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Biocatalytic Profiling of Free and Immobilized Partially Purified Alkaline Protease from an Autochthonous *Bacillus aryabhattai* Ab15-ES

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Abstract: Partially purified alkaline protease produced by an indigenous bacterial strain, *Bacillus aryabhattai* Ab15-ES, was insolubilized in alginate beads using an entrapment technique. Maximum entrapped enzyme activities of 68.76% and 71.06% were recorded at optimum conditions of 2% (*w*/*v*) sodium alginate and 0.3 M calcium chloride. Biochemical profiling of free and immobilized proteases was investigated by determining their activity and stability as well as kinetic properties. Both enzyme preparations exhibited maximum activity at the optimum pH and temperature of 8.0 and 50 °C, respectively. However, in comparison to the free enzyme, the immobilized protease showed improved pH stability at 8.0–9.0 and thermal stability at 40–50 °C. In addition, the entrapped protease exhibited a higher Vmax and increased affinity to the substrate (1.65-fold) than the soluble enzyme. The immobilized protease was found to be more stable than the free enzyme, retaining 80.88% and 38.37% of its initial activity when stored at 4 °C and 25 °C, respectively, for 30 d. After repeated use seven times, the protease entrapped in alginate beads maintained 32.93% of its original activity. These findings suggest the efficacy and sustainability of the developed immobilized catalytic system for various biotechnological applications.

Keywords: *Bacillus aryabhattai* Ab15-ES; protease; alkaline protease; immobilization; alginate beads; characterization

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1. Introduction

Proteases are enzymes that catalyze the breakdown of proteins into simpler subunits by hydrolysis of the peptide bond that exists between two amino acids in a polypeptide chain [1–4]. They occupy a pivotal position, constituting more than 65% of the total industrial enzyme market [5–7]; their dominance of which is expected to increase further in the coming years [8]. Proteases have versatile applications in various industries, including the detergent, pharmaceutical, leather, silk, food, silver recovery and agriculture industries, as well as in wastewater treatment and organic chemical synthesis [7,9–13].

Proteases are ubiquitous in nature and are produced by plants, animals and microbes [14]. The inability of plant and animal proteases to meet contemporary global demands has led to an increased interest in microbial proteases. Microbial proteases serve as an excellent source of commercial enzymes since microorganisms can be cultured in large quantities in relatively short times through an established fermentation process for the generation of abundant yields of the desired metabolite. These properties are owed to their broad biochemical diversity and susceptibility to genetic manipulation [15]. Microbial proteases account for approximately 40% of the total worldwide production of enzymes [16–18]. Microorganisms such as bacteria, molds and yeasts have been reported for extracellular protease production [19,20].

Free enzymes are soluble and susceptible to harsh reaction conditions, including extreme temperatures, a very high or low pH, a high ionic strength, a high concentration of reactants and the presence of inhibitors [21]. Enzymes may not function optimally under such extreme conditions, which are often encountered in bioprocesses [22]. In order to ameliorate the aforementioned challenges, the immobilization of protease in suitable support materials represents a promising alternative [8,23–25].

The use of immobilized enzymes in bioprocesses has many advantages, including increased stability and activity over a broad range of pH and temperatures, localization, ease of product separation and handling, recovery yield and reusability, rapid termination of reactions, controlled product formation, adaptability to various engineering designs with a consequent decrease in running cost and autolysis rate [21,26,27]. However, the selection of suitable support materials and immobilization techniques is paramount to overcoming the drawbacks of immobilization. The general methods employed for enzyme immobilization include physical adsorption, ionic binding, covalent binding, cross-linking and entrapment methods [28]. Among the different immobilization techniques, the entrapment method has been considered the most often due to its simplicity and non-toxicity [29].

The entrapment method involves the physical restriction of the enzyme within a confined space or network in a semi-permeable capsule, obtained by the gelation of polyanionic or polycationic polymers in the presence of multivalent counterions, thus retaining enzyme catalytic activity and its capacity to be used continuously [8,30,31]. It is carried out in a three-dimensional gel lattice, made of either natural (agar, cellulose, alginate, carrageenan) or synthetic (polyacrylamide, polyurethane, polyvinyl, polypropylene) polymers [32,33]. However, calcium alginate beads are mostly preferred owing to their mild gelling properties, easy formulation, biocompatibility, cost-effectiveness and potential to improve enzyme stability and functional properties [34–36]. Alginate is a water-soluble anionic linear polysaccharide composed of sequential arrangements of 1,4-linked β-D-mannuronic acid and α-L-glucuronic acid in different proportions and can be precipitated by the addition of Ca^{2+} ions, producing microspheres with good strength and flexibility [37,38]. It is employed in many biotechnological applications as a support material for releasing and encapsulating cells and enzymes [39].

