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Immobilization of Little Millet *(Panicum sumatrense)* α-amylase

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Abstract: ***Alpha amylase (1, 4-α-D-glucan glucanohydrolase, EC 3.2.1.1) from germinating little millet seeds purified to homogeneity was entrapped in 2% calcium alginate beads and gave immobilization yield of 65%. It was observed that entrapment of α-amylase was not affected by pH. Temperature optima of immobilized enzyme was higher compared to free enzyme. The enzyme on immobilization showed an apparent K_m of 2.8×10^{-3}g/ml. Immobilized enzyme showed the highest operational stability for upto 7 reuses by retaining 72.5% of residual activity.***

Keywords: α-amylase, little millet, immobilized, calcium alginate, entrapment

1. Introduction

Alpha amylases (1,4-α-D glucan-4-glucanohydrolases, EC 3.2.1.1), are a family of endoamylases (glycosyl hydrolase 13 family) that catalyse the hydrolysis of α-D-(1,4) glycosidic linkages in starch and its components, i.e. amylose, amylopectin and glycogen releasing malto-oligosaccharides in the α-anomeric form. They are ubiquitous, produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. These enzymes are of great significance in present day biotechnology and find applications in areas including starch saccharification, baking, brewing, textile, fermentation and distilling industries.

Soluble enzymes are widely used for various industrial applications. However, these enzymes can be used only once since they cannot be separated from the reaction mixture. Further, most often the presence of enzyme in the final product is undesirable. Immobilization of the enzymes onto solid supports that are either organic or inorganic is a very effective way to increase enzyme stability and operational lifetime [1-3]. Besides it facilitates the separation of enzymes from reaction media easily, the recovery and purification of the final products from enzymes become more reliable, simple and efficient [4].

It is preferable that the method employed for immobilization of enzyme should cause as little trauma to the enzyme as possible. Entrapment is often the most desirable method because it results in high recovery of enzyme activity after immobilization, long enzyme stability in the immobilization matrix and protection from microbial degradation [5, 6]. Alginate, a naturally occurring copolymer comprising mannuronic acid and glucouronic acid, is a polymer of choice for entrapment of cells and enzymes due to its cost effectiveness.

In this study, immobilization conditions were investigated for *Panicum sumatrense* α-amylase as a model enzyme. Some properties of immobilized enzyme were determined and compared with those of free enzyme.

2. Literature Survey

Various studies have been carried out on constraining the enzyme on to a solid support thereby immobilizing it and increasing its stability towards temperature [7, 8]. For many industrial applications, enzymes and cells have to be immobilized, *via* very simple and cost effective methods, in order to be reused over very long period of time [9]. There have been many reports about immobilization of α-amylase used for the hydrolysis of starch and production of maltose. Some examples involve glass beads and glutaraldehyde fixation [10], polymeric microspheres [11], UV-curable polymer [12], functionalized silica [13], adsorption on zirconia [14], covalent binding [15, 16, 17], ionic binding [16, 17] etc.

3. Materials and Methods

Sodium alginate was obtained from Sigma Chemical Co. (St. Louis, MO, USA), little millet seeds were purchased from the local market, Visakhapatnam, India. All other chemicals used were of analytical grade. *Panicum sumatrense* α-amylase was purified according to Usha and Hemalatha [18].

3.1 Entrapment

Entrapment in calcium alginate beads was carried out according to Fraser and Bickerstaff [19]. Different concentrations of sodium alginate (1.0, 2.0 and 3.0 %) were prepared by dissolving alginate in distilled water and then the enzyme solution (200 U) was added. The mixture obtained was extruded drop wise through a pasture pipette (1mm diameter) into a gently stirred 2 % (w/v) $CaCl_2$ solution to give bead size of 2mm. The beads were left for further 20-30 min before collecting, washed with acetate buffer (0.05 M, pH 5.0) and kept in the same buffer for a suitable time to remove the unbound enzyme. The calcium alginate beads containing the enzyme were thoroughly washed with distilled water and used for further studies.

3.2 Immobilized Enzyme Assay

The reaction mixture, containing 20 ml of 2% (w/v) starch solution in acetate buffer (0.05 M, pH 5.0) and 2.0 g of

carrier were incubated at 45°C in a water bath shaker. After the enzymatic reaction had proceeded for 20 min, 0.5 ml of the hydrolysed products were assayed for amylase activity using DNS according to Bernfeld method [20]. One unit is defined as the amount of amylase that produced 1 µmole of reducing sugar under assay condition per gram of carrier.

3.3 Determination of immobilization efficiency

Immobilization efficiency was determined from the difference in enzyme activity in the solution before and after the immobilization.
Immobilization yield (%) = (I/A-B) ×100
Where A = added enzyme (U/g of bead);
B = unbound enzyme (U/g of bead);
I = immobilization enzyme (U/g of bead).

3.4 Effect of pH on the activity of immobilized α-amylase

The pH optimum for the calcium alginate immobilized α-amylase was determined by assaying at different pH values using different buffers *viz*., 0.05 M Glycine-HCl (pH 2-3), Sodium acetate buffer (pH 4-5), Sodium phosphate buffer (pH 6-7) and Tris buffer (pH 8-9). The substrate 2% starch was prepared in respective pH buffers and % maximum enzyme activity was calculated. The pH of the immobilized enzyme was compared with the soluble enzyme.

3.5 Effect of temperature on the activity of immobilized α-amylase

The optimum temperature for soluble and calcium alginate immobilized enzyme was determined by assaying the enzyme at temperature from 20°C to 80°C. Maximum activity was calculated as stated above at their optimal pH.

3.6 Effect of substrate concentration on immobilized α-amylase

Effect of substrate concentration on α-amylase activity was carried out at 45°C in 0.05 M acetate buffer pH 5 at different substrate concentrations (0.2 to 2×10^{-3} g/ml starch) at optimum pH for immobilized enzymes. The kinetic constants K_m and V_{max} were calculated using Lineweaver-Burk plot [21].

3.7 Operational stability of the immobilized α-amylase

After each assay of amylase activity, the beads were removed, washed thoroughly with distilled water and stored at 4°C. Then the beads were reassayed for amylase activity and the same process was repeated till the tenth use.

4. Results

Immobilization of purified little millet α-amylase, was attempted in order to assess the activity retained in entrapped calcium alginate beads in comparison to the free α-amylase. Figure-1 shows the immobilization of α-amylase by entrapment at different concentrations of sodium alginate. The highest immobilization yield (65%) was obtained with sodium alginate