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Enzyme Entrapment and Cross-Linking of Psychrophilic Lipase from *Serratia marcescens* VT 1

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Abstract

Immobilization has gained importance in industrial biotechnology as an efficient approach for modifying the characters ensuring the reusability of biocatalysts. The current study focuses on the potential application of entrapment and cross-linking techniques to immobilize the lipase from *Serratia marcescens* VT 1 (SMVT 1). Purified SMVT 1 lipase was immobilized by entrapment onto calcium alginate beads and cross-linking with glutaraldehyde. The effect of different factors affecting entrapment and cross-linking was determined. Further, the immobilized lipase was subjected to characterization and reusability studies. The calcium alginate entrapment and glutaraldehyde cross-linking resulted in an immobilization yield of 65.72% and 77.19% respectively. Optimal conditions for entrapment were 3% sodium alginate, 3 M calcium chloride ($CaCl_2$), and an immobilization time of 45 min, while, for cross-linking it was 0.4% glutaraldehyde and a cross-linking time of 45 min. Characterization and reusability studies highlighted cross-linking as superior with enhanced activity and stability at higher temperatures, a shift in optimum pH to 8, good stability in basic pH, and 58.5% residual activity after eight cycles of reactions. Fourier transform infrared spectroscopy analysis provided insights into the bond formations during calcium alginate entrapment and glutaraldehyde cross-linking. Cross-linking was identified as an ideal mode of immobilization for SMVT 1 lipase.

Keywords: Calcium alginate, Cross-linking, Glutaraldehyde, Immobilization, Lipase, *Serratia marcescens.*

Introduction

Industrial biocatalysis harnesses biological catalysts to drive industrial reactions, offering advantages such as operating under gentle conditions, exhibiting high specificity, minimizing side reactions, and maximizing productivity while keeping environmental toxicity to a minimum (1). Lipases (EC 3.1.1.3), a ubiquitous enzyme known for their role in lipid digestion, have garnered significant interest for their catalytic abilities in esterification and transesterification, making them indispensable in industrial biocatalysis (1, 2). It forms the third most commercially utilized biocatalyst, generally originating from microbes, and finding applications in the production of food, dairy, leather, textiles, detergent, pharmaceuticals, biodiesel, bio-lubricants, and bioremediation (2). Psychrophilic lipases/cold-adapted lipases exhibiting optimal activity and stability at low temperatures produced by organisms adapted to cold environments with biotechnological applications requiring low temperatures like food processing, detergent formulations, biodiesel production, and pharmaceuticals are gaining importance (1).

Despite numerous studies, the foremost reason for the underusage of lipase includes the cost and loss of enzymes during reactions (1). This can be solved by immobilization of free enzymes onto a suitable carrier. Enzyme immobilization involves attaching enzymes onto solid support or within porous matrices without significantly varying their catalytic activity, thereby enhancing their stability and reusability. Immobilization imparts desirable properties comparable to conventional catalysts like enhanced thermal and chemical stability, improved handling, recovery, reusability, and quick cessation of the reaction, thereby lowering contamination, catalyst consumption, cost, and time (3). The three common techniques adopted for lipase immobilization are entrapment, support binding, and cross-linking (1).

Entrapment involves the physical trapping of enzymes within a matrix or polymer network like calcium alginate, agar-agar, K-carrageenin, agarose, gelatine, polyacrylamide,

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chitosan, collagen, and porous ceramics, which restrict their movement while allowing substrates and products to diffuse freely. Cross-linking involves cross-linkage between enzymes or to a solid support using crosslinkers forming covalent bonds with specific amino acids in the enzyme (3, 4). The cross-linking can occur through chemical cross-linking, using crosslinkers like glutaraldehyde and carbodiimides, physical cross-linking via interactions such as hydrogen bonds and hydrophobic reactions, and enzyme-mediated cross-linking, exemplified by transglutaminase (3). Entrapment and cross-linking methods offer several advantages, including retention of enzyme activity**,** enhanced stability, reusability, ease of separation, scalability, design flexibility, environmental compatibility, simplicity, and cost-effectiveness.

The present study involves the immobilization of a purified psychrophilic lipase from SMVT 1 by entrapment in calcium alginate and chemical cross-linking using glutaraldehyde, as well as the investigation of the various factors affecting immobilization, characterization of immobilized lipases and their reusability. The glutaraldehyde cross-linking is expected to give chemical stability, alter optimum pH and temperature, and improve the reusability along with a slight reduction in activity, while calcium alginate entrapment could offer physical stability and reusability with minimal impact on activity.

Materials and Methods

Lipase

The extracellular psychrophilic lipase used was produced by submerged fermentation from a mesophilic soil strain, SMVT 1 (2). The lipase was purified using DEAE cellulose and Sephadex G-75 columns following ammonium sulphate precipitation and dialysis. The purified lipase was lyophilized and stored at -20°C.

Entrapment of SMVT 1 lipase

The bead formation was initially standardized to avoid loss of enzyme. Sodium alginate 1, 2 and 3% was dissolved in phosphate buffer (0.1 M) pH 7 by boiling. The cooled solution was released into pre-chilled $CaCl_2$ solution (1, 2, 3, and 4 M) dropwise using a burette from a height of 10 cm. The formed beads were allowed to harden for 1 h and later separated by filtration. The minimum concentrations resulting in the best beads were used for lipase entrapment.

For entrapment, to 2% sodium alginate solution (boiled and cooled near to room temperature) 1 mg of lyophilized lipase mixed in 1 ml of 0.1 M phosphate buffer (pH 7) was added and stirred to spread uniformly. This solution was taken in a 50 ml burette and suspended dropwise into pre-chilled 2 M $CaCl_2$ taken in a petri plate from a height of 10 cm one drop at a time. The calcium alginate beads containing lipase were kept for 1 h to allow hardening. The beads were filtered and washed in distilled water twice and stored at 4°C in phosphate buffer (pH 7) for further use. The $CaCl_2$ solution was analyzed to calculate lipase activity to determine immobilization yield (5).

