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Research paper

Evaluation of immobilization and hydrolytic properties of α-amylase onto chitosan-PVA copolymer

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Abstract

The immobilization of α-amylase onto the chitosan-PVA (Chs-co-PVA) copolymer was successfully achieved through physical adsorption from its suspension in tris-HCl buffer (0.1 M, pH 8.0, with an activity of 2.89 U/mL). The process resulted in a maximum protein binding efficiency of 79.28% at pH 8.0, with 20.72% of the enzyme remaining unbound. The hydrolysis of starch by both free α-amylase and copolymer-bound α-amylase was evaluated under various reaction conditions. It was found that the immobilized α-amylase exhibited enhanced activity and stability compared to the free enzyme, particularly at elevated temperatures, extended incubation times (50 minutes), and an optimal pH of 8.0. The presence of specific salt ions, including Mg^{2+}, Co^{2+}, Fe^{3+}, Ca^{2+}, K^{+}, Zn^{2+}, Na^{+}, Pb^{2+}, Hg^{2+}, and Cu^{2+}, further improved the hydrolytic activity of the immobilized enzyme compared to the free enzyme. Additionally, immobilized α-amylase also demonstrated greater thermo stability, reusability, and storage stability.

Keywords: *Chitosan-PVA Copolymer; α-Amylase; Immobilization; Hydrolysis; Starch.*

1. Introduction

Enzymes are vital biocatalysts widely utilized in chemical and pharmaceutical industries [1]. Among them, α-amylase plays a crucial role in hydrolyzing starch into oligosaccharides [2], which have significant applications in the pharmaceutical, starch processing [3], and textile industries [4]. However, the commercial application of enzymes is often constrained by their high cost and limited reusability. To address these challenges, immobilization techniques are employed, where enzymes are bound to solid matrices or supports to enhance their stability and reusability [5]. Various materials have been explored for the immobilization of α-amylase, including superparamagnetic carboxymethyl chitosan/sodium alginate nanosphere [6], calcium chloride solution matrices [7], and blended PVA with pectin cross-linked by glutaraldehyde [8]. Other supports include cellulose ultrafiltration membranes [9], hydrogels made from poly(vinylamines) or poly (vinyl formamides) [10], mesoporous silica, and palm wood chips [11]. These immobilization strategies have demonstrated improvements in starch hydrolysis efficiency [7], [9 - 11] and polysaccharide digestion in artificial systems such as rumen models [8].
Natural polymers like chitosan are frequently used for enzyme immobilization due to their biocompatibility and favorable physicochemical properties [12]. Chitosan degradation products are non-toxic, non-immunogenic, and non-carcinogenic, making it an ideal material for biological applications [13]. Similarly, polyvinyl alcohol (PVA) is a cost-effective, non-toxic, biodegradable, and biocompatible synthetic polymer [14]. PVA gels have been effectively used to encapsulate enzymes such as aldolase and lactase, enabling their application in medicine due to PVA's compatibility with human tissues [15].
Combining chitosan's natural advantages with PVA's synthetic properties produces a hybrid biomaterial suitable for enzyme immobilization. Studies have shown that chitosan-PVA copolymers improve enzyme binding efficiency, stability, and hydrolytic activity [16 - 19]. Applications of these copolymers include the immobilization of various enzymes, such as pectinase [16], urease [17], phytase [18], and carboxylesterase [19], where the immobilized forms exhibit enhanced performance in terms of activity, stability, and suitability for industrial uses. For example, immobilization on PVA-chitosan nanofibers has shifted the optimal pH and temperature for enzymatic activity, making these materials suitable for applications in agriculture, pharmaceuticals, and food processing [16 - 19]. Amphiphilic membranes from PVA can be used for enzyme immobilization [20].
Building on these advancements, this study investigates the immobilization of α-amylase onto a chitosan-PVA copolymer (Chs-co-PVA) via physical adsorption. The hydrolytic activity of the bound enzyme on starch, as well as its stability and reusability, is systematically evaluated, showcasing the potential of this copolymer for industrial enzyme applications.

2. Experimental

2.1. Materials and method

Pristine copolymer, Chs-co-PVA prepared [16], is used as a material for enzyme immobilization. α-amylase (Department of Biotechnology, H.P. University Shimla, H.P., India), tris base (Hi-Media), HCl (Ranbaxy India), dinitrosalicylic acid (DNSA) (Hi Media), and starch (Hi Media) were used as received. Distilled water was used throughout the study. Metal salts $CuSO_4$, $FeSO_4$, $CoSO_4$, and $NiSO_4$ (S.D. Fine Chemicals, Boisar, India) were used as received.

