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Physicochemical properties of free and calcium alginate immobilized alkaline pectin lyase from *Bacillus cereus*

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Purified pectin lyase from *Bacillus cereus* was successfully immobilized in alginate beads with a high binding efficiency of 84.55%. The optimal immobilization was achieved using 2.5% (w/v) alginate concentration. Both free and immobilized enzyme showed optimum pH of 10.0 and temperatures of 40 and 45°C respectively. Pectin lyase gave maximum activity at a substrate concentration of 0.5% w/v for free and 0.75% w/v for the immobilized enzyme and relatively similar V_{max} values were obtained for both free (3.3 µmol/min) and immobilized pectin lyase (3.6 µmol/min). The K_m for the immobilized pectin lyase (0.19 mg/ml) was slightly higher than that of the free (0.16 mg/ml) enzyme. The maximum inhibition of 50.2% was observed in the presence of Hg^{2+} ion for free pectin lyase and immobilized enzyme showed maximum inhibition of 67.32% in the presence of Na^{+} ion with statistically significant p-value ($p < 0.05$). Thermal stability was not significantly altered by immobilization. Thermal stability of free and immobilized pectin lyase was relatively similar at different temperatures. The immobilized pectin lyase retained almost 53% of its original activity up to 7th cycle. Furthermore, during storage at 4°C, immobilized pectin lyase retained relative activity of 79.77% and free enzyme retained 63.63% relative activity upto 35 days of storage, this indicated that the immobilization improved stability of the enzyme.

Keywords: Pectin lyase, immobilization, *Bacillus cereus*, calcium alginate, characterization.

Introduction

Microbial enzymes have many valuable characteristics such as specificity, activity and selectivity under optimized conditions[1]. High cost and low operational stability in harsh conditions often hampered the industrial applications of enzymes[2]. To avoid these problems, enzymes have been immobilized to improve their catalytic activity, stability and recovery from reaction mixture. Pectinases are the commercial enzymes and they use pectin as a substrate which is organic polysaccharides of higher plants having galacturonic acid chains with residues of carboxyl groups and varying degree of methyl esters[3-4]. The pectinases have been immobilized on different supports like corn starch microspheres, chitosan-tethered silica, entrapment in calcium alginate, agar-agar, covalent immobilization on magnetite nanoparticles nylon, ion exchange resin, silk and chitin[5-6]. Various support materials such as microporous polyacrylamide microspheres, activated agar-agar gel maintained by multipoint attachment, porous polyethylene terephthalate by surface assimilation and poly (6-caprolactam) activated by glutaraldehyde have been used for pectinase immobilization[7-8]. The various methods used for immobilization of pectinase are physical adsorption, binding to solid carrier, entrapment/ microencapsulation, cross-linking of enzyme accumulates (CLEAs) resulting in messenger-familiar macromolecules[9-12]. Encapsulation has been found potent with alginate gelatin-calcium hybrid carriers that provided increased mechanical stability and prevented enzyme leakage[13]. Electro spun nanofibers and primitive materials have been used as nanostructure supports for enzyme entrapment with their extended ranging applications in the field of refined chemistry, biomedicine, biosensors and biofuels[14-15]. Enzyme side chain amino acids have different functional groups, assist to covalent union of enzyme and peptide-modified surfaces with restraint protein orientation regulate enzyme reactivity[16]. Most often used process is covalent immobilization, when the reaction procedure does not prescribe enzyme in the product[17]. To diminish the damage of enzyme into the substrate solution, the enzymes are immobilized through cross-linking of proteins to an insolvable support[18]. Pectinases including diverse biotechnological applications such as bioscouring of

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cotton fibres, clearing of fruit juices and wine[19] oil extraction, plant protoplast separation, food colouring dye extaction from red grapes[20] and pectic waste water treatment[21]. Pectin lyase is very unique among pectinases because of its direct action on pectin and it is finding great demand in fabric industry and plant fiber processing industry. Using distinct support materials such as EUDRAGIT L100-55 polymer, bentonite, γ-alumina, nylon and chitin pectin lyase has been immobilized[22]. Since very few details are profitable on pectin lyase immobilization which makes it essential to appraise functional constancy of enzyme under industrial processes. Therefore, in the present study, the immobilization of purified pectin lyase from *Bacillus cereus* onto various support materials was analyzed and other immobilization parameters were determined and compared with free enzyme.