Proteases are classified as acidic proteases, neutral proteases or alkaline proteases based on their optimal pH of activity [14,40]. Alkaline proteases are proteases that are active at neutral to alkaline pH conditions. They occur as serine or metalloproteases and are commonly exploited for various commercial applications due to their higher activity and stability at broad pH and temperature ranges [41–43]. Substrates, including casein, azocasein, hemoglobin, etc., are employed for the determination of the catalytic activity of proteases. However, azocasein is mostly preferred due to its high solubility and sensitivity [44]. Protease degrades the substrate to yield trichloroacetic acid-soluble azopeptide, which is measured spectrophotometrically. Azocasein has been used as a reliable substrate for protease assays by several co-workers [45–47]. Many microbial species are known to produce alkaline protease. However, among bacteria, members of the genus *Bacillus*, including *Bacillus pumilus* [48], *Bacillus licheniformis* [49], *Bacillus caseinilyticus* [50], *Bacillus subtilis* [51,52], *Bacillus megaterium* [53], *Bacillus coagulans* [54] and *Bacillus alveayuensis* [7], are notable for the commercial scale production of alkaline proteases for various biotechnological applications [9,55]. Proteases from *Bacillus aryabhattai* have been reported [56,57]; however, to the best of our knowledge, this is the first report on a comparative study of the biochemical properties of free and immobilized proteases from *Bacillus aryabhattai*.

Due to the emergent market and potential applications of proteases, there is a continuing interest in bioprospecting for indigenous hyperactive proteolytic bacteria with robust catalytic activity suitable for various industrial and environmental applications. In the present study, extracellular protease from *Bacillus aryabhattai* Ab15-ES was partially purified and immobilized in alginate beads through entrapment. Thereafter, a comparative

study of the biochemical properties of the free and immobilized proteases was carried out to ascertain their possible biotechnological applications.

2. Materials and Methods

2.1. Materials

Azocasein (used as a substrate for protease assay) and sodium alginate (used as support material for enzyme immobilization) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Partial Purification of Protease

Protease-producing *Bacillus aryabhattai* Ab15-ES, previously isolated from poultry processing wastewater, was cultivated in an optimized fermentation medium consisting of (in g/L): maltose, 12.35; beef extract, 5.30; NaCl, 0.5; $CaCl_2.2H_2O$, 0.1; K_2HPO_4, 0.3; KH_2PO_4, 0.4; and $MgSO_4.7H_2O$, 0.1, with the pH adjusted to 7.8. The inoculated culture medium was then incubated at 40 °C for 48 h at 150 rpm [4]. The cell-free culture supernatant (crude protease), obtained through centrifugation at 10,000 rpm for 10 min at 4 °C, was partially purified by ammonium sulfate precipitation and dialysis. Solid ammonium sulfate was added to the crude enzyme by continuous stirring up to 60% saturation. The saturated solution was kept undisturbed at 4 °C overnight. The precipitate was collected via centrifugation at 10,000 rpm for 10 min at 4 °C, re-dissolved in a small amount (1:50) of 0.05 M Tris-HCl buffer (pH 7.2) and then dialyzed overnight against the same buffer at 4 °C.

2.3. Immobilization of Partially Purified Protease in Calcium Alginate Gel Beads

Immobilization of protease was carried out according to the entrapment method of Guleria et al. [8] with modifications to use sodium alginate as support material. Sodium alginate solution was prepared by gradually adding varying concentrations (1–5%, *w*/*v*) of sodium alginate in 0.05 M Tris-HCl buffer (pH 7.2). A two-milliliter aliquot of sodium alginate suspension was mixed with 0.5 mL of a partially purified protease. This mixture was extruded dropwise into a 5 mL $CaCl_2$ solution (0.1–0.5 M) using a hypodermic syringe from a 5 cm height with constant stirring. The beads obtained were kept for curing at 4 °C for 1 h. Thereafter, the cured beads of about 3 mm diameter were recovered from the solution through filtration, washed 3–4 times under mild agitation with 0.05 M Tris-HCl buffer (pH 7.2) and distilled water to remove unbound enzymes and then stored at 4 °C until use [30]. A similar method was used for the preparation of control alginate beads without the enzyme.

2.4. Determination of Immobilization Efficiency

The immobilization efficiency, *Y*, described as the percentage of bound enzyme activity observed in the immobilization, was calculated using the equation illustrated in Equation (1) [58]:

$$Y\ (\%) = \frac{\text{Observed activity}}{\text{Immobilized activity}} \times 100 \quad (1)$$

where immobilized activity was determined by measuring the total residual enzyme activity that remained in the enzyme solution after immobilization and subtracting this from the total starting activity [58].