2.2. Synthesis of chitosan-co-PVA copolymer

The copolymer Chs-co-PVA was synthesized employing APS as a radical initiator, following the established method [16].

2.3. Immobilization of α-amylase

Pristine Chs-co-PVA copolymer has been used as a support for binding the enzyme α-amylase. The activity of the immobilized enzyme for the hydrolysis of starch was studied as a function of various reaction parameters and compared with that of the free enzyme.

Procedure

To assess the hydrolytic activity, 0.50 mL of starch solution, 0.47 mL of tris-HCl buffer (0.1 M, pH 8.0), and 0.03 mL of enzyme solution were combined in a test tube. A control sample, omitting the enzyme, was also prepared. The mixtures were incubated at 50°C for 50 minutes, and then cooled to room temperature. Subsequently, 2 mL of DNSA reagent was added to each tube. For the control, 0.03 mL of denatured enzyme solution (boiled for 30 minutes at 100°C) was introduced, and absorbance (O.D.) was measured at 540 nm against a blank.

2.4. Enzyme assay and protein estimation

α-Amylase assay was done by the standard colorimetric method using starch as substrate. Protein concentration in the cell-free culture broth was estimated [17] using the Lowry et al. method

2.5. Immobilization of α-amylase onto Chs-co-PVA copolymer

α-Amylase was immobilized onto the Chs-co-PVA copolymer via physical adsorption. A powdered copolymer sample (5 g) was incubated with 2 mL of tris-HCl buffer (0.1 M, pH 8.0) at 37°C for 1 hour in a water bath. Subsequently, 10 mL of purified enzyme solution (2.89 U/mL activity; protein content: 10.27 mg/mL) was added to the matrix and incubated for 50 minutes at 50°C. The matrix was washed 3-4 times with tris-HCl buffer to remove unbound enzyme. To study hydrolytic activity, 1.5% (v/v) starch was added to the enzyme-bound matrix and incubated at 37°C for 1 hour for cross-linking. Residual traces of the activating agent were removed by washing the matrix three additional times with tris-HCl buffer. Immobilized protein content was determined by calculating the difference between the total protein used and the unbound protein in the supernatant.

2.6. Evaluation of hydrolytic properties of copolymer-bound α-amylase

The hydrolytic properties of the copolymer-bound α-amylase (1.5mg) as a hydrolase for starch were determined. Reaction parameters such as temperature, incubation time, pH, substrate concentration, and the presence of metal ions were examined. Thermo stability, reusability, and storage stability were also assessed and compared to the activity of the free enzyme.

3. Results and discussion

3.1. Immobilization of α-amylase onto Chs-co-PVA copolymer

In the present study, the immobilization of α-amylase onto the Chs-co-PVA copolymer, is carried out through physical adsorption from its suspension in tris-HCl buffer (0.1 M, pH 8.0, 2.89 U/mL activity). Maximum protein binding efficiency at pH 8.0 (79.28%) with 20.72% of enzyme unbound efficiency, was observed.

3.2. Evaluation of the hydrolytic properties of the pristine copolymer-bound α-amylase

The hydrolytic activity of both free α-amylase and copolymer-bound α-amylase was studied under varying conditions, including temperature, incubation time, pH, substrate concentration, and the presence of metal salts. The stability and reusability of the immobilized enzyme were also evaluated.

3.2.1. Effect of the temperature

The activity of free and immobilized α-amylase for starch hydrolysis was assessed at different temperatures (Fig. 1). The free enzyme showed maximum activity (2.91 U/mL) at 50°C, which decreased sharply at higher temperatures, reaching 0.72 U/mL at 75°C. In contrast, the immobilized enzyme exhibited higher thermal stability, achieving maximum activity (3.36 U/g) at 65°C before declining. These results indicate that immobilization enhances the enzyme's thermal stability, consistent with previous studies on immobilized α-amylase using polyimide materials [21] and bentonite-chitosan composites [22].