Materials and Methods

Materials

Pectin, thiobarbituric acid (TBA), other chemicals used were of high quality analytical grade and used as received. The chemicals used in the present investigation were either obtained from Sigma Aldrich (U.S.A) or Himedia (Mumbai, India).

Pectin lyase from *Bacillus cereus* was precipitated by ammonium sulphate saturation and purified after using techniques of DEAE-cellulose and sephadex G-75 column chromatography.

Pectin Lyase Assay

Pectin lyase assay was performed by spectrophotometeric method using pectin as substrate and thiobarbituric acid (TBA) as coloring agent at 550 nm[23]. The principle of the activity determination is based on measuring the absorption of colored derivatives which was obtained by reaction of unsaturated uronic acid ester and TBA at 550 nm. One unit of enzyme activity was defined as the amount of enzyme which formed l µmol of unsaturated galacturonic acid with a molar extinction coefficient of 5500 $M^{-1}cm^{-1}$ per minute under standard assay conditions.

Protein Estimation

The concentration of protein was estimated by dye binding method using bovine serum albumin (BSA) as standard[24].

Immobilization of Pectin Lyase on Silica and Celite-545 Matrix

The matrix (5 g) was dipped in buffer (pH 10.0). It was centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant was discarded and 4-5 washings were given. The matrix dipped in buffer was kept at 4°C overnight. Glutaraldehyde (cross linking agent) solution (4%) was added and kept at 37°C under shaking conditions for 1 h. The gel was washed twice with buffer to remove unbound glutaraldehyde. The purified pectin lyase was incubated with the matrix for 1 h under shaking condition. The supernatant was collected in vial and the bound enzyme was separated. Enzyme activity and protein content of bound enzyme and supernatant was determined. The binding efficiency was calculated.

Immobilization of Enzyme on Chitosan-PVA Copolymer

Chitosan-polyvinyl alcohol (PVA) copolymer (5 g) was incubated with 20 ml of glycine-NaOH buffer (50 mM, pH 10.5) for 12 h in a glass vial. Ten ml of purified pectin lyase was added to chosen matrix and incubated for 1 h at 37°C. The matrix was then given 1-2 washings with buffer to get rid of unbound enzyme. The matrix-bound pectin lyase was then cross-linked using glutaraldehyde (1%, v/v) for 1 h at 37°C in a water-bath. The matrix-bound biocatalyst was further given 1-2 washings with glycine-NaOH buffer (50 mM, pH 10.5) to get rid of traces of activating agent.

Immobilization of Pectin Lyase in Calcium Alginate Beads

The alginate beads used as immobilization support was prepared by dissolving 3.0% sodium alginate in distilled water. After solubilization, the purified enzyme was added at proportion 3:10 (v/v) to the sodium alginate solution. The gel obtained was dripped in 100 ml of 0.2 M $CaCl_2$ solution using 5 ml syringe under constant agitation. The beads formed of alginate were kept in the solution of $CaCl_2$ at 4°*C* for 12 h. The beads were washed with deionized distilled water until no enzyme activity was detected. Finally, the calcium alginate beads were dried, weighted and stored in desiccator at 4°*C*.

Binding Efficiency

The binding efficiency was calculated as the ratio of activity expressed by the bound enzyme to the total activity used for immobilization.

Effect of Support Concentration on Immobilization of Pectin Lyase

The matrix was used in varied concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5% w/v) with the enzyme solution in order to find the effect of matrix concentration on pectin lyase immobilization.

Characterization of Free and Immobilized Pectin Lyase

Effect of Buffer pH on Free and Immobilized Pectin Lyase

Effect of pH of buffer (glycine-NaOH) was studied by incubating free as well as immobilized enzyme in buffer of different pH values (7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5 and 12.0). The pectin lyase activity was estimated by standard assay method.

Effect of Temperature on Free and Immobilized Pectin Lyase

In order to study the effect of reaction temperature on the pectin lyase activity, the free and bound pectin lyase was incubated in the reaction buffer at different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C) under shaking conditions and enzyme activity was determined.

Effect of Incubation Time on Free and Immobilized Pectin Lyase

The effect of incubation time on the activity of free and immobilized pectin lyase was determined by incubating the enzyme at previously optimized conditions for different time intervals of 5, 10, 15, 20, 25, 30 and 35 min and enzyme activity was checked.