2.5. Protease Assay

Protease activity was determined according to the modified method of Secades and Guijarro [59], using azocasein as a substrate. Enzyme solution (120 µL) was added to 480 µL of azocasein solution (consisting of 1%, *w*/*v* of azocasein in 100 mL of 0.05 M Tris-HCl buffer, pH 7.2), and the mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 600 µL of 10% chilled trichloroacetic acid (TCA), followed by further incubation in ice for 30 min to allow the undigested protein to precipitate. Thereafter,

coagulated protein was removed from the reaction mixture through centrifugation at 10,000 rpm for 10 min at 4 °C using high-speed refrigerated centrifuge (Avanti J-26 XP, Beckman Coulter, Brea, CA, USA). The obtained supernatant (800 µL) was neutralized by addition of 200 µL of 1.8 N NaOH. The absorbance was measured at 440 nm using a UV-Vis spectrophotometer. The blank consisted of TCA–substrate solution. The activity of immobilized protease was determined using the procedure described above, except that 0.5 g of microspheres was taken for assay. Lowry method was used for the estimation of total protein using bovine serum albumin as a standard [60]. One unit (U) of enzyme activity was defined as the amount of enzyme that yielded an increase in absorbance (440 nm) of 0.01 per minute under defined assay conditions.

2.6. Characterization of Free and Immobilized Protease

2.6.1. Effect of Temperature and pH on Free and Immobilized Protease Activity

The temperature at which optimum protease activity was achieved for both free and immobilized proteases was determined by carrying out enzyme assays at various temperatures (30–80 °C) for 30 min in 0.05 M Tris-HCl buffer (pH 7.2). Protease activity was measured, as described previously. The optimum pH for free and immobilized protease activity was determined at the optimum temperature for 30 min at different pH values ranging from 3.0–11.0. The buffers used include: 0.05 M citrate buffer (pH 3.0–4.5), 0.05 M acetate buffer (pH 5.0–5.5), 0.05 M phosphate buffer (pH 6.0–6.5), 0.05 M Tris-HCl buffer (pH 7.0–9.0), and glycine–NaOH buffer (pH 9.5–11.0). Azocasein was dissolved in the respective buffers and then used to assay for protease activity, as described above. The relative protease activities as percentage (%) of maximum enzyme activity were calculated and plotted against the respective temperature or pH [30]. All experiments were carried out in triplicate.

2.6.2. Effect of Temperature and pH on Free and Immobilized Protease Stability

The thermal stability of the free and immobilized proteases was determined by pre-incubating the enzyme in 0.05 M Tris-HCl buffer (pH 8.0) at different temperatures (40, 45, 50 °C) for up to 3 h. Aliquots were taken at desired intervals and assayed for residual activity under standard assay conditions. Unheated enzyme was considered as a control (100%). The pH stability of the enzyme was assessed by pre-incubating the enzyme sample in the respective buffers (0.05 M) for up to 3 h at optimum temperature [30]. Aliquots were taken at desired intervals and assayed for residual protease activity under standard experimental conditions. All experiments were performed in triplicate.

2.6.3. Kinetics Properties of Free and Immobilized Protease

The influence of substrate (azocasein) concentrations ranging from 1 to 10 mg/mL on free and immobilized protease activity was studied by measuring enzyme activity, as previously described under standard assay conditions. Lineweaver–Burk plot was constructed by plotting reciprocals of substrate concentrations against the reciprocals of enzyme velocity. From the plot, Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values were determined [61].

2.6.4. Storage Stability of Free and Immobilized Protease

The storage stability of free and immobilized enzymes was determined by preserving the enzymes at 4 and 25 °C. At intervals of 5 d for up to 30 d, samples of the stored enzyme were assayed for protease activity [30]. Residual activity was calculated against the initial enzyme activity taken as 100%.

2.6.5. Reusability of Immobilized Protease

In order to assess the reusability of the protease entrapped in the calcium alginate beads, the azocasein hydrolysis reaction was repeated several times at 37 °C for 30 min. Thereafter, the beads were separated, washed with Tris-HCl buffer (pH 7.2) and used to

start a cycle using fresh substrate solution [30]. Protease activity was measured after every reaction time under standard assay conditions. The relative activity was calculated by taking the enzyme activity of the freshly prepared beads in the first cycle as 100%.

2.6.6. Scanning Electron Microscopy of Beads with Immobilized Protease

The surface morphology of alginate beads containing immobilized protease was studied using ZEISS EVO LS15 scanning electron microscope.

3. Results and Discussions

3.1. Partial Purification of Protease

Bacillus aryabhattai Ab15-ES was cultivated in a previously optimized, cheap fermentation medium for extracellular protease production [4]. After centrifugation of the medium, the obtained culture supernatant, regarded as the crude enzyme, was partially purified by ammonium sulfate precipitation at a 0–60% (*w*/*v*) saturation, followed by dialysis. These techniques are useful and reliable for enzyme purification since they enable the quick and in-bulk precipitation of cellular proteins from the crude enzyme [62]. The rationale for the partial purification of the enzyme was to reduce the downstream processing costs of the biocatalyst for exploitation in large-scale biotechnological applications. The results of the purification procedures are summarized in Table 1. The partially purified protease recorded a 2.19-fold increase in specific activity in comparison to the culture supernatant and a recovery of 60.98%. Sahin et al. [25] and Zhu et al. [63] reported a 2.1-fold purification and 64% recovery, respectively, following ammonium sulfate precipitation of proteases from *Bacillus subtilis* M-11 and *Geobacillus* sp. YMTC 1049.