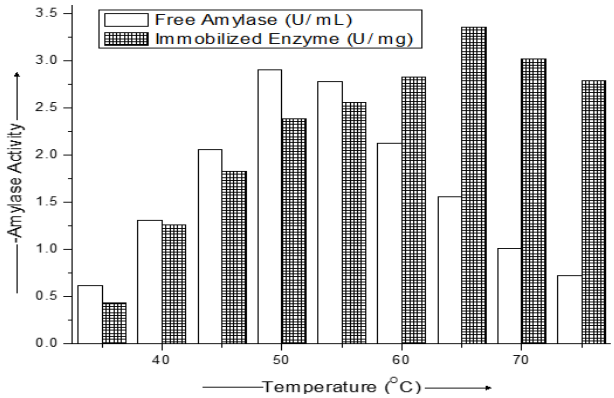

Fig. 1: Effect of Temperature on the Activity of Free and Immobilized Amylase pH=8.0, [Buffer]=0.1M.

3.2.2. Effect of the incubation time

The influence of incubation time on hydrolytic activity was studied (Fig. 2). Both free and immobilized enzymes showed increased activity with longer incubation times, reaching a maximum at 50 minutes. The free enzyme exhibited peak activity at 2.89 U/mL, while the immobilized enzyme demonstrated a higher maximum activity of 3.37 U/g. Beyond this point, activity declined but remained higher for the immobilized enzyme compared to the free enzyme.

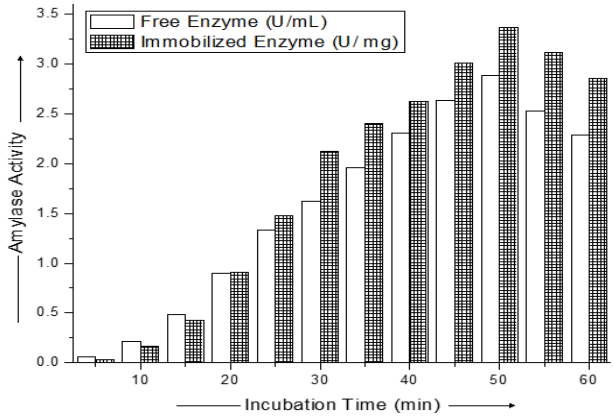

Fig. 2: Effect of Incubation Time on the Activity of Free and Immobilized Amylase pH = 8.0, [Buffer]= 0.1 M.

3.2.3. Effect of the pH

The effect of pH on enzyme activity during starch hydrolysis was analyzed in Fig. 3. It is observed from the figure that the immobilized enzyme shows higher maximum activity (3.35 U/g) as compared to a free enzyme (2.9 U/mL) at the same pH (8.0). Similar observations have been made by Baysal et.al. [22] for α-amylase immobilized onto bentonite-chitosan composite. Beyond the optimal pH, activity decreased for both forms, but the immobilized enzyme maintained greater activity at pH 10.0 (2.06 U/g) compared to the free enzyme (1.43 U/mL). This difference may be attributed to proton partitioning effects between the solution and the copolymer surface.

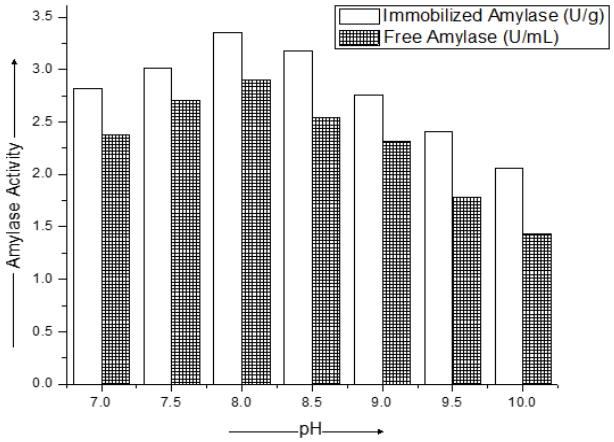

Fig. 3: Activity of Free and Immobilized α-Amylase at Different pH.