Determination of Kinetic Parameters (K_m and V_{max}) of Free and Immobilized Pectin Lyase

The varying concentrations {0.25%, 0.5%, 0.75%, 1%, 1.25% and 1.5% (w/v)}of selected substrate were used to conduct the reaction with purified free and immobilized pectin lyase from *Bacillus cereus* under optimum assay conditions. The reciprocal of the reaction velocity (1/V) was plotted against the reciprocal of the substrate concentration (1/[S]) to determine the K_m and V_{max} values by the Line Weaver-Burke plot.

Effect of Metal Ions on Free and Immobilized Pectin Lyase

Various metal ions such as Ca^{2+}, Mn^{2+} Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}, K^+, Na^+ and Hg^{2+} were used at concentration^2 of 1 mM in reaction volume to check their effect on the activity of pectin lyase.

Thermal Stability

Thermal stability of the free and immobilized pectin lyase was investigated by measuring the relative residual activity after incubating them at different temperatures (25°C, 40°C, 45°C and 65°C) including optimum temperature and enzyme activity was calculated at interval of 30 min upto 8 h.

Reusability of The Immobilized Pectin Lyase

The reusability (operational stability) of the immobilized pectin lyase was determined after successive batch cycles of a defined amount of enzyme used repetitively upto 11 reaction cycles on the hydrolysis of pectin at previously optimized assay conditions. After each cycle of reaction, the enzyme was recovered and this enzyme was used to catalyze the fresh hydrolytic reaction.

Storage Stability

To determine the effect of storage conditions on the activity, the free and immobilized pectin lyase was stored at 4°C in a refrigerator and enzymatic activity was determined after intervals of 5 days upto 35 days to follow the stability of immobilized catalyst during the storage.

Statistics

All of the tests were conducted in triplicates for determination of pectin lyase activity. Statistical analysis was done using Student's t-test and data were expressed as means ± standard deviations.

Results

Purified Pectin Lyase

The pectin lyase from *Bacillus cereus* was purified about 23.77-fold after using DEAE and Sephadex G-75 gel filtration chromatography with a yield of 18.45% (Table 1). The specific activity of purified pectin lyase was found to be 38.75 U/mg which was used for the immobilization purpose.

Binding Efficiency of Pectin Lyase onto Various Supports

The binding efficiency of purified alkaline pectin lyase from *Bacillus cereus* on silica, celite-545, chitosan-PVA copolymerand sodium alginate was found to be 61%, 73%, 69% and 85%, respectively (Table 2).

Table 1 — Summary of the purification of alkaline pectin lyase from *Bacillus cereus.*

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	412	672	1.63	1	100
Ammonium sulphate saturation	15.7	406	25.85	15.85	60.41
DEAE-cellulose column chromatography	6.1	185	30.32	18.6	27.52
Sephadex G-75 column chromatography	3.2	124	38.75	23.77	18.45

Effect of Sodium Alginate Concentration on Immobilization Process

The effect of alginate concentration on immobilization of the pectin lyase from *Bacillus cereus* has been shown in Table 3 and maximum immobilization was obtained at 2.5% sodium alginate concentration.

Characterization of Free and Sodium Alginate Immobilized Pectin Lyase

Effect of pH of Buffer (Glycine-NaOH)

Both free and immobilized pectin lyase showed optimum activity at pH 10.0 of glycine-NaOH buffer (50 mM) (Fig. 1).

Table 2 — Binding efficiency of purified pectin lyase from *Bacillus cereus* onto various matrices.

Immobilization matrix	Binding efficiency (%)
Celite-545	72.54
Chitosan-PVA	69.04s
Sodium alginate	84.55
Silica	61.13

Table 3 — Effect of sodium alginate concentration on the immobilization of pectin lyase from *Bacillus cereus*.

Sodium alginate concentration (%, w/v)	Enzyme activity (U/mg)
0.5	1.13 ± 0.021
1	1.45 ± 0.019
1.5	2.74 ± 0.035
2	2.98 ± 0.021
2.5	3.14 ± 0.041
3	2.87 ± 0.039
3.5	2.42 ± 0.036
4	2.11 ± 0.051
4.5	1.89 ± 0.043
5	1.47 ± 0.026