Table 1. Summary of purification steps of protease from *Bacillus aryabhattai* Ab15-ES.

Purification Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Recovery (%)	Purification Fold
Crude enzyme	24,784	190.31	130.23	100	1.00
$(NH_4)_2SO_4$ precipitation (60%)	15,114	52.90	285.71	60.98	2.19

3.2. Immobilization of Protease in Calcium Alginate Gel Beads

Enzyme entrapment depends on sodium alginate and calcium chloride concentrations for the formation of beads with high permeability and rigidity [64]. In the present study, the effects of sodium alginate concentrations ranging from 1–5% (*w*/*v*) and 0.2 M $CaCl_2$ were investigated on the immobilization efficiency of protease from *Bacillus aryabhattai* Ab15-ES. The maximum immobilization efficiency (68.76 ± 2.42%) was recorded with beads prepared from 2% sodium alginate. After this, there was a gradual decrease in the immobilization efficiency, with the lowest immobilization (22.78 ± 2.07%) obtained with 1% sodium alginate (Table 2). This is probably due to the maximum leakage of the enzyme from the beads, resulting from the larger pore sizes of the beads and less tight cross-links with calcium chloride, leading to lower immobilization efficiency [24]. This corroborates the findings of Geethanjali and Subash [64] and Anwar et al. [23], in which the maximum immobilization efficiency was recorded at 2% sodium alginate. In addition, sodium alginate concentrations of 2% have been reported by several co-workers to be suitable for enzyme immobilization [8,24,65–67].

Since calcium chloride serves as a cross-linking agent, its concentration affects the activity and density of immobilized biomolecules [27,68]. This also influences the mechanical strength of alginate beads as well as the efficiency of immobilized systems [69]. Therefore, the concentration of calcium chloride is essential for the stability and pore size of the beads [70]. The influence of calcium chloride concentrations (0.1–0.5 M) on the immobilization efficiency was studied at 2% sodium alginate since 2% showed the highest immobilization efficiency [30]. The highest entrapped enzyme activity was obtained from beads prepared from 0.3 M calcium chloride with an immobilization efficiency of 71.06 ± 1.30% (Table 3). This supports the findings of Geethanjali and Subash [64], in

which the maximum immobilization efficiency was recorded with beads formed from 0.3 M calcium chloride.

Table 2. Immobilization efficiency of alkaline protease from *Bacillus aryabhattai* Ab15-ES at varying sodium alginate concentrations.

Sodium Alginate Concentration (%)	Immobilization Efficiency (%)
1	22.78 ± 2.07
2	68.76 ± 2.42
3	53.90 ± 3.86
4	45.35 ± 1.62
5	39.74 ± 0.88

All values are expressed as mean from triplicate values; ± indicates SD.

Table 3. Immobilization efficiency of alkaline protease from *Bacillus aryabhattai* Ab15-ES at varying calcium chloride concentrations.

$CaCl_2$ Concentration (M)	Immobilization Efficiency (%)
0.1	46.81 ± 2.41
0.2	62.96 ± 0.58
0.3	71.06 ± 1.30
0.4	55.92 ± 2.40
0.5	42.87 ± 1.78

All values are expressed in mean; ± indicates SD.

3.3. Scanning Electron Microscopic Analysis

Scanning electron microscopy of the morphology of alginate gel beads revealed the presence of irregular pores of various dimensions on the bead surface, coupled with a stable microstructure with evenly distributed layers in the epicenter of the beads (Figure 1). The porosity of the beads helps to enhance their specific surface area and increase substrate diffusion [71].

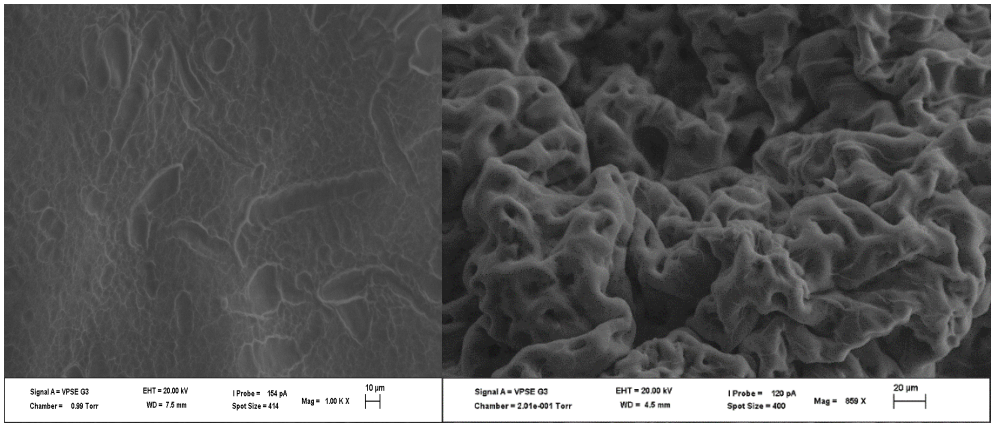

Figure 1. Scanning electron microscopy of surface morphology of alginate beads.