3.2.4. Effect of salt ions on the pristine copolymer-bound α-amylase activity

The hydrolytic activity of free and immobilized α-amylase was evaluated in the presence of various salt ions (10 mM solutions of Zn^{2+}, Mg^{2+}, Na^{+}, Pb^{2+}, Co^{2+}, Hg^{2+}, Fe^{3+}, Cu^{2+}, and K^{+}) (Fig. 4). Metal ions such as Mg^{2+}, Co^{2+}, Fe^{3+}, Ca^{2+}, and K^{+} enhanced the activity of both enzymes, with immobilized α-amylase showing superior activity (e.g., 3.96 U/g for Ca^{2+}) compared to the free enzyme (3.48 U/mL for

Ca^{2+}). In contrast, ions like Zn^{2+}, Pb^{2+}, Hg^{2+}, and Cu^{2+} inhibited activity, with immobilized enzymes exhibiting higher resilience. The observed inhibition may be due to interactions between metal ions and enzyme active sites, reducing hydrolysis efficiency.

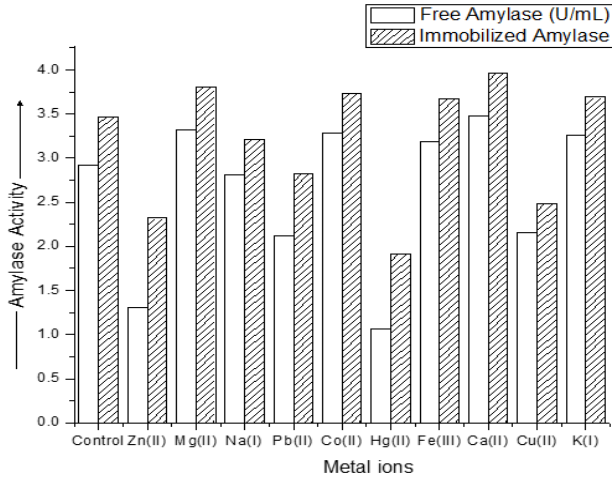

Fig. 4: Effect of Metal Ions on the Activity of Free and Immobilized α-Amylase.

3.2.5. Effect of substrate concentration

The effect of concentration of the substrate (starch) on hydrolytic activity of the free and the copolymer bound enzyme was studied and the results are presented in Fig. 5. The immobilized enzyme demonstrated higher activity across all substrate concentrations, peaking at 3.47 U/g, compared to the free enzyme's maximum of 2.87 U/mL at 0.15% starch. These findings suggest that immobilized enzymes remain active across a broader substrate concentration range, unlike free enzymes, which require an optimal concentration for maximum activity.

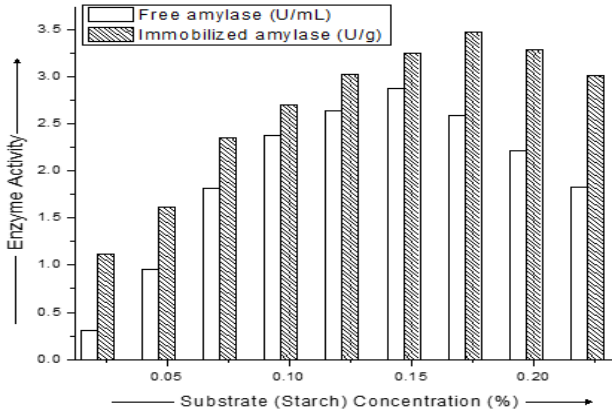

Fig. 5: Effect of Substrate (Starch) Concentration on the Activity of Free and Immobilized Amylase pH=8.0, [Buffer]=0.1M.

3.2.6. Effect of thermo-stability

The hydrolytic activity of free α-amylase and immobilized α-amylase as a function of thermo-stability measured at different temperatures has been studied up to 8 h (with a gap of 1 h), and the results are presented in Fig. 6, and Fig. 7, respectively. Both enzyme forms exhibited a decline in activity with increasing temperature and incubation time, but the immobilized enzyme retained significantly higher activity. After 8 hours, the activity loss was minimal for immobilized α-amylase at 4°C (2.32% loss) compared to the free enzyme (6.50% loss). At higher temperatures, immobilized α-amylase exhibited greater stability, with reduced activity loss compared to free enzyme, confirming that immobilization enhances thermal tolerance.