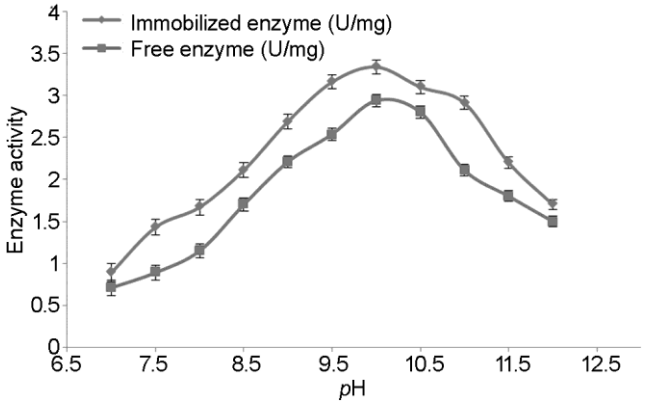

Fig. 1 — Effect of buffer pH on activity of free and immobilized pectin lyase from *Bacillus cereus.*

Effect of Temperature

The activity of pectin lyase increased gradually as the temperature was increased from 25°C to 40°C for free enzyme and for bound enzyme it increased gradually from 25-50°C. It started decreasing with the further rise in the temperature. The reaction temperature of 40°C for free enzyme and 45°C for bound enzyme was found to be the most suitable temperature for the optimal activity of the enzyme (Fig. 2).

Effect of Incubation Time

In this study, pectin lyase from *Bacillus cereus* showed maximum activity after the incubation of 15 min for both free and immobilized enzyme (Fig. 3).

Determination of Kinetic Parameters (K_m and V_{max})

The kinetic parameters (K_m and V_{max}) of the free and immobilized enzyme were determined using different concentrations of substrate citrus pectin under optimum conditions and it was found that pectin lyase gave maximum activity at concentration of 0.5% w/v for free and 0.75% w/v for the

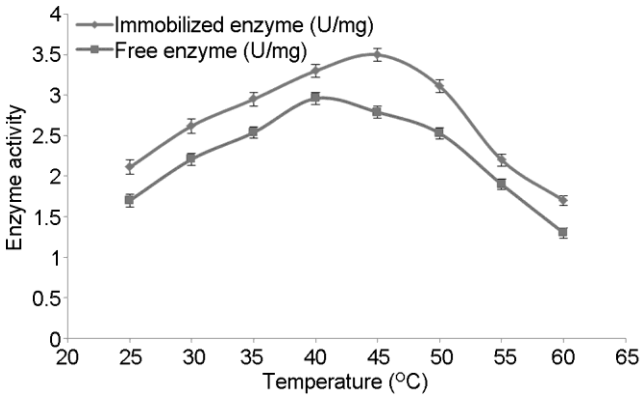

Fig. 2 — Effect of temperature on activity of free and immobilized pectin lyase from *Bacillus cereus.*

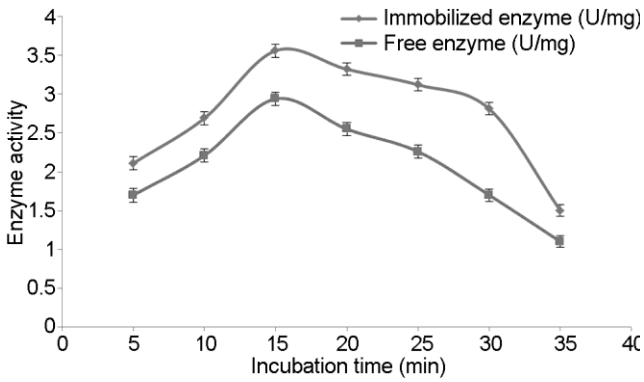

Fig. 3 — Effect of incubation time on activity of free and immobilized pectin lyase from *Bacillus cereus.*

immobilized enzyme (Table 4). The kinetic parameters (K_m and V_{max}) were calculated from Line Weaver-Burk double reciprocal plots for free and immobilized pectin lyase (Fig 4). The V_{max} value indicates the intrinsic characteristics of the enzyme and relatively similar V_{max} values were obtained for both free (3.3 µmol/min) and immobilized pectin lyase (3.6 µmol/min). The K_m for the immobilized pectin lyase (0.19 mg/ml) was slightly higher than that of the free (0.16 mg/ml) enzyme.

Effect of Metal Ions

The maximum inhibition of 50.2% was observed in the presence of Hg^{2+} ion for free pectin lyase and immobilized enzyme showed maximum inhibition of 67.32% in the presence of Na^{+} ion with statistically significant p-value of $p < 0.05$ (Table 5).