Cross-linking of SMVT 1 lipase

Cross-Linked Enzyme Aggregates (CLEAs) were prepared using glutaraldehyde, a cross-linking agent. One mg of lyophilized lipase was mixed with 2 ml phosphate saline buffer pH 7.2 in a 25 ml conical flask. The dissolved lipase was precipitated by adding 6 ml of acetone by stirring for 5 min at 120 rpm in a shaker at room temperature. To this, 0.5% of glutaraldehyde (v/v) was added and further stirred for 1 h at 150 rpm at room temperature. The CLEAs were recovered by centrifuging at 4000 rpm for 15 min at 4°C. The supernatant was discarded; the aggregates were resuspended in a buffer, and vortexed gently for 5 min followed by centrifugation. This step was repeated until the protein concentration (A280) was zero in the supernatant. The CLEAs were stored in phosphate saline buffer pH 7.2 at 4°C for further studies (6, 7).

Estimation of activity of immobilized lipase

The activity of free and immobilized lipases was quantified spectrophotometrically using Para-nitrophenyl palmitate (PNPP) as substrate. The reaction mixture had 9 ml of 50 mM Tris HCl, pH 7.5 containing 40 mg Triton X- 100 and 10 mg Gum arabic mixed with 3 mg of PNPP in 1 ml propane-2- ol. Entrapped lipase (1 g) and CLEAs (0.1 g) were added to the reaction mixture and the process was carried out at 37°C for 30 min. The formation of p-nitrophenol was estimated at 410 nm. One unit of lipase activity was defined as the amount of

Immobilized lipase activity, immobilization yield, and retained activity

Immobilized lipase activity was calculated based on the equation.

$$Immobilized\ lipase\ activity\ (U/g) = \frac{Enzyme\ activity\ of\ the\ immobilized\ lipase}{Quantity\ of\ the\ immobilized\ lipase}$$

The immobilization efficiency was evaluated based on immobilization yield (%) and retained activity (%).

$$Immobilization\ yield\ (\%) = \frac{Amount\ of\ protein\ immobilized}{Amount\ of\ protein\ introduced} \times 100$$

where protein immobilized = amount of protein introduced for immobilization – the amount of protein leached.

$$Retained\ activity\ (\%) = \frac{Enzyme\ activity\ of\ the\ immobilized\ lipase}{Enzyme\ activity\ of\ the\ free\ lipase} \times 100$$

immobilized enzyme resulting in 1 µmol of p-nitrophenol per min per gram under standard conditions.

Factors affecting the entrapment of lipase

Factors like concentrations of sodium alginate and $CaCl_2$ and immobilization time were studied. Sodium alginate concentrations of 2-5% were studied. The bead formation was done with 2 M $CaCl_2$ and 1 mg lipase for 1 h. The various concentrations of $CaCl_2$ investigated included 1-5 M. The remaining parameters were 2% sodium alginate, 1 mg lipase, and an immobilization time of 1 h. The immobilization time was studied for 30, 45, 60, and 75 (min). Sodium alginate and $CaCl_2$ concentration were based on the above studies, and 1 mg lipase was used.

Factors affecting the cross-linking of lipase

The effect of varying concentrations of glutaraldehyde and cross-linking time were studied. The different concentrations of glutaraldehyde studied include (0.2-1%). The rest of the parameters were maintained as such. The different cross-linking times studied include 30, 45, 60, 75, and 90 (min). The best glutaraldehyde concentration was selected, and no other parameters were varied.

Characterization of immobilized lipase

The immobilized lipases were studied for their optimum temperature, thermal stability, optimum pH and pH stability. The optimum temperature for the immobilized lipase was calculated by carrying out the PNPP hydrolysis at different temperatures (10-80°C) for 30 min.

Thermal stability was determined by incubating the immobilized lipase in different temperatures; 10, 30, 50, and 70 (°C) for 2 h in Tris-HCl buffer pH 7.5, and calculating the residual activity by carrying out the PNPP hydrolysis for 30 min. The p-nitrophenol formed in both cases was measured at 410 nm.

To study the effect of pH (4-11) on immobilized lipase the reaction mixture was prepared with buffers of specific pH (sodium acetate buffer 4 and 5, sodium phosphate buffer 6-8, and sodium carbonate buffer 9-11) and the hydrolysis of PNPP was carried out. To study the pH stability the immobilized lipase was incubated in buffer solutions with pH ranging from 6, 7, 8, 9, and 10 for 2 h and residual activity was calculated, PNPP was used as the substrate. In both cases, the reaction time was 30 min and p-nitrophenol formed was determined at 410 nm.

Reusability

To evaluate the reusability, the enzyme-entrapped beads and CLEAs were subjected to 8 continuous cycles of PNPP hydrolysis. Each reaction step was 30 min. After every reaction, the immobilized lipase was filtered or centrifuged out and washed with 50 mM Tris-HCl buffer pH 7.5 before subjecting it to a new reaction. The activity during the first step was considered 100%.

Fourier transform infrared spectroscopy (FTIR) analysis

The presence of functional groups was examined using FTIR (Shimadzu Prestige 21). FTIR analysis was performed for calcium alginate beads, calcium alginate beads with lipase, glutaraldehyde, CLEAs, and free lipase. The absorbance spectra were measured between 400 and 4000 cm^{-1}.

