran lipase in the presence of 10% (v/v) propanol, where only 58% relative activity was reported in entrapped lipase (Kanmani et al., 2015). The ability to retain the lipase inside the alginate beads decreases with an increase in the hydrophilic character of the solvent (Hertzberg et al., 1992). Non polar solvents, such as petroleum ether and *n*-hexane, also inhibited the activity of rice bran lipase (Kanmani et al., 2015). Although, the possible reason behind the loss of lipolytic activity in the presence of polar solvents has not been investigated before, a logical explanation could be stripping of essential water molecules from the enzyme, resulting in instability. Although no loss of activity was observed for calcium alginate entrapped lipase from both sources in presence of non-polar solvents; no gain in relative activity compared to free lipase was observed. As non-polar solvents are known to bind to the lid of the lipase, keeping the lipase in the open confirmation and further enhancing the lipolytic activity; entrapment might hinder this interaction due to reduced structural flexibility (Priyanka et al., 2018). As the enzyme in the alginate beads cannot move as freely as the enzyme in solution, non-polar solvents cannot interact with the enzyme lid and hence activation of lipolytic activity was observed (Table 3).

Reusability and stability

No significant loss of activity was observed after 3 cycles of reuse with lipase entrapped calcium alginate beads from both sources, however after the 3rd cycle a significant loss of lipolytic activity was observed for both the H1 and H3 lipase (see Fig. 7). This decrease in activity could be due to loss of enzyme from the carrier due to repetitive bead washing at the end of each cycle (Kumar et al., 2014). Another possible reason could be the damage to beads from repeated washing, as reported in case of entrapped lipase from *Candida rugosa* (Knezevic et al., 2002). This limitation can be overcome by coating the surface of alginate bead with

Table 3. The effect of various solvents on the stability of entrapped and free purified lipases from H1 (*P. reinekei*) and H3 (*P. brenneri*) was examined.

Condition	Additive	Lipase from H1 (*P. reinekei*)		Lipase from H3 (*P. brenneri*)	
		Free	Entrapped	Free	Entrapped
28°C 24 hr	Control (no additive)	100.00	100.00	100.00	100.00
	10% Methanol	92.71 ± 2.36	91.12± 2.31	98.64 ± 2.12	99.64± 2.24
	20% Methanol	97.86 ± 2.42	78.26 ± 2.27*	80.019 ± 2.59	70.92 ± 2.90*
	30% Methanol	70.61 ± 2.21	57.32 ± 2.49*	65.01 ± 2.94	51.01 ± 2.94*
	10% Ethanol	92.27 ± 2.02	76.41 ± 2.33*	84.45 ± 2.33	73.45 ± 2.33*
	20% Ethanol	92.07 ± 2.54	54.75 ± 2.83***	16.60 ± 1.99	0.00
	30% Ethanol	24.50 ± 1.33	56.85 ± 2.78	18.00 ± 1.08	0.00
	50% Hexane	193.28 ± 2.93	97.83 ± 2.61****	131.02 ± 2.99	93.02 ± 2.95***
	50% Cyclohexane	275.95 ± 2.14	95.65 ± 2.21****	122.48 ± 2.74	102.77 ± 2.35***
	50% heptane	324.37 ± 2.96	96.74 ± 2.91****	128.80 ± 2.71	98.97 ± 2.13***
40°C 24 hr	Control (no additive)	100.00	100.00	100.00	100.00
	10% Methanol	89.29 ± 2.79	85.86 ± 2.95	102.50 ± 2.02	101.00± 2.09
	20% Methanol	36.23 ± 1.32	55.11 ± 1.86*	91.55 ± 2.40	89.55 ± 2.44
	30% Methanol	26.5 ± 1.59	50.00 ± 1.21*	6.10 ± 0.12	26.10 ± 1.21
	10% Ethanol	44.89 ± 2.65	74.64 ± 2.76*	103.023 ± 2.78	103.03 ± 2.66
	20% Ethanol	37.77 ± 1.97	65.62 ± 2.52*	53.15 ± 2.22	69.29 ± 2.09
	30% Ethanol	33.97 ± 1.61	52.03 ± 2.89*	1.26 ± 0.13	31.31 ± 2.11**
	50% Hexane	399.57 ± 4.94	97.83 ± 2.61****	111.78 ± 2.58	101.44 ± 2.69**
	50% Cyclohexane	461.63 ± 4.24	95.65 ± 2.22****	114.94 ± 2.57	104.94 ± 2.04**
	50% Heptane	378.03 ± 4.14	96.74 ± 2.91****	124.91 ± 2.04	104.38 ± 2.21**