Thermal Stability

At the optimum temperature of 40ºC and 45ºC for free and immobilized pectin lyase, the half-life obtained was 4 h and 5 h respectively at 25°C, enzyme activity was almost stable and half-life of 6 h and 5.5 h was obtained for free and immobilized pectin lyase respectively, however, at 65°C the half-life of free and immobilized pectin lyase was observed to be 2 h and 3.5 h respectively (Fig.5 & 6).

Table 4 — Effect of substrate concentration on the activity of free and immobilized pectin lyase from *Bacillus cereus.*

Substrate concentration (citrus pectin (%, w/v)	Immobilized enzyme activity (U/mg)	Free enzyme activity (U/ml)
0.25	2.11 ± 0.017	1.81 ± 0.015
0.5	2.69 ± 0.043	2.95 ± 0.032
0.75	3.56 ± 0.029	2.76 ± 0.027
1	3.12 ± 0.031	2.37 ± 0.019
1.25	2.77 ± 0.045	1.98 ± 0.034
1.5	2.39 ± 0.019	1.43 ± 0.021

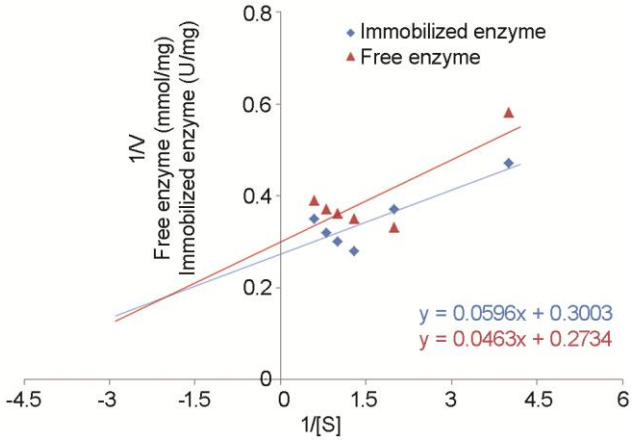

Fig. 4 — Lineweaver-Burk plot for free and immobilized alkaline pectin lyase from *Bacillus cereus.*

Table 5 — Effect of metal ion on the activity of free and immobilized pectin lyase from *Bacillus cereus.*

Metal ion (1 mM)	Free enzyme activity (U/ml)	Immobilized enzyme activity (U/mg)
Ca^{2+}	2.86 ± 0.1***	2.11 ± 0.05*
Mn^{2+}	1.95 ± 0.04*	1.76 ± 0.2*
Mg^{2+}	3.11 ± 0.04*	3.07 ± 0.06**
Zn^{2+}	2.23 ± 0.05**	3.11 ± 0.07*
Cu^{2+}	1.86 ± 0.06*	2.67 ± 0.07*
Hg^{2+}	1.48 ± 0.05*	1.42 ± 0.05
K^{+}	1.54 ± 0.03*	1.32 ± 0.06*
Na^{+}	1.67 ± 0.06*	1.15 ± 0.07*
Fe^{3+}	2.94 ± 0.07*	2.13 ± 0.04*
Control	2.97 ± 0.02	3.52 ± 0.03

Values are mean ± S.D. of 3 replicates *** $p < 0.001$; **$p < 0.01$ and *$p < 0.05$ as compared to the control

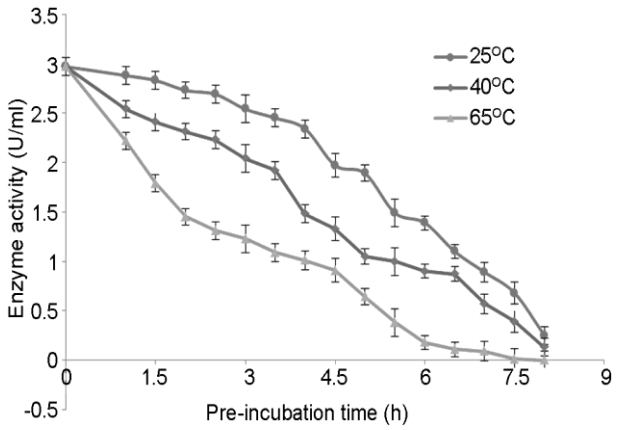

Fig. 5 — Thermal stability of free pectin lyase from *Bacillus cereus* at different temperatures.