3.4. Effect of Temperature and pH on Free and Immobilized Protease Activity

The optimum temperature of free and immobilized protease activity was determined by carrying out enzyme assays at different temperatures ranging from 30 to 80 °C. As shown in Figure 2, there was an increase in the relative activities of both the soluble and

immobilized enzymes as the temperature increased from 30 to 50 °C, with the maximum activity recorded at the optimum temperature of 50 °C. Thereafter, the relative activities of both free and immobilized proteases decreased up to 80 °C. However, the entrapped protease demonstrated higher relative activity in comparison to the free enzyme, especially at high temperatures (55–80 °C). Similar results were also obtained from protease immobilized on polysulfone membranes [25] and Fenugreek β-amylase covalently attached to functionalized graphene sheets [72]. At 80 °C, the relative activity of the immobilized protease is 1.6-fold (31.01%) higher than that of the free enzyme. This clearly indicated that the support material retained the tertiary structure of the enzyme at high temperatures [23]. In other words, multi-interactions between the matrix and enzyme play a crucial role in the temperature tolerance of the immobilized enzyme [30,73].

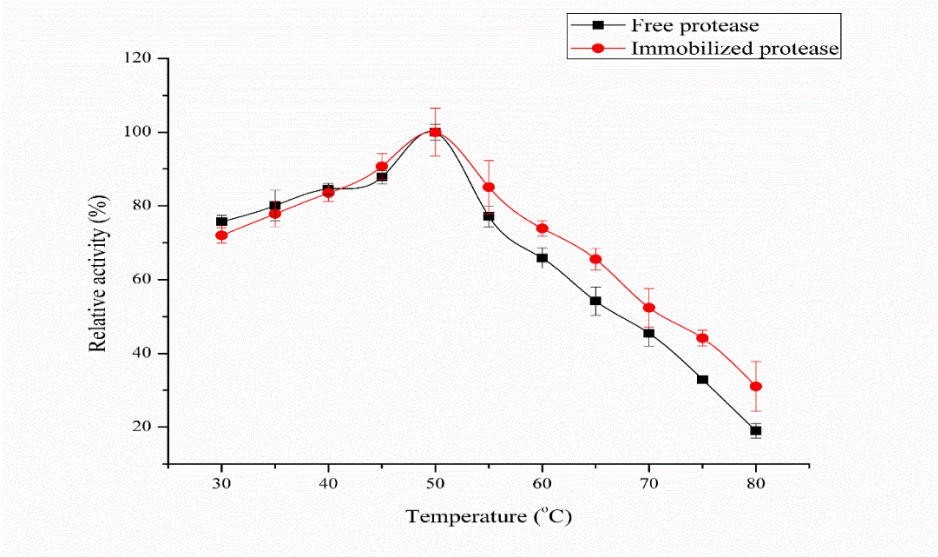

Figure 2. Effect of temperature on activity of free and immobilized proteases from *Bacillus aryabhattai* Ab15-ES. Protease activity was measured under standard assay conditions at various temperatures (30–80 °C) at pH 7.2. Values indicate the average of triplicate values, while the error bars represent the standard deviation.

The effect of pH on the activity of soluble and entrapped proteases was investigated under varied pH values (3.0–11.0) in different buffers at 50 °C, and the results are presented in Figure 3. The catalytic activity of the enzyme increased as the pH increased, reaching its maximum activity at pH 8.0 for both free and immobilized proteases. This suggested that the chemical coupling of the enzyme to the polymer resulted in no change in its pH profile [30]. Anwar et al. [23] and Geethanjali and Subash [64] have reported similar results on the immobilization of proteases from newly isolated *Bacillus subtilis* KIBGE-HAS and *Labeo rohita*, respectively, where no change in the pH profile of the enzyme was observed before and after entrapment in calcium alginate. This phenomenon has also been observed by other authors [74–76]. In addition, there was a significant increase in the relative activity of the immobilized protease at a wide pH range (8.5–11.0) in comparison to the free enzyme, an indication of its resistance to the alkaline changes in the medium [77]. Similar results have also been reported in the literature for enzymes immobilized onto/in a solid matrix [66,76,78].