All samples were incubated in solvents at 28°C and 40°C for 24 hr. Relative activity (%) was calculated relative to that of entrapped lipase after 24 hr of incubation at same temperature but without the corresponding organic solvent. Data represented here are the mean of three independent assays with standard deviation. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p < 0.0001 represents significant, very significant and extremely significant difference in lipase stability (after entrapment) based on t-test.

chitosan or silicate (Won et al., 2005). Storage stability of calcium alginate beads confirmed their stability at 4°C for up to18 days with no statistically significant loss in lipolytic activity (see Fig. 8). Hence, calcium alginate

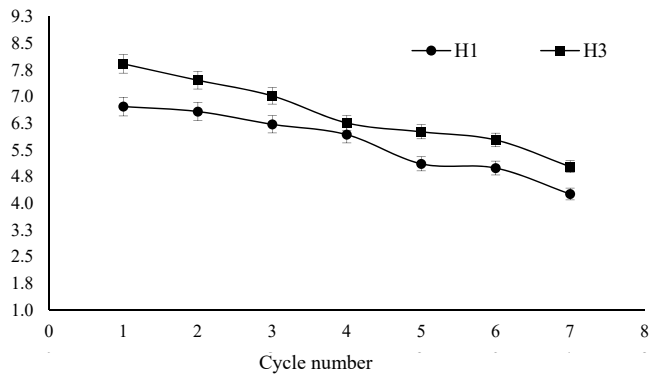

Figure 7. The effect of reusability on the activity of entrapped lipase was monitored over 7 cycles. Enzyme activity was calculated in terms of residual IU. Data represented here are the mean of three independent experiments and error bars represent standard deviation.

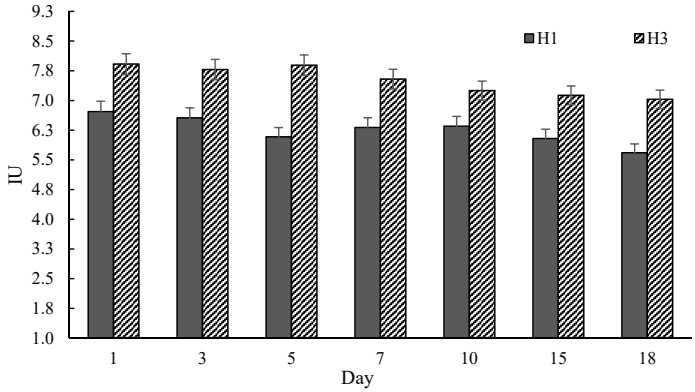

Figure 8. The effect of storage stability at 4°C on the activity of entrapped lipase was monitored over the course of 18 days. Enzyme activity was calculated in terms of residual IU. Data reported here are the mean of three independent experiments and error bars represent standard deviation.

beads entrapped lipase can be re-used for 3 cycles and can be stored at 4°C, for a minimum of 18 days increasing the range of their potential applications.

Conclusion

Entrapment of lipase from H1 (*P. reinekei*) and H3 (*P. brenneri*) in calcium alginate beads reduced the rate of reaction by two-fold due to mass transfer limitations. Furthermore, no significant change in the stability of calcium alginate lipase compared to their respective free form was observed in different pH and temperatures. However, the calcium alginate entrapped lipase showed significant stability in presence of 1mM EDTA; this stability decreased with increased EDTA concentration. As the stability of the two novel lipases after calcium alginate entrapment in presence of organic solvents was also negatively affected, entrapment cannot be considered as an immobilization method to enhance thermal or solvent stability of these lipases.

However, the primary advantage of enzyme recovery noted in this study is a tempting parameter to justify further exploration and application of calcium alginate entrapped lipases for catalytic reactions, including the traditional trans-esterification reaction, to reduce overall catalysis costs.

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Competing Interests

The authors declare that they have no competing interests.

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