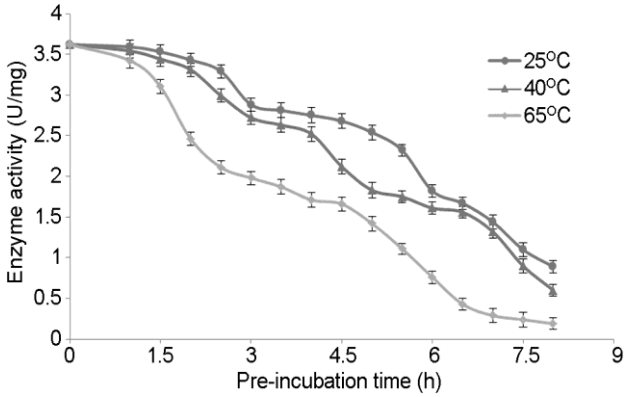

Fig. 6 — Thermal stability of immobilized pectin lyase from *Bacillus cereus* at different temperatures.

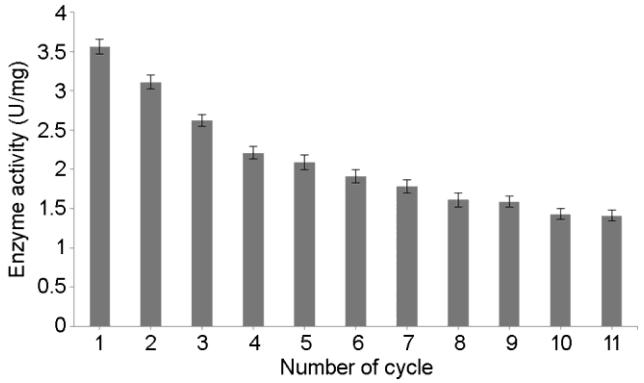

Fig. 7 — Reusability of immobilized pectin lyase from *Bacillus cereus.*

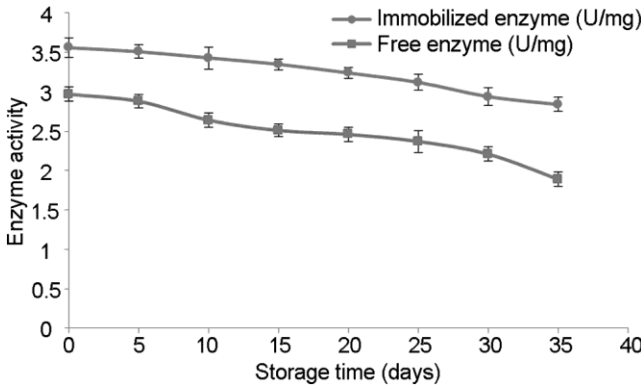

Fig. 8 — Storage stability of free and immobilized pectin lyase from *Bacillus cereus.*

Reusability

The immobilized pectin lyase retained almost 53% of its original activity up to 7th cycle (Fig. 7).

Storage Stability

The immobilized pectin lyase can be stored at 4°C in refrigerator for a long time and during storage at 4°C upto 35 days, immobilized pectin lyase retained relative activity of 79.77% and free enzyme retained relative of 63.63% activity upto 35 days of storage (Fig. 8).

Discussion

The yield of immobilization means the effective activity of the supported enzyme[25] and effectiveness of immobilization address to the amount of enzyme theoretically incorporated to the support. In the present study, maximum binding efficiency was achieved in the presence of sodium alginate and it was used as matrix in the further studies. The immobilized enzymes have shown better thermal and pH stability, have good reusability and easy to separate[26]. Moreover, their result appearance to be the most suitable for practical applications. Recently in a report, hybrid inorganic matrix alginate/ gelatin/calcium oxalate (AGOCa) showed highest immobilization efficiency of 61.7% for immobilization of pectinase from *Aspergillus niger* ATCC 9642[27]. It was attributed to the formation of biomimetric layer in the matrix, increasing the amount of enzyme retained in the matrix. In a similar study, pectinase was immobilized in alginate by simple inclusion, but it had lower activity and stability which made it difficult to be used on an industrial scale[28]. Using PEI-coated polymer supports[29-30] immobilized pectinase prospect for recycle ability. Similarly commercial preparations of pectinases Pectinex Ultra SP-L exhibited an immobilization efficiency of 76% when conjugated to alginate by non-covalent interactions[31]. In case of commercial pectin lyase preparations, the maximum amount of enzyme was immobilized using an alginate concentration of 0.17 g alginate per milliliter (Ipsita *et al*., 2003). In a different study, polyvinyl alcohol (10%) gel was used in the form of Lenti Katsto immobilize pectinase enzyme preparations Panzym Yield-MASH and Panzym Smash-XXL which retained the maximum polygalacturonase and pectin lyase activities respectively[32]. Previously, the concentration of glutaraldehyde 0.2% (v/v) was found to be optimal when pectinase was immobilized onto sodium alginate[33].