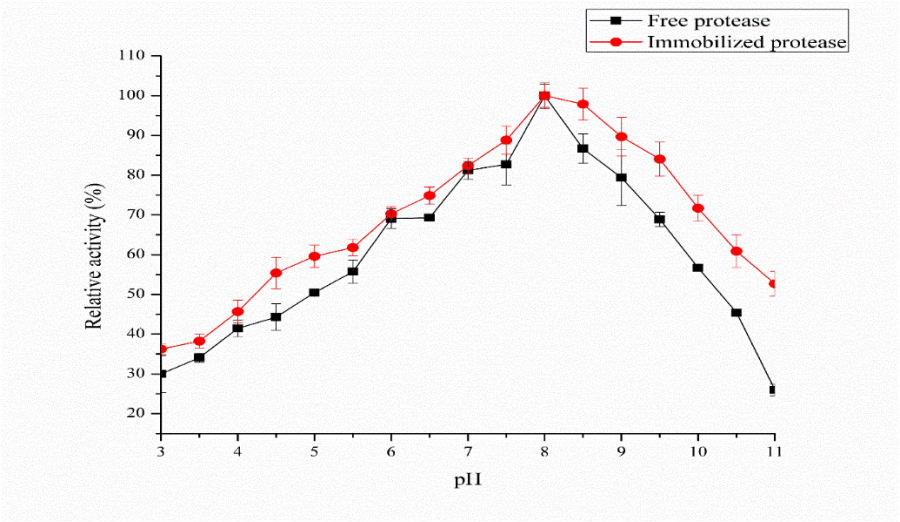

Figure 3. Effect of pH on activity of free and immobilized proteases from *Bacillus aryabhattai* Ab15-ES. Enzyme activity was determined at 50 °C. Values indicate the average of triplicate values, while the error bars represent the standard deviation.

3.5. Effect of Temperature and pH on Free and Immobilized Protease Stability

The thermal stability of free and immobilized proteases was investigated in the temperature range of 40–50 °C for 0–180 min. As shown in Figure 4, the protease immobilized in alginate beads demonstrated better thermal stability in comparison to the free enzyme. After incubation at 50 °C for 3 h, the immobilized enzyme retained 60.58% of its initial activity, whereas the free soluble enzyme maintained only 28.41%. The significant improvement in the thermal stability of the entrapped protease may be due to its immobilization in the support material, which protects the enzyme's tertiary structure and prevents the conformational transition of the enzyme upon heating [30,79,80]. The enhancement of enzyme stability after immobilization in alginate beads has been reported by several authors [66,81–83].

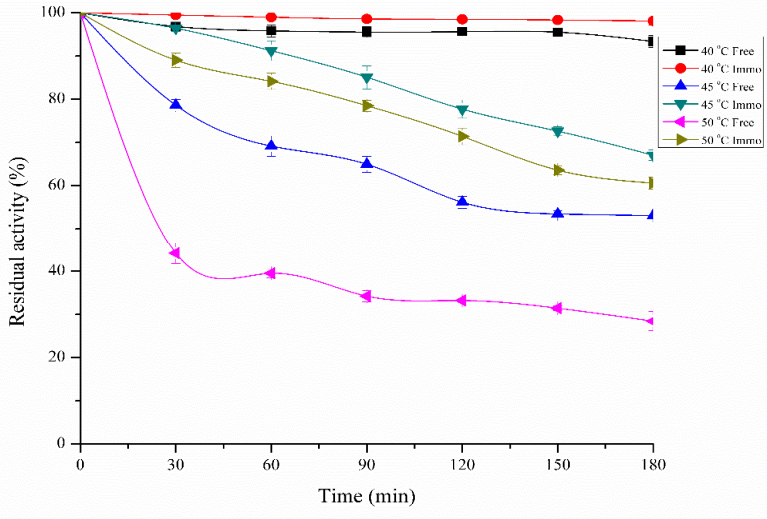

Figure 4. Thermal stability of free and immobilized proteases from *Bacillus aryabhattai* Ab15-ES. The enzyme was pre-incubated at different temperatures (40–50 °C) for 3 h at pH 8.0, and the residual enzyme activities were estimated at regular intervals under standard assay conditions. The non-heated enzymes were taken as 100%. Values indicate the average of triplicate values, while the error bars represent the standard deviation.

The pH stability was investigated by pre-incubating the free or immobilized proteases in different buffers (pH 8.0–9.0) for 3 h at optimum temperatures. Samples were taken at 30 min intervals for the determination of the residual enzyme activity. The results revealed that the immobilized protease in alginate beads demonstrated higher stability than the free enzyme, especially at pH 8.5 and 9.0, with retentions of 69.6% and 63.53% activity, respectively, after 3 h (Figure 5). These findings can be attributed to the enzyme protection provided by the microenvironment structure and properties of the alginate beads. Similar results have been reported during the immobilization of thermostable β-glucosidase in alginate gel beads and κ-carrageenase onto magnetic iron oxide nanoparticles [66,76].

