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Research Article

IMMOBILIZATION OF α-AMYLASE FROM *Pennisetum typhoides* INSIDE GELATIN BEADS AND ITS CHARACTERIZATION

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Abstract: α-Amylase isolated from *Pennisetum typhoides* (specific activity 578.8 U/mg protein) was used for immobilization within gelatin beads. The optimum percent immobilization obtained was 88%. The pH optimum of immobilized α-amylase was 7.0 without any shift from the pH optimum of soluble enzyme. The value of K_m for soluble starch in gelatin beads is 4.5 mg/ml. The V_{max} value for gelatin bead catalyzed reaction is 926.3 µmol/min/mg. The optimum temperature of immobilized α-amylase was 70°C, however the soluble enzyme showed a fall in activity beyond 60°C. The activation energy determined from Arrhenius plot was 3.98 kcal/mol., lower than that of soluble enzyme. The immobilized α-amylase retained 60.0% activity when incubated for 10 min at 65°C. The gelatin α-amylase bead when stored at 4°C and assayed for activity at different intervals (days) retained 78.6% of enzyme activity at 90 days of storage. The same enzyme bead could be reused up to 8 cycles with 60.7% retention of activity. Immobilization clearly imparts advantages to the industrial enzyme α-amylase.

Keywords: α-Amylase; *Pennisetum typhoides*; immobilization; gelatin; reusability

Introduction

Enzyme immobilization refers to limitation of movement of enzyme by physical or chemical means. It is now gaining attention due to its potential applications in different industries and construction of biosensors for diagnostic purposes. Enzymes are purified to homogeneity and the cost of production is high. Enzymes in soluble form catalyze the reaction in presence of substrates but cannot be recovered from the reaction mixture. The enzyme, if immobilized, can be separated from the reaction mixture. The major advantage of immobilization is the reuse of immobilized enzyme for several cycles and hence production cost is reduced. Enzymes can be immobilized on surface of an inert matrix or can be entrapped inside a gel bead (Kennedy, 1995). Improvement in the field of enzyme immobilization took place through development of robust immobilized enzymes that can function nicely under hostile conditions (Kise and Hayakawa, 1991; Khmelnitsky and Rich, 1999).

α-Amylases find extensive applications in different industries such as paper, dyeing, textile, brewing, food and detergents. α-Amylases have been immobilized on different matrices like chitosan beads (Tripathi *et al.*, 2007; Kumari and Kayastha, 2011), glass beads (Dvali *et al.*, 1978; Ramesh and Singh, 1981; Kahraman *et al.*, 2007; Rani *et al.*, 2007), alginate beads (Dey *et al.*, 2003; Kumar *et al.*, 2006; Tee and Kaletunc, 2009; Riaz *et al.*, 2009), Amberlite beads (Tripathi *et al.*, 2007; Kumari and Kayastha, 2011), cellulose fibers (Varavinita *et al.*, 2002), silichrome (Dvali *et al.*, 1978), celite (Ertan *et al.*, 2006), coconut fibres (Dey *et al.*, 2002), paramagnetic polyacrolein beads (Varlan *et al.*, 1996), poly (methyl methacrylateacrylic acid) microspheres (Aksoy *et al.*, 1998), activated plastic supports (Roig *et al.*,

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1993) and Fuller's earth (Rath *et al.*, 2012). A soybean oil based resin has been developed for improved immobilization of α-amylase (Kahraman *et al.*, 2006).