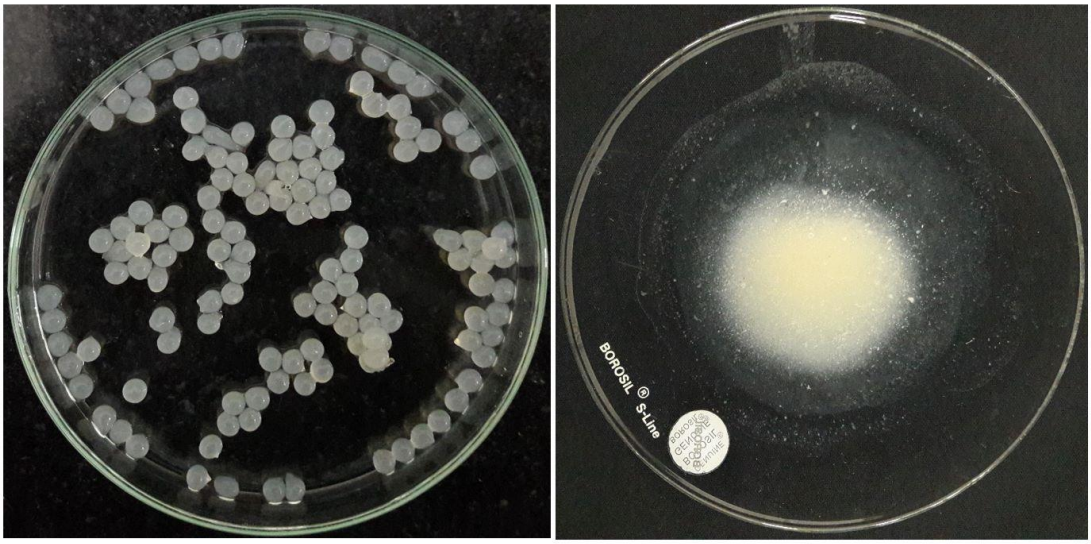
Statistical analysis

All experimental runs were conducted in triplicate and the results were analysed using MS Excel.

Results and Discussion

Entrapment and cross-linking of SMVT 1 lipase

Enzyme immobilization is a vital step for the industrial adoption of enzymes. Out of the different concentrations studied 2% and 3% sodium alginate were effective in bead formation in all concentrations of $CaCl_2$, leading to the use of 2% sodium alginate and 2 M $CaCl_2$ for lipase entrapment. The resulting beads were slightly yellowish, predominantly spherical but with some showing droplet shapes, averaging 0.3-0.4 cm in diameter (Fig. 1A). During the entrapment process, calcium cations crosslink with anionic guluronic acid in alginate via glycosidic bonds (8). The droplet shape formed can be attributed to rapid water release within the alginate network due to high osmotic pressure from $CaCl_2$ concentrations exceeding 1 M (9). Calcium alginate entrapment of SMVT 1 lipase yielded 62.29% immobilization, while SMVT 1 CLEAs (Fig. 1B) achieved 77.19% immobilization (Table 1). CLEAs are formed when glutaraldehyde cross-linking occurs through Schiff base formation with protein amino groups, offering economic production, high stability, and easy optimization (10).



A B

Figure 1: (A) Calcium alginate entrapped lipase, and (B) Cross-linked enzyme aggregates in phosphate saline buffer

Table 1: Immobilization of SMVT 1 lipase by calcium alginate entrapment and cross-linking

SMVT 1 lipase	Enzyme activity	Protein (mg/ml)	Immobilization yield (%)	Retained activity (%)
Free	134.75 U/ml	0.671	-	-
Alginate beads	98.43 U/g	0.23	65.72	73.04
CLEAs	115.08 U/g	0.153	77.19	85.4

Factors affecting SMVT 1 lipase entrapment

The most effective concentration of sodium alginate was 3%, despite the high immobilization yield observed at 5%, it resulted in low retained activity (Table 2). The optimal $CaCl_2$ concentration was 3 M, showing the highest immobilization yield and the ideal immobilization time was 45 min with 3% sodium alginate and 3 M $CaCl_2$. However, the highest enzyme activity and retained activity occurred at 30 min, which might have resulted from incomplete gelation, causing enzyme leaching. Lipase-entrapped beads formed under these conditions showed an increased activity of 3 U/g (101.63 U/g). Similar observations were

previously reported, alginate concentration (2-4%) and $CaCl_2$ concentration (2-5%) were found effective (11). Increased alginate and $CaCl_2$ concentrations can decrease enzyme contact with reactants due to reduced pore size of the beads (12). Adequate gelation and hardening are crucial, depending on the calcium ion source and concentration, to preserve bead structure and shape (13).

Factors affecting SMVT 1 lipase cross-linking

The results suggest the crosslinker concentration to be directly proportional to immobilization yield, and inversely proportional to retained activity. Maximum retained activity was obtained with 0.4% glutaraldehyde and the lowest with 1% (Table 3). When compared to the initial cross-linking time of 1 h, 45 min led to an increased retained activity with 0.4% glutaraldehyde. The immobilization yield was almost similar at 45, 60, and 75 min. The CLEAs generated with 0.4% glutaraldehyde and 45 min of cross-linking time resulted in an activity of 121.17 U/g. The results of cross-linking studies reach agreement with the observation of previous studies (10). Reduced activity despite higher immobilization yield may result from enzyme denaturation and larger aggregate formation due to both inter and intra cross-linking during the reaction (14). Smaller glutaraldehyde molecules can penetrate the protein, affecting active site amino groups (15). Kartal and Kilinc noted that shorter periods resulted in inadequate cross-linking, while longer durations led to reduced protein flexibility and larger particle sizes, decreasing substrate-enzyme contact (10). Long and coworkers observed maximum retained activity with 0.5% glutaraldehyde for *Aspergillus flavus* lipase (14). Pan and colleagues observed 60% residual activity following a 10 min glutaraldehyde cross-linking with *S. marcescens* lipase (16).