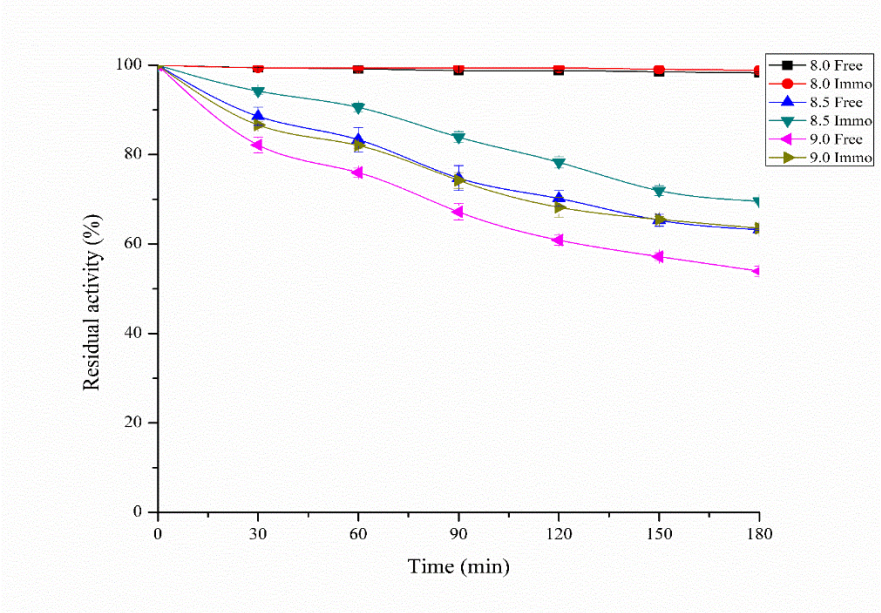

Figure 5. pH stability of free and immobilized proteases from *Bacillus aryabhattai* Ab15-ES. The enzyme was pre-incubated at pH values 8.0–9.0 for 3 h at 50 °C, and the residual enzyme activities were estimated at regular intervals under standard assay conditions. The non-heated enzymes were taken as 100%. Values indicate the average of triplicate values, while the error bars represent the standard deviation.

3.6. Kinetics Properties of Free and Immobilized Protease

The kinetic properties represent a key parameter for determining the efficiency of the immobilization process. In the present study, the kinetic parameters (K_m and V_{max}) were estimated from a Lineweaver–Burk plot using azocasein as a substrate at pH 8.0 and 50 °C. The calculated K_m of free and immobilized protease were 2.023 and 1.225 mg/mL, respectively. The lower K_m value of the immobilized enzyme indicates an increased affinity (1.65-fold) of the enzyme to the substrate upon immobilization in alginate gel beads (Figure 6). Furthermore, the V_{max} of the immobilized protease was higher (250 U/mL) than that of the free enzyme (232.56 U/mL), suggesting a more efficient conformation of the immobilized enzyme in the solid matrix [30,84]. In addition, the V_{max}/K_m value of the immobilized enzyme was found to be higher than the corresponding value of the free counterpart (204.08 U/mg vs. 114.96 U/mg), an indication of the high catalytic efficiency of the immobilized enzyme [85].

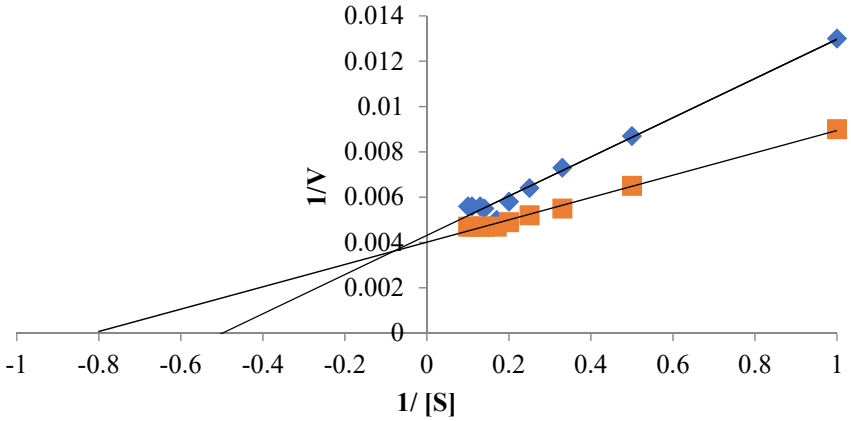

Figure 6. Lineweaver–Burk plot of free (◆) and immobilized (■) proteases from *Bacillus aryabhattai* Ab15-ES. The enzyme activity was measured at various azocasein concentrations (1.0–10.0 mg/mL) at pH 8.0 and 50 °C. The K_m and V_{max} values were determined using Lineweaver–Burk plot. S: substrate concentration; V: protease-specific activity.

3.7. Storage Stability of Free and Immobilized Protease

Storage stability represents a prominent index for the evaluation of the properties of enzymes for possible commercialization [86]. The immobilization of enzymes in a suitable support matrix is an important tool for a more rigid molecule that can resist conformational changes [72]. In essence, soluble enzymes are not stable during storage, leading to a reduction in catalytic activity [87]. In the present study, free and immobilized proteases were stored separately at 4 and 25 °C for 30 d and residual activity was determined at 5 d intervals. At 4 °C, the immobilized enzyme was found to be more stable, retaining 80.88% of its original activity after 30 d in comparison to the free enzyme, which maintained 64.6%. However, no significant loss in activity was recorded for the free enzyme for up to 5 d (Figure 7). Remarkably, while the soluble enzyme was almost completely inactivated when stored at 25 °C for 30 d, the immobilized protease still retained 38.37% of its initial activity. This improved storage stability may be due to a reduction in the denaturation of the enzyme or better structural stabilization as a result of the immobilization of the enzyme. Similar results have been reported after the immobilization of enzymes in alginate gel beads [30,66,88,89].