Gelatin is a mixture of proteins and peptides derived from hydrolysis of collagen. It forms a solution of high viscosity when dissolved in water. It forms gel under cold condition, but melts around 37°C, exhibiting thermo-reversible property. Gelatin is also rich in glycine, proline and hydroxyproline, but lack tryptophan. Gelatin finds extensive application in food and pharmaceutical industry. Further, it is another matrix used widely for immobilization of enzymes (Srivastava *et al.*, 2001; Munjal and Sawhney, 2001; Kumar *et al.*, 2005; Nagatomo *et al.*, 2005; Naganagouda *et al.*, 2007; Jaiswal and Prakash, 2011; Jaiswal *et al.*, 2012). Gelatin being a protein stabilizes the enzyme protein. The immobilization of enzymes inside gelatin involves both physical entrapment and covalent cross-linking (Alteriis *et al.*, 1988). Formaldehyde and glutaraldehyde are used as cross-linkers that bind enzyme with gelatin (Kumar *et al.*, 2005). Glutaraldehyde has been used in dual capacities as cross-linking agent as well as enzyme coupling agent (Kennedy *et al.*, 1984). Immobilization of α-amylase into photographic gelatin films by cross-linking with chromium (III) acetate, chromium (III) sulfate, potassium chromium (III) sulfate and formaldehyde has been reported (Bayramoglu *et al.*, 1992 a). Chromium (III) acetate has been used as cross-linking agent to immobilize glucose oxidase onto gelatin for the construction of a biosensor (Emregul *et al.*, 2005). Photographic gelatin was found to be very efficient natural polymer due to its diffusion characteristics (Bayramoglu *et al.*, 1992 b). Polydopamine coated magnetic-chitin (DMCT) particles as a new matrix for enzyme immobilization has been introduced with further enhanced immobilization efficiency in the presence of glutaraldehyde (Sureshkumar and Lee, 2011). Gelatin and alginate together have been employed for purification and immobilization (Fadnavis *et al.*, 2003). Immobilized α-amylase inside gelatin beads has found application as detergent additive (Jaiswal and Prakash, 2011).

α-Amylases from microbial sources have drawn considerable attention with respect to immobilization (Dvali *et al.*, 1978; Ramesh and Singh, 1981; Kvesitadze and Dvali, 1982; Dey *et al.*, 2002; Varavinita *et al.*, 2002; Ertan *et al.*, 2006; Rani *et al.*, 2007; Riaz *et al.*, 2009 and Demirkan *et al.*, 2011). However, limited information is available on immobilization of α-amylases from plants (Kumar *et al.*, 2006; Tripathi *et al.*, 2007; Kumari and Kayastha, 2011; Jaiswal and Prakash, 2011; Rath *et al.*, 2012). In the present paper, we report immobilization and characterization of α-amylase from *P. typhoides* inside gelatin beads.

Materials and Methods

Gelatin, soluble starch, sodium potassium tartrate, 3,5-dinitrosalicylic acid, sodium hydroxide (Hi Media, India); dehydrated alcohol (Bengal Chemicals & Pharmaceuticals Ltd., India); formaldehyde (Loba Chemie, India) and maltose, imidazole (Sisco Research Laboratory, India) were procured from the sources mentioned within parenthesis. The α-amylase isolated from *P. typhoides* was used for the present immobilization studies have been described earlier (Kharkrang and Ambasht, 2012). All solutions were prepared in de-ionized water from Milli-Q system (Millipore, USA).

Soluble α-amylase assay and protein quantification- The α-amylase activity was assayed by discontinuous procedure using 3,5-dinitrosalicylate (DNS) reagent (Bernfeld, 1955). The 1.0 ml assay mixture contained 0.5 ml soluble starch, 0.4 ml 50 mM imidazole buffer, pH 7.0 maintained at 37°C. The reaction was initiated by addition of an aliquot 0.1 ml of enzyme and incubated for 3 min. The formation of 3-amino-5-nitro-salicylate was monitored in presence of the reducing sugar as described earlier (Kharkrang and Ambasht, 2012). One unit of α-amylase was defined as the amount of enzyme releasing one µmol maltose equivalent per minute under the assay conditions. Protein content was quantified according to method by Bradford (1976) with minor modifications.