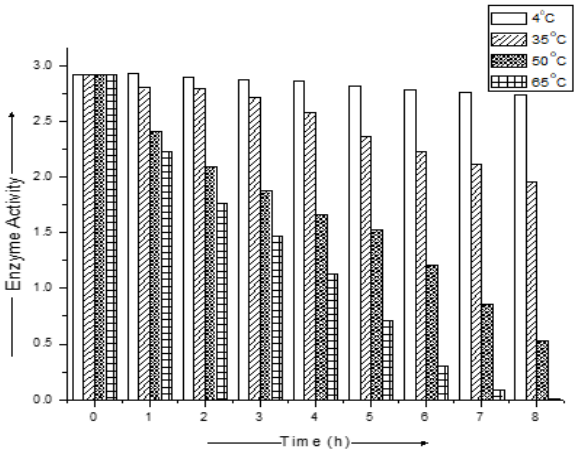

Fig. 6: Effect of Temperature on the Thermostability of Free α-Amylase pH=8.0, [Buffer]=0.1M.

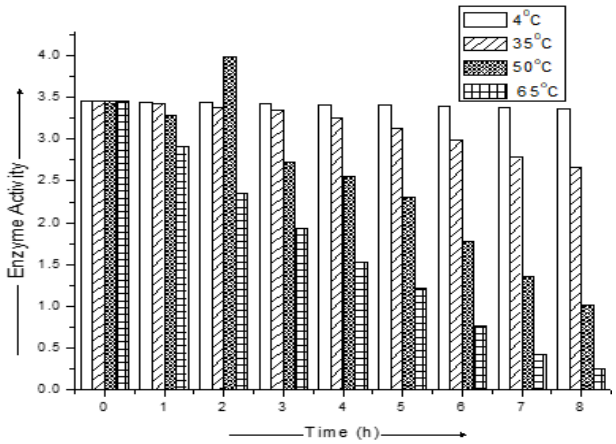

Fig. 7: Effect of Temperature on the Thermostability of Immobilized α-Amylase pH = 8.0, [Buffer]= 0.1 M.

3.2.7. Reusability

The reusability of immobilized α-amylase was assessed across multiple cycles of starch hydrolysis (Table 1). The immobilized enzyme retained 100% activity after the first cycle, but subsequent cycles showed reduced activity, with decreases of 64.26%, 34.29%, and 12.39% after the second, third, and fourth cycles, respectively. These results highlight the practicality of immobilized enzymes in repeated reactions, albeit with some loss of efficiency over time.

Table 1: Reusability of Immobilized α-Amylase

Number of Cycles	Immobilized α-Amylase Activity (U/g)
1	3.47
2	2.23
3	1.19
4	0.43

3.2.8. Storage stability of copolymer-bound α-amylase

The storage stability of immobilized α-amylase was examined by incubating it at 60°C in a water bath and comparing its activity to free enzyme over time (Fig. 8). While, free enzyme activity declined drastically to 6.19 % after 40 days, immobilized α-amylase retained 50.86% activity after the same period and 39.30% after 50 days. This demonstrates the superior storage stability of immobilized enzymes, making them suitable for long-term applications.

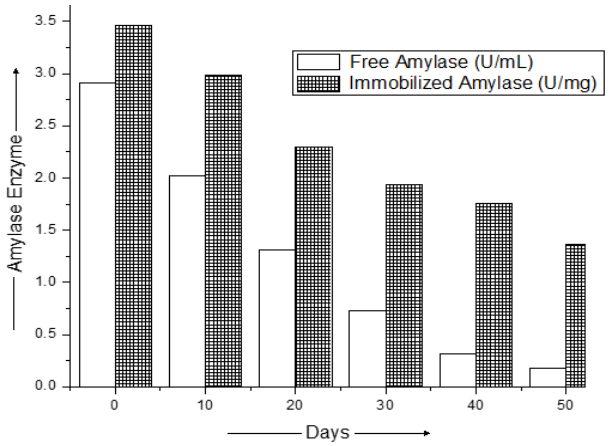

Fig. 8: Stability of Free and Immobilized α-Amylase pH = 8.0, [Buffer]= 0.1 M.

4. Conclusion

For α-amylase immobilization onto Chs-co-PVA, the activity increased for all the variables studied as compared to the free α-amylase activity, indicating that Chs-co-PVA offers an excellent support for the α-amylase. A notable observation was the consistent activity of the immobilized enzyme across all starch concentrations, whereas the free enzyme required an optimal starch concentration to achieve maximum activity. The immobilized enzyme demonstrated greater thermo stability and superior hydrolytic efficiency compared to its free counterpart, making it a more robust and effective catalyst under varying conditions. These findings highlight the advantages of immobilizing α-amylase on Chs-co-PVA copolymer, emphasizing its potential for enhanced performance in industrial applications.

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