New microenvironment created due to the entrapment of the enzyme in the matrix might buffer and accordingly, protect the protein structure of the enzyme (Barbosa *et al*., 2015). It has been reported that immobilization of enzymes serve to turn the optimum pH for variability of enzyme molecules under varying pH[34]. Pectinase from *Aspergillus aculeatus* showed that the free enzyme had a pH-optimum of 5.0 while the immobilized enzyme maintained high level of activity in a broad pH range of 3.0-7.0 probably due to ionically charged surface of the PEI-functionalized polymer (Rajdeo *et al*., 2016). A significant decrease in immobilization was observed at neutral pH values and at pH 8.8 which might be due to electrostatic repulsion between the neutralize charges of alginate and enzyme molecules[35]. After immobilization, increment in temperature improve molecular dispersion rate due to the alterations of natural properties of the enzyme[36]. Moreover, interactions between enzyme and support improve the molecular inflexibility and conformational stabilty of the immobilized enzymes (Li *et al*., 2008). The adequate high energy is

necessary to reach a sufficient agreement to make possible the binding with the substrate, making the enzyme more resistant to heat denaturation (Wang *et al*., 2013). In a similar study, an ideal temperature of 50°C for the soluble and 60°C for immobilized pectin lyase from *Penicillium italicum* had been reported[37]. At 35°C all the pectinase preparations had shown their highest activity and when further increased in temperature, immobilized preparations reveal slightly higher resistance to temperature change[38].

The optimal reaction incubation time is necessary to achieve maximum enzyme activity in enzyme catalyzed reactions and further incubation beyond optimum value causes a reduction in enzyme activity, which might be due to product inhibition or due to the denaturation of the enzyme at assay temperature, when incubated for a longer duration of time[39]. This is comparable to a study, where calcium alginate entrapped pectinase showed maximum pectinolytic activity after 10 min of reaction as compared to free pectinase which accomplish maximum enzymatic activity after 5 min of reaction time (Rehman *et al*., 2016). Rapidly decreased residual activity of free pectinase measured as 49% of its initial activity at the end of 24 h incubation time at 35°C, however, the pectinase immobilized onto florisil and nanoparticle silica preserve 60 and 70% of their initial activities, respectively (Alkorta *et al*., 1996). In other reports, it was describe that the pectinase from *Bacillus licheniformis* KIBGE-IB21 entrapped in calcium alginate gel protected more than 80% and 70% of its initial activity at 30°C and 40°C respectively after 120 h incubation time[40] and the immobilized polygalacturonase from *Aspergillus niger* gave maximum activity at 15 min incubation time. Using chitosan-co-PVA in case of pectinase immobilization increased copolymer percent swelling with time of reaction and maximum swelling (730.18% in 510 min) was achieved at 4 h of reaction time; however, increase in the time of reaction beyond the optimum might affect the copolymer structures due to chain scission reactions affecting swelling characteristics of the copolymer chitosan-co-PVA[41].

Increase in the value of K_m may be caused by the low accessibility of the substrate to the catalytic site of the enzyme, as a result of diffusion limitation, as well as the conformational changes of the enzyme, resulting in the loss of conformation of enzyme-substrate complex[42]. Both soluble and immobilized enzyme showed a linear Michaelis-Menten behaviour, in agreement with other studies, where immobilization of pectin lyase from *Aspergillus niger* on bentonite activated by glutaraldehyde did not change its apparent K_m but this value increased when pectin lyase was immobilized on polyamides[43-44]. Likewise, alkaline pectin lyase from *Bacillus clausii* had estimated K_m value of 0.87 mg/ml for hydrolyzing apple pectin[45] at pH 10.0 and pectin lyase from *B. pumilus* had K_m and V_{max} values as 0.298 mg/ml and 132.6 µmol/min respectively[46]. Similarly, pectinase from *B. firmus* resulted in V_{max} and K_m value of 90.09 U/ml and 0.27% respectively[47]. Immobilization procedure did not affect the accessibility of substrate to active sites significantly and generally, the maximum rate of reaction catalyzed by the immobilized pectin lyase was lower than that of free enzyme[48].