Table 2: Factors affecting SMVT 1 lipase entrapment in calcium alginate beads

Sodium alginate				
Concentrations (%)	Enzyme activity (U/g)	Protein (mg/ml)	Immobilization yield (%)	Retained activity (%)
2	98.42	0.215	67.95	73.03
3	106.68	0.161	76.01	79.16
4	104.35	0.164	75.55	77.43
5	95.2	0.157	76.6	70.64

Calcium chloride				
Concentrations (M)	Enzyme activity (U/g)	Protein (mg/ml)	Immobilization yield (%)	Retained activity (%)
1	97.15	0.219	67.36	72.09
2	98.37	0.211	68.55	73
3	105.26	0.163	75.71	78.11
4	100.7	0.175	73.91	74.73
5	92.41	0.169	74.81	68.57

Immobilization time				
Time (min)	Enzyme activity (U/g)	Protein (mg/ml)	Immobilization yield (%)	Retained activity (%)
30	108.66	0.165	75.4	80.63
45	102.27	0.163	75.7	75.89
60	101.65	0.168	74.96	75.46
75	101.78	0.168	74.96	75.53

Table 3: Factors affecting SMVT 1 lipase cross-linking with glutaraldehyde

Glutaraldehyde				
Concentrations (%)	Enzyme activity (U/g)	Protein (mg/ml)	Immobilization yield (%)	Retained activity (%)
0.2	102.49	0.178	73.47	76.05
0.4	114.97	0.155	76.9	85.32
0.6	97.88	0.146	78.24	72.63
0.8	81.31	0.125	81.37	60.34
1	54.9	0.113	83.4	40.74

Cross-linking time				
Time (min)	Enzyme activity (U/g)	Protein (mg/ml)	Immobilization yield (%)	Retained activity (%)
15	99.55	0.168	74.96	73.87
30	109.74	0.16	76.15	81.43
45	121.17	0.151	77.49	89.92
60	115.2	0.153	77.19	85.49
75	101.63	0.149	77.79	75.42

Characterization of immobilized lipase

Immobilization can modify the secondary structure, thereby enhancing or diminishing the enzyme characteristics (17). But, in this study, no significant variation in optimum temperature or drastic change in thermal stability was detected. However, SMVT 1 CLEAs had better relative activity and stability. The optimum temperature of both entrapped and CLEAs was estimated to be 30°C similar to the free enzyme (Fig. 2A). At higher and lower temperatures also, a similar profile can be observed, but the relative activity at every studied temperature improved. In the immobilized form the SMVT 1 lipase retained more than 50% activity at 60 °C in comparison to 30% for the free enzyme. The immobilized SMVT 1 lipase was highly stable at the studied lower and optimum temperatures of 10°C and 30°C (Fig. 2B). The CLEAs showed improved thermal stability at 50°C and 70°C retaining 45-30% of the initial activity, while the free and entrapped SMVT 1 lipase were fully inactivated at 70°C. A rise in optimum temperature after cross-linking from 35 to 45°C and excellent stability of *Rhizopus oryzae* lipase was reported (10). Similar observations during calcium alginate entrapment were reported (18, 19). Reduced activity and minimal stability improvements of calcium alginate entrapped lipase were previously reported (20).

While studying optimum pH the alginate beads were found to lose their rigidity. So, the stability of calcium alginate beads in specific buffers was studied for 5 h. Results are shown in Fig. 3. In pH 8-11 the beads got completely dissolved (Fig. 3E, 3G, 3I, 3J). The sodium phosphate buffer pH 8 and 9 was replaced with Tris-HCl buffer. The beads maintained their structure in pH 8 but in 9 the beads got dissolved. This structural instability observed while studying the optimum pH of alginate beads in the basic buffers 8-11 is due to the calcium ion exchange between the alginate beads and the phosphate and carbonate ions in the buffer generating calcium phosphate and calcium carbonate (21). So, the optimum pH and pH stability were studied in the range of 4-8 for entrapped lipase and 4-11 for CLEAs.

The optimum pH for CLEAs shifted towards the alkaline pH 8 in comparison to pH 7 of free and entrapped lipase (Fig. 4A). At all studied basic pH CLEAs showed elevated relative activity, around 60% of its optimal activity was observed at pH 11 and 30% activity at acidic pH 4 and 5. In comparison, the free and entrapped lipase were fully inactivated at extreme acidic pH and basic pH. The pH stability study was carried out with CLEAs only as alginate beads were unstable at high pH and entrapped lipase was inactive at acidic pH.

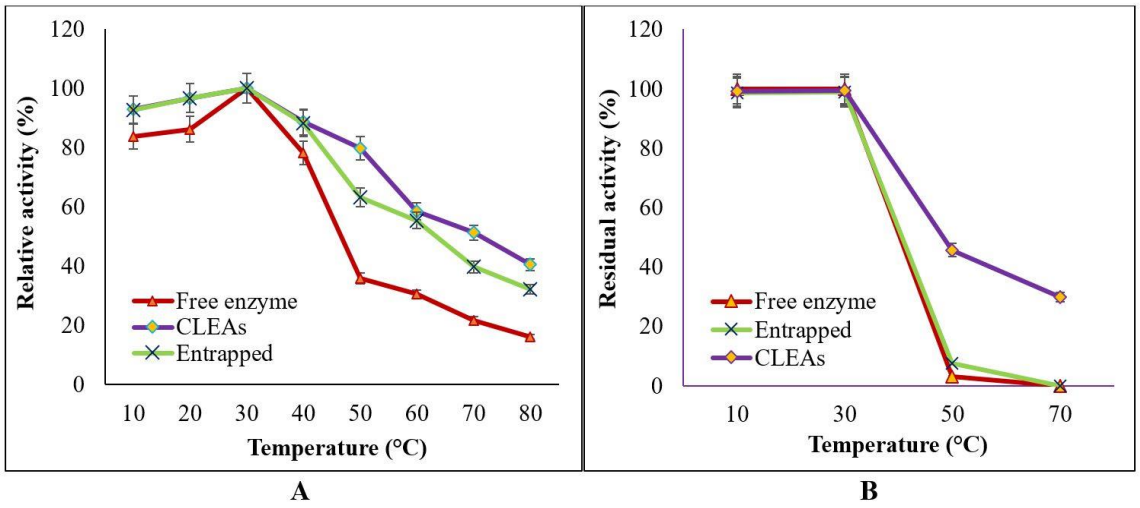


Figure 2: (**A**) Optimum temperature of immobilized lipase, and (**B**) Thermal stability of immobilized lipase