Entrapment of α-amylase inside gelatin beads- The α-amylase (171.8 U/ml) was diluted 12-fold (14.32 U/ml) for the entrapment experiment. A 10% gelatin solution was prepared by dissolving 10.0 g gelatin in 100 ml de-ionized water with constant stirring at 50°C. A hardening solution

was prepared by taking formaldehyde, ethanol and water in the ratio of 2:5:3, respectively. A 0.25 ml enzyme aliquot (3.58 U) was mixed to a 5.0 ml, 10% gelatin solution in a test tube, followed by addition of 1.0 ml of hardening solution. The gelatin-enzyme hardening solution (6.25 ml) was taken in a pipette and transferred in used clean tablet strips. These strips were then kept in the refrigerator till the gel formation occurred. The gel beads formed were removed from the strips, washed with distilled water to remove unbound enzyme and stored in assay buffer under refrigeration. Enzyme entrapped inside the beads were used for α-amylase activity assay.

Activity assay of immobilized enzyme: The 1.0 ml assay mixture contained 0.5 ml soluble starch (1%) and 0.5 ml assay buffer, maintained at 37°C. The reaction was initiated with addition of three immobilized enzyme beads and was incubated for 10 min. The enzyme beads were taken out after 10 min. and DNS reagent was added. The further steps were according to as described earlier (Kharkrang and Ambasht, 2012). The rate of reaction is expressed as enzyme unit per bead in all the experiments.

The immobilization of enzyme is expressed in terms of % immobilization that is determined from the following formula (Eq. i).

$$\%\,immobilization = \frac{Activity\ in\ immobilized\ beads}{Activity\ in\ soluble\ enzyme} \times 100 \quad \text{(i)}$$

Kinetic characterization of immobilized enzyme: The variation of enzyme activity with pH has been studied using 50 mM imidazole buffers (pH 5.0-9.0) at saturating soluble starch concentration. From the data, a plot of % relative activity versus pH was made along with that of soluble enzyme to determine pH optima. The rate of enzyme gelatin bead catalyzed reaction was monitored at various concentrations of starch. From the data, Lineweaver Burk plot (1/v versus 1/[Starch]) was made and values of K_m and V_{max} were determined. Effect of temperature on rate of enzyme gelatin bead catalyzed reaction was studied in the range of 40-70°C maintaining the reaction mixture at a particular temperature. From the data, a plot of relative % activity versus temperature was made to determine optimum temperature and was compared with that of soluble enzyme. Further from the data, Arrhenius plot was made and value of energy of activation was determined. In another experiment, the enzyme gelatin beads were maintained at different temperatures (45-65°C) for 10 min. and a plot of relative % residual activity versus temperature was made and compared with that of soluble enzyme.

Storage and reusability of immobilized α-amylase: Immobilized α-amylase beads were routinely stored in assay buffer at 4°C. In one set of experiment, enzyme activity was routinely tested on different days using different beads. A plot of % residual activity versus days was made. In another set of experiment, activity assay of a particular bead was performed for several cycles and a plot of % residual activity versus cycles of reuse was made.

Results and Discussion

The total number of enzyme beads obtained from 6.25 ml of gelatin enzyme hardening solution was 38. The percent immobilization of enzyme inside gelatin beads was determined from the equation (i) mentioned earlier and was found to be 88% with 0.0828U/bead. The % immobilization is very high in the present case like the one immobilized in celite (Ertan *et al.*, 2006). It suggests that there is little leaching of enzyme from gelatin beads. Better enzyme retention has been reported when only formaldehyde was used as cross-linking agent (Alteriis *et al.*, 1985). The magnified photograph (5x) of α-amylase entrapped inside gelatin bead recorded with Kodak digital camera is shown in Fig. 1.

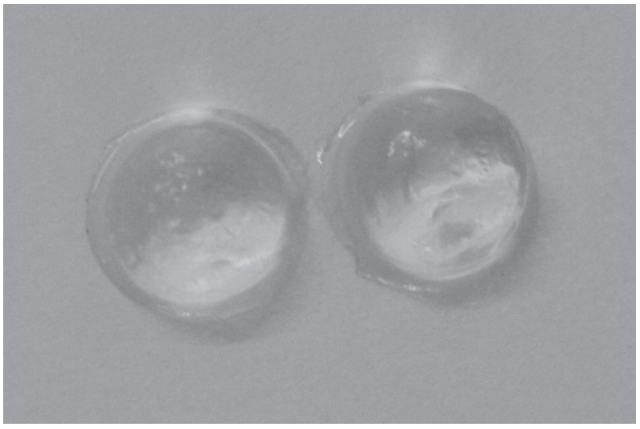

Figure 1 : **Magnified photograph (5x) of α-amylase entrapped inside gelatin beads using Kodak digital camera**