The maximum activity of immobilized enzyme is disclosed without any metal ion due to the fact that some metal restrictive allosteric sites inflict difficulty to metal access to enzyme active site for intensify its activity and pectin lyase from *B. pumilus* (P9) was completely inhibited by 10 mM of Zn^{2+}, Ca^{2+} and Mn^{2+} ions (Hayrunnisa *et al*., 2010). Moreover, this decrease in the activity might be due to the reason that the metal ions interact with the active functionalities on the enzyme thus blocking the active sites involved in the hydrolysis and hence lower efficiency was observed[49].

Immobilized pectin lyase, showed good stability at 45ºC as compared to free enzyme. Thermal stability of free and immobilized pectin lyase was relatively resembled at other temperatures and the thermal stability was lower at higher temperature of 65°C. The calcium alginate entrapped pectin lyase obtained a smaller rate of thermal inactivation pertaining to the free enzyme. Thermally strong form of immobilized pectinase is highly preferable to improve its operating limit and facilitate storage and transport. It was reported that both free and polymer immobilized pectinase were found to be constant upto 40ºC and lost activity rapidly beyond 50ºC, also, the half-life times of the free and immobilized pectinase protect 60 and 70% of their initial activities respectively at 35ºC (Rajdeo *et al*., 2016). The stability of the immobilized enzyme could be increased and attribute to the augmentation of enzyme inflexibility and conformational flexibility by immobilization, preventing the conformational change at high temperatures (Ling *et al*., 2016).

The immobilized pectin lyase showed good reusability upto 7 cycles. After immobilization, every enzyme ultimately loses its activity due to native denaturation, aggregation of inhibited compounds and has to be disposed and it was reported that pectinases immobilized onto nanoparticle silica and florisil retained 80% and 76% of their initial activities particularly after 10 reuses (Alkorta *et al*., 1996). The entrapped enzymes usually tolerate a slow damage of activity after successive catalytic cycles (Esawy *et al*., 2013) and similar results were also possess for immobilized pectin lyase from *Aspergillus niger* SA6 which retained about 20% of activity (Buga *et al*., 2010). The loss of enzyme activity can be attributed to the lixiviation of the enzyme from the support as a result of successive washings after each cycle, in addition to possible conformational alterations and mechanical damages after repeated cycles (Won *et al*., 2005). After 10 reuses, the pectinase enzyme from *Bacillus licheniformis* KIBGE IB-21 entrapped in agar-stabilizer matrix retained 20% of its initial activity. The commercial pectinase immobilized covalently onto amino functionalized silica coated attractive nanoparticles protected 50% of its initial activity after 12 reuses (Seenuvasan *et al*., 2013). One of the most significant aspect of immobilized enzymes are reusability for industrial applications and pectinases enzymes are immobilized onto modified submicroscopic silica and florisil retained 80 and 76% of their initial activities respectively after 10 reuses (Rajdeo *et al*., 2016).

Immobilized pectin lyase showed high relative activity as compared to free enzyme upto 35 days of storage. Achieving high storage stability is one of the most important parameters which should be considered in industrial processes[50]. Storage stability of immobilized pectinase is improved over free pectinase and has been shown in a study, where immobilized pectinase conserved 60% of their initial and the free pectinase conserved 51% of their initial activity after storing them upto 24 days (Seenuvasan *et al*., 2013). The results of a study had indicated that the immobilization with magnetic nanoparticles improved the stability of pectinase (Fang *et al*., 2016). During the first and second months of storage, the purified alkaline pectin lyase from *Aspergillus niger*_WHAK1 had maximum enzymatic activity and it retained 88% of its initial activity up to 17 months at 4°C (Poturcu *et al*., 2017).

Conclusion

The immobilization strategy of purified pectin lyase gave good results in terms of its pectinolytic activity. A considerably high immobilization efficiency of 85% was obtained using alginate beads. The results demonstrate that a highly active and durable immobilized enzyme preparation was developed by immobilizing pectin lyase in calcium alginate beads that can be used for sustainable industrial processes. Moreover, the immobilization procedure on alginate is not only inexpensive but very easy to carry out and provides extremely mild conditions, so that the potential for industrial application is considerable. Nevertheless, while free pectinases are being extensively used in many industries, the immobilized forms are still today the object of many research projects.

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Conflict of Interest

There is no conflict of interest for the publication.

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