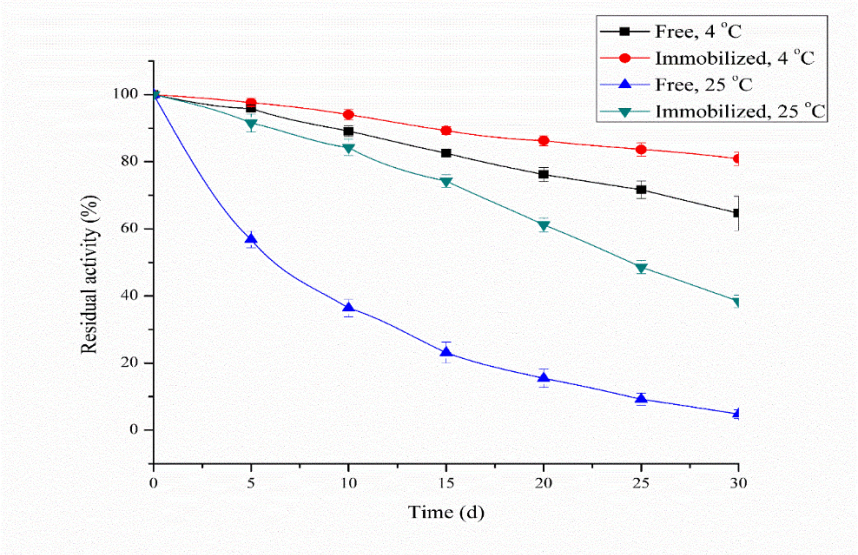

Figure 7. Storage stability of free and immobilized proteases at 4 and 25 °C. Values indicate the average of triplicate values, while the error bars represent the standard deviation.

3.8. Reusability of Immobilized Protease

The reusability of immobilized biocatalysts is recognized as one of the most important features that influences their cost-effective application for various bioprocesses [78]. In the present study, the reuse potential of immobilized protease was investigated in several cycles for substrate hydrolysis. The immobilized protease activity decreased gradually with increasing reuse cycles. The immobilized enzyme retained 32.93% of its original activity after continuous use for up to seven cycles, indicating good operational stability of the biocatalyst [30] (Figure 8). The loss in activity may be due to repeated use, which weakens the binding strength between the matrix and the immobilized enzyme, resulting in the physical loss of the enzyme from the support material [90]. In addition, the decrease in the enzyme's activity can be linked to the distortion of the active site due to intermittent encounters with the substrate, causing a partial or complete loss of catalytic efficiency [72]. Furthermore, the pore size of the alginate beads becomes larger after repeated use, leading to increased leakage of the enzyme and reduced biocatalytic activity [90,91]. Gupta et al. [66] reported on the reusability of immobilized β-glucosidase in alginate beads. The immobilized enzyme was reused four times with a residual activity of 17.85%. Similarly, the continuous use of protease and urease immobilized in alginate beads for up to three and five cycles, respectively, has been reported [23,64,92].

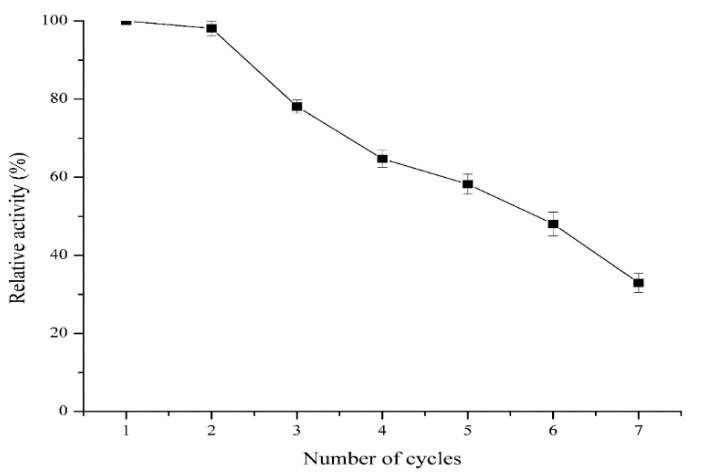

Figure 8. Reusability of protease immobilized in alginate gel beads. Values indicate the average of triplicate values, while the error bars represent the standard deviation.

4. Conclusions

Alkaline protease from an indigenous *Bacillus aryabhattai* Ab15-ES was successfully immobilized in alginate gel beads at optimum conditions of 2% sodium alginate and 0.3 M calcium chloride. Both free and immobilized proteases recorded optimum activity at a pH and temperature of 8.0 and 50 °C, respectively. Entrapment in alginate gel beads considerably improved the thermal and pH stability of the enzyme, as well as its storage stability in comparison to the soluble enzyme. Furthermore, the immobilized protease exhibited an increased affinity to the substrate and a higher catalytic efficiency, as well as good operational stability for up to seven reaction cycles, suggesting its significance for sustainable and economic biosynthetic processes.

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