The effect of pH on rate of enzyme gelatin bead and soluble enzyme catalyzed reactions (% relative activity) is shown in Fig. 2. The optimum pH is observed to be 7.0, like the soluble enzyme suggesting that entrapment of enzyme inside the bead did not change the property of the enzyme. In some other cases also, immobilized enzymes do not show any change in pH optima (Arica e*t al.*, 1995; Ertan *et al.*, 2006; Rani *et al.*, 2007). Further, the immobilized and soluble enzymes behaved similarly between pH 6.5-7.5. The soluble enzyme however, showed better activity below pH 6.5 and above 7.5.

The immobilized α-amylase inside gelatin beads was used to study effect of [starch] on rates of reaction catalyzed by them at pH 7.0. The Lineweaver Burk plot of the data is shown in Fig. 3. The K_m value for starch was 4.5 mg ml^{-1} α-amylase gelatin bead catalyzed reaction. The K_m value is higher with respect to the soluble α-amylase (0.5 mg ml^{-1}) determined at pH 7.0 (Kharkrang and Ambasht, 2012). Similar results have been obtained with other immobilized enzymes (Tee and Kaletunc, 2009; Tripathi *et al.*, 2007; Kumari and Kayastha, 2011). K_m value increases with the size of the bead (Dey *et al.*, 2003). The increase in the value of K_m is due to diffusional limitation of substrate (Reddy and Kayastha, 2006). The V_{max} value determined was 926.3 µmol. $min.^{-1}$ mg^{-1} protein, for α-amylase gelatin bead catalyzed reaction.

The effect of temperature (40-70°C) on rate of reaction catalyzed by α-amylase immobilized

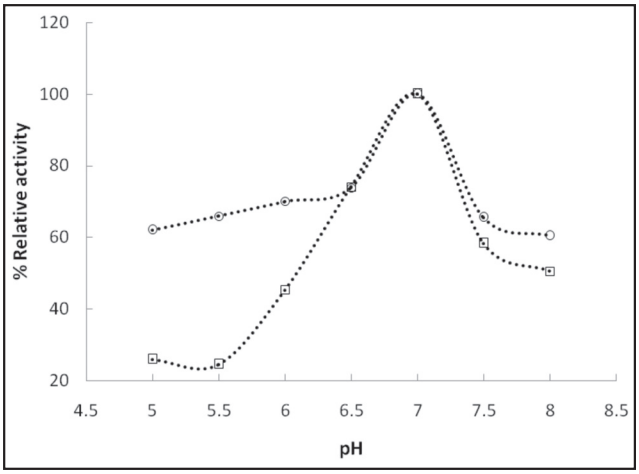

Figure 2: **Effect of pH on relative activities of soluble (o) and gelatin immobilized α-amylase (□) bead catalyzed reaction**

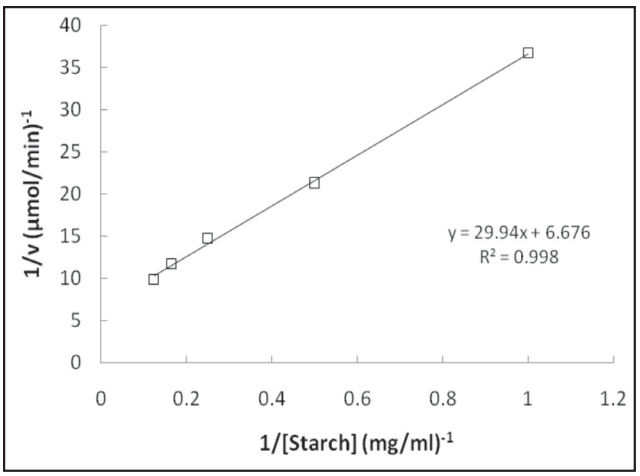

Figure 3: **Lineweaver Burk plot of gelatin immobilized α-amylase bead catalyzed reaction rate at different starch concentrations**