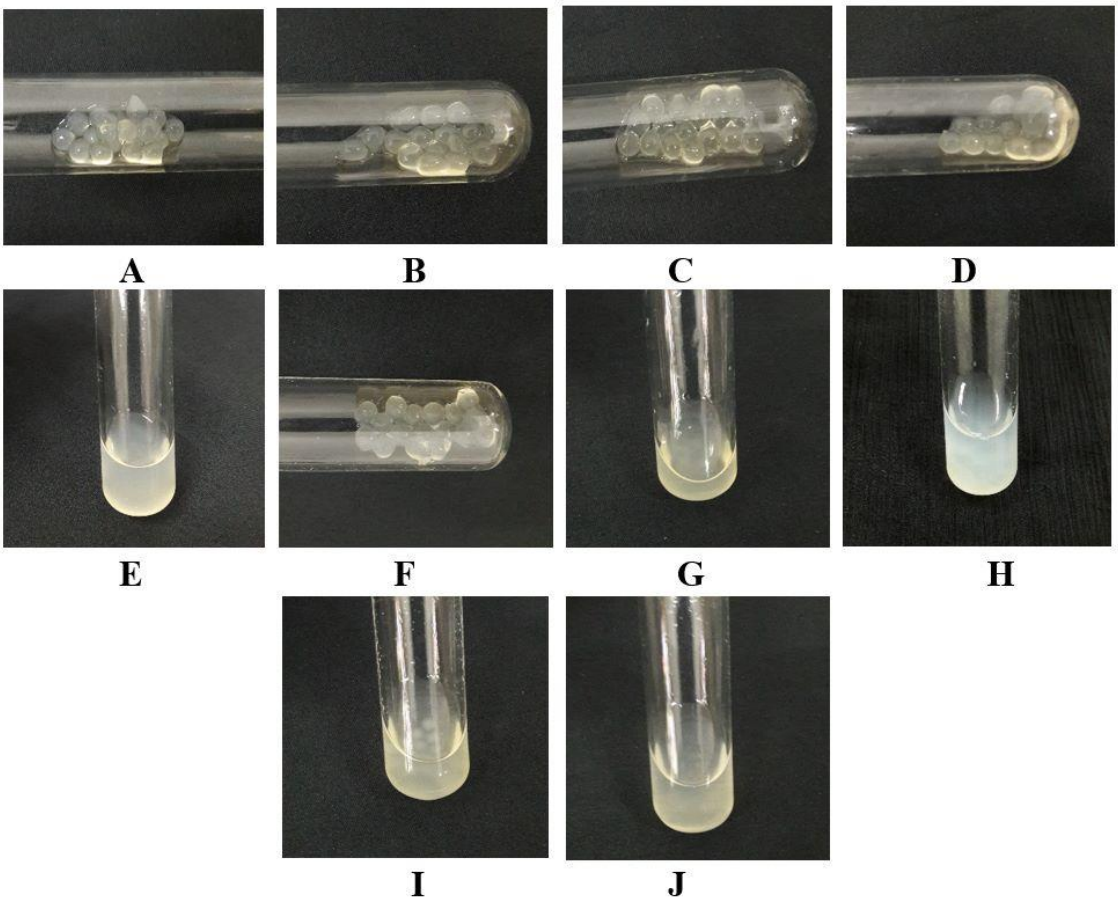


Figure 3: Effect of different pH buffers on calcium alginate beads (**A**) pH 4, (**B**) pH 5, (**C**) pH 6, (**D**) pH 7, (**E**) pH 8, (**F**) pH 8 (Tris HCl), (**G**) pH 9, (**H**) pH 9 (Tris HCl), (**I**) pH 10, and (**J**) pH 11

The CLEAs were detected to have improved stability at basic pH, retaining 87% of initial activity in pH 8 and 65% in pH 7 by the end of the study. The free enzyme was fully inactivated in pH 9 and 10, whereas CLEAs had 69.11% and 56.59% activity left. (Fig. 4B). The results of the optimum pH and pH stability studies are in accordance with previous works (10, 16). A similar shift toward basic pH was observed in the CLEAs of *Rhizopus* lipase resulting from the polyanionic nature of the protein caused by a change in the electrostatic charge of the enzyme after cross-linking (10).

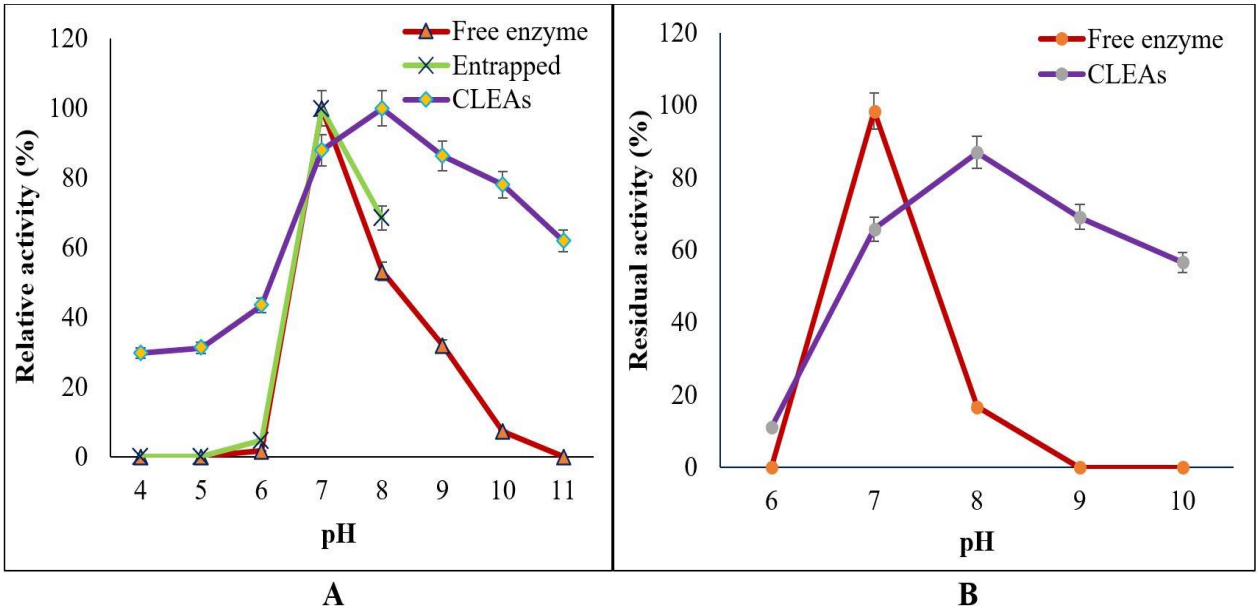


Figure 4: (**A**) Optimum pH of immobilized lipase, and (**B**) pH stability of cross-linked enzyme aggregates (CLEAs) in comparison to free lipase

Reusability

Enzyme immobilization aims for reusability and easy separation from the reaction medium (2). The entrapped SMVT 1 lipase retained 42.96% of its initial activity, in comparison the CLEAs lost nearly 42% of its original activity. However, the entrapped lipase maintained more than 93% activity after the first 3 cycles and showed the highest residual activity till the 4th cycle. When compared regarding the overall activity (CLEAs- 121.17 U/g and alginate beads- 101.63 U/g) CLEAs retained 70.91 U/g activity which was almost 27 U/g more than the entrapped lipase (Fig. 5). The loss in activity can be attributed to physical loss or enzyme denaturation (22). The reuse of alginate beads suffers from enzyme leaching, which can be resolved by chitosan and silicate coating of enzyme-loaded beads (23). For CLEAs, activity decline in reuse results from enzyme leakage due to unstable Schiff base rupture formed with protein amino groups (10). Hara and coworkers estimated a 28% loss in activity after the second cycle for CLEAs from *Pseudomonas* lipase (24).

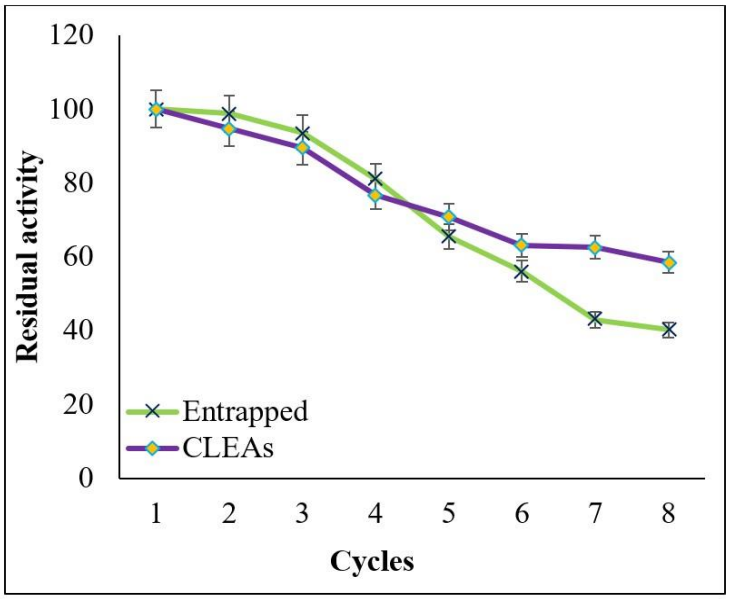


Figure 5. Reusability of immobilized SMVT 1 lipase

Based on the above findings, it can be concluded that calcium alginate entrapment had minimal impact on enzyme structure and activity as revealed by the least amount of variation during characterization studies. Activity loss may result from the pore size of the beads affecting substrate entry, product release, or enzyme leakage. Activity loss during cross-linking may arise from CLEAs physical loss or enzyme leakage due to weak bond breakage. Both methods did not affect substrate selectivity. CLEAs showed altered pH optimum and enhanced thermal stability resulting from structural modification but had little effect on PNPP catalysis. Encapsulation of the CLEAs in an encapsulating medium could mitigate physical loss. The CLEAs, exhibiting optimal and enhanced activity in alkaline pH conditions, coupled with excellent cold adaptability and stability, as well as reusability, could prove invaluable in industries such as detergents, biofuels, and food processing, where there's a demand for reusable psychrophilic biocatalysts (1).

FTIR analysis

FTIR analysis of lipase-entrapped beads revealed an overall rise in transmittance percentage, suggesting potential changes in band frequencies, the emergence of new bands, or alteration of existing ones, indicating the encapsulation of the enzyme within calcium alginate (26). The FTIR spectrum of the lipase (Fig. 6) had characteristic strong peaks in the single bond (3500-3200 cm^{-1} and 2950-2850 cm^{-1} - O–H, N–H and C–H stretch), triple bond (2700-2000 cm^{-1}), and double bond (1735.93 and 1666.50 cm^{-1} - C=O) region. The spectrum of calcium alginate and calcium alginate entrapped lipase (Fig. 7) were found to be almost similar as the reaction in both cases is formation of calcium alginate, exhibiting strong bands in the single bond region (3500-3200 cm^{-1} - O–H) and double bond region: 1649.14 and 1460.11 cm^{-1} - C=O for calcium alginate and 1647.21 cm^{-1} - C=O for the entrapped lipase.