inside gelatin bead was studied at pH 7.0. The plot (% relative activity versus temperature) is shown in Fig. 4a along with soluble enzyme. It is evident from the plot that optimum temperature for immobilized enzyme is 70°C, higher with respect to soluble enzyme (60°C). Invariably, the immobilized enzymes show higher temperature optima in comparison to soluble enzymes (Varavinita *et al.*, 2002; Kumar *et al.*, 2006; Tripathi *et al.*, 2007; Rani *et al.*, 2007). From the above data, Arrhenius plot was made for immobilized enzyme catalyzed reaction and is shown in Fig. 4b. The energy of activation was determined and was found to be 3.98 $kcalmol^{-1}$. The value is lower than that of soluble enzyme (6.9 kcal mol^{-1}) determined at the same pH 7.0 (Kharkrang and Ambasht, 2012). The energy of activation for immobilized α-amylases on different matrices was found to be in between 5.0-6.0 kcal/mol (Dey *et al.*, 2003; Rani *et al.*, 2007). However, in one case, the energy of activation of soluble enzyme was very high (40.9 kcal/mol) and increased further upon immobilization in alginate beads (51.7kcal/mol) (Kumar *et al.*, 2006).

The effect of temperature on relative % residual activity of immobilized and soluble enzyme was studied at different temperatures (45-65°C) for fixed time (10 min.). At any temperature, there is higher activity retention in immobilized enzyme in comparison to soluble enzyme. The immobilization of enzyme by entrapment within the gel matrices provides a

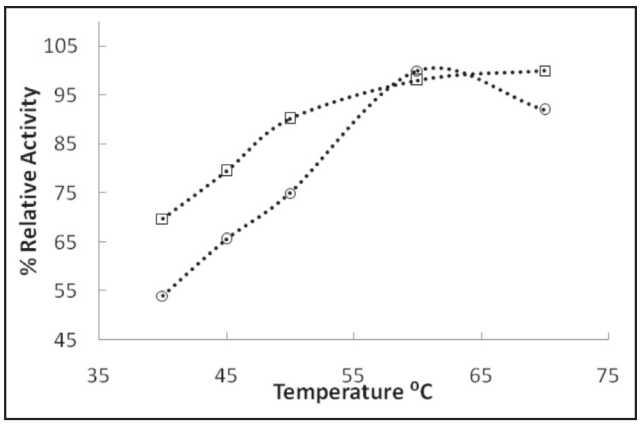

Figure 4a: Effect of temperature on soluble α-amylase (o) and gelatin immobilized α-amylase (□) bead catalyzed reactions

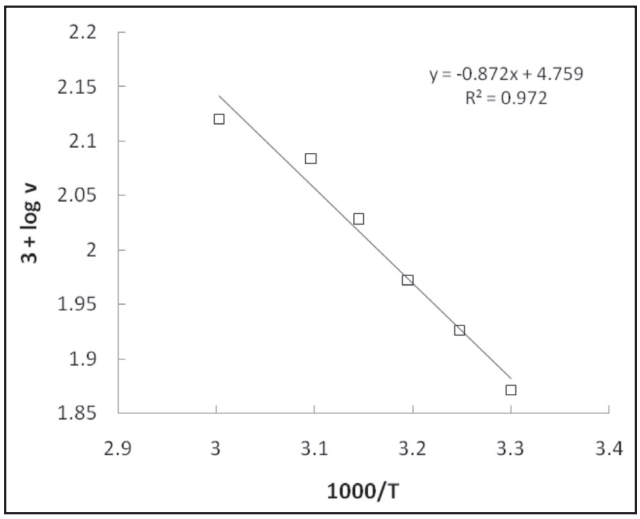

Figure 4b: Arrhenius plot of gelatin immobilized α-amylase bead catalyzed reaction at different temperatures

kind of protection or shield to the enzyme, and hence the enzyme could withstand the adverse effects under higher temperatures making it more thermal stable.

The storage stability of immobilized α-amylase was studied at 4°C. The result is shown in Fig. 5. More than 80% activity is retained after 90 days. Soluble enzyme however, retained only 50% activity when stored at 4°C after 90 days (Kharkrang and Ambasht, 2012). Under similar conditions more activity loss has been reported in other immobilized α-amylases (Kvesitadze and Dvali, 1982; Tripathi *et al.*, 2007; Rani *et al.*, 2007; Kumari and Kayastha, 2011). In one case there was no loss in enzyme activity up to 180 days (Dvali *et al.*, 1978). At high temperatures also immobilized enzymes have been stored without

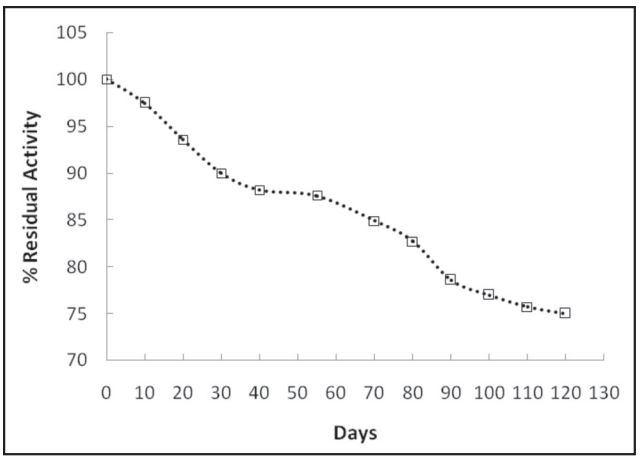

Figure 5: **Storage stability of gelatin immobilized α-amylase beads at 4°C**

loss in activity (Nagatomo *et al.*, 2004; Tee and Kaletunc, 2009).

The number of cycles of the reuse of same bead was tested for immobilized gelatin enzyme bead. The results are shown in Fig. 6. The gelatin enzyme beads retained 80% activity after 5th time reuse and activity retention dropped to 45% on 10th reuse. Losses have been observed in some other immobilized α-amylases (Kumar *et al.*, 2006; Ertan *et al.*, 2007; Tripathi *et al.*, 2007; Kumari and Kayastha, 2011; Sureshkumar and Lee, 2011). The loss in activity may be due to weakening in the strength of binding between matrix and enzyme and on further repeated use, the enzyme may leach out from the matrix.

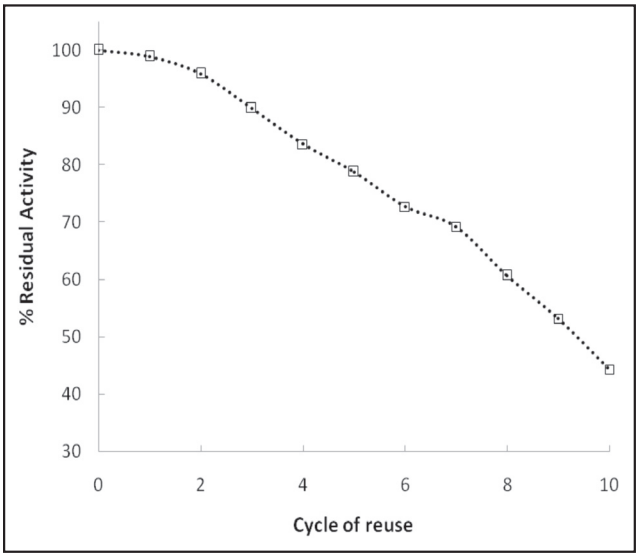

Figure 6: **Reusability of the same gelatin immobilized α-amylase bead at 4°C**

Conclusion

The present work on immobilization of α-amylase inside gelatin beads suggests important role in industries. The properties of enzyme do not change as a result of immobilization (pH optima 7.0). The immobilized enzyme is thermally more stable, shows little loss in activity when stored at 4°C and can be used for several cycles without significant loss in activity.

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Abbreviation

DNS, 3,5-dinitrosalicylate.

Note

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