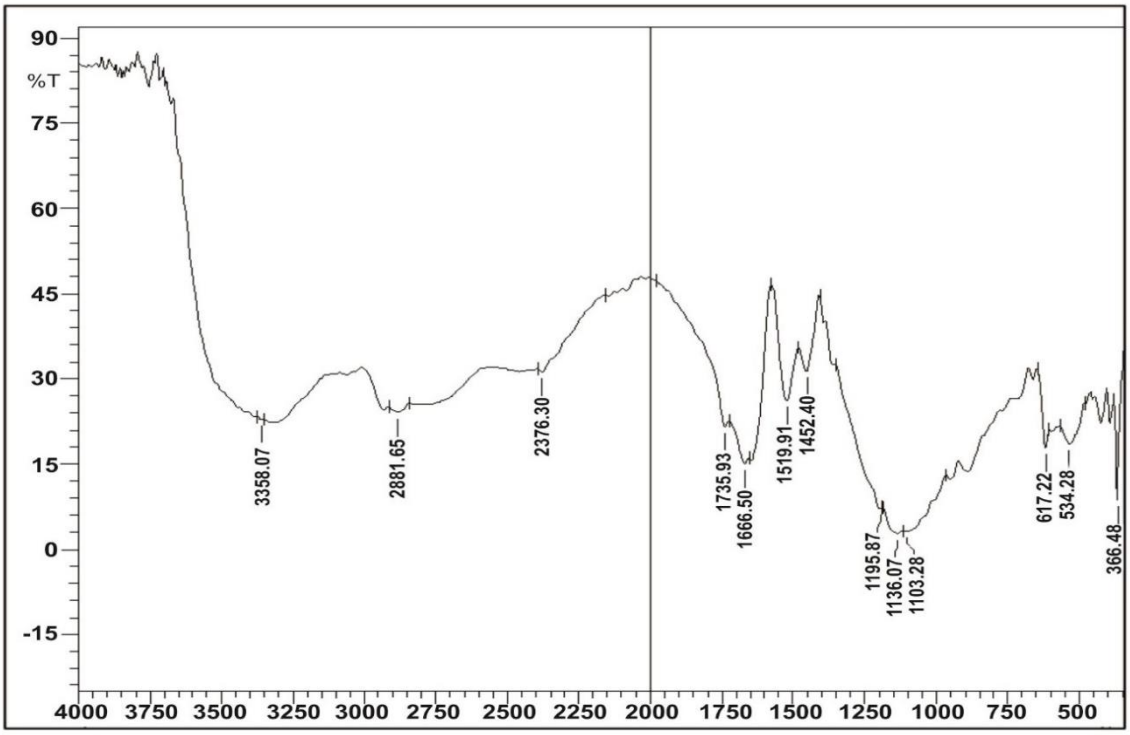


Figure 6: FTIR spectrum of free SMVT 1 lipase

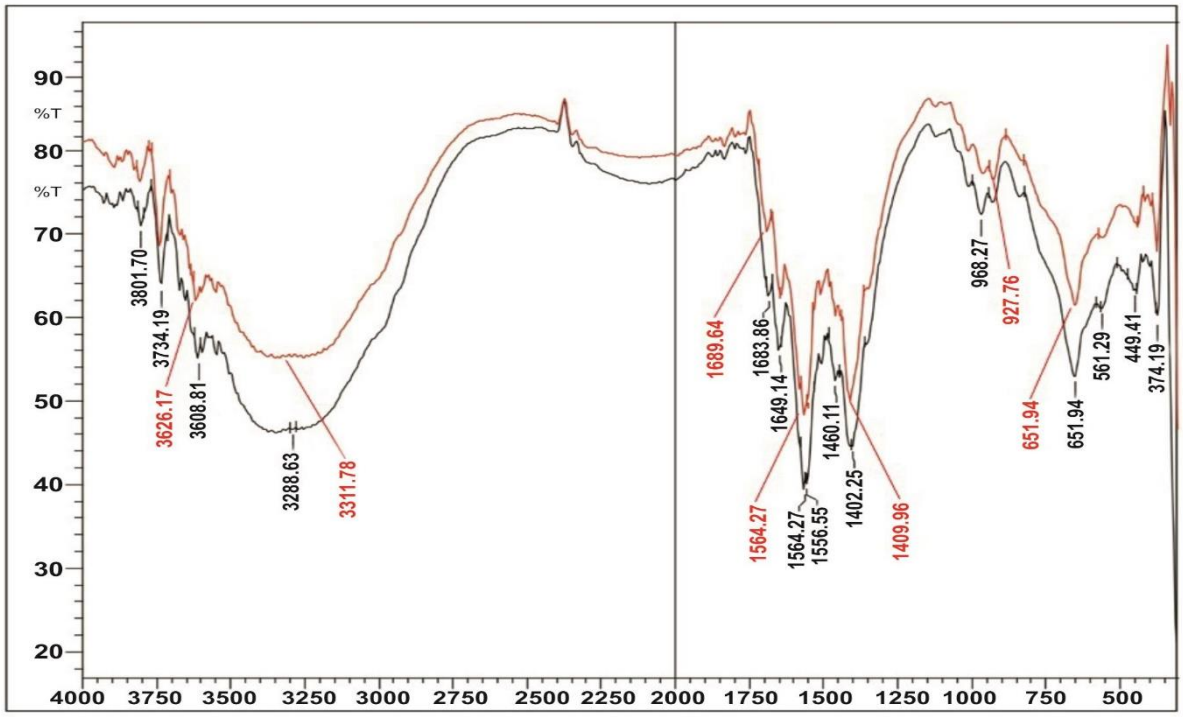


Figure 7: FTIR spectrum of calcium alginate (black) and calcium alginate entrapped SMVT 1 lipase (red)

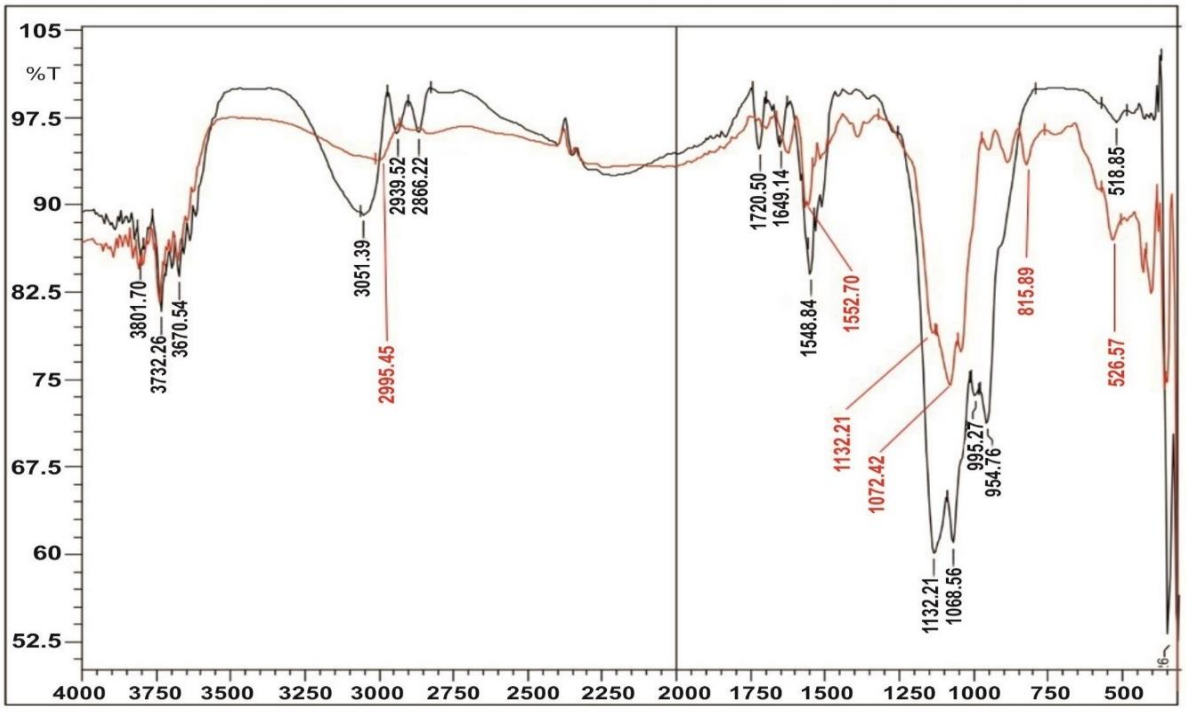


Figure 8: FTIR spectrum of glutaraldehyde (black) and cross-linked enzyme aggregates of SMVT 1 lipase (red)

The FTIR spectrum of the SMVT 1 lipase entrapped beads showed widening of the O–H stretch (3000–3600 cm^{-1}) attributed to the hydrogel nature of calcium alginate (27). The calcium alginate beads arise from the interaction between the COO– groups of sodium alginate, from β-D-mannuronic acid and α-L-guluronic acid (1649.14 and 1460.11 cm^{-1}) with Ca^{2+} through ionic bonding (28). The peaks at 1647.21 cm^{-1}, 1552.70 cm^{-1} and 1460-1409 cm^{-1} might signify carboxylate ions or potentially correspond to protein N–O and amide I and amide II clusters post-entrapment (26).

The FTIR spectra of the glutaraldehyde (Fig.8) had a band in the single bond region specific to O–H stretch (3051.39 cm^{-1}), C–H stretch (2939.52 cm^{-1} and 2866.22 cm^{-1}), C=O stretch (1720.50 cm^{-1}, and 1649.14 cm^{-1}). The inverted peaks at 1600-1725 cm^{-1} designate the aldehyde group. The FTIR spectra of CLEAs (Fig. 8) showed bands similar to lipase and glutaraldehyde with specific variation. Glutaraldehyde's aldehydic groups react vigorously with amines, thiols, phenols, hydroxyl, and imidazole-like functional groups, forming covalent bonds (29). Band intensity and frequency shift at 1600-1750 cm^{-1} result from the interaction between glutaraldehyde's aldehyde group and lipase amide groups. Changes in bands within the 1000-1250 cm^{-1} range (C-N stretch) suggest covalent bond formation via amine and carboxyl reactions (30). Amine and hydroxyl groups of nitrogen and oxygen atoms bond, facilitating enzyme-glutaraldehyde cross-linking. Shifts in O-H, C-H, C=O, and C-N stretch bands, alongside absorption intensity changes, reflect these variations (31, 32).

Conclusion

The results disclosed the successful immobilization of SMVT 1 lipase. CLEAs had improved pH stability in the basic pH with appreciable levels of activity, stability, and reusability. Unlike CLEAs, entrapment resulted in effective immobilization without any characteristic improvement, but can be reused with 80% residual activity for four repeated cycles which is beneficial compared to loss of purified enzyme in a single process. Study shows cross-linking to be a simple and economic protocol resulting in effective immobilization with enhancement of characteristic properties and reusability for enzyme immobilization in comparison to entrapment. The choice between these techniques depends on the specific requirements of the application and the desired balance between stability, reusability, and activity. Further study is needed to enhance and optimize the formation and usability of immobilized lipase along with its application.

Abbreviation

Serratia marcescens VT 1 [SMVT 1], Cross-Linked Enzyme Aggregates [CLEAs], Fourier Transform Infrared Spectroscopy [FTIR], Para-nitrophenyl palmitate [PNPP], calcium chloride [$CaCl_2$]

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Author Contributions

Vivek K conceptualized the study, collected the literature and methodology, performed the experiments, wrote, and edited the manuscript.

Conflicts of Interest

I Vivek K hereby declare that I have no personal or financial conflicts of interest regarding the publication of the manuscript.

Ethics Approval

Not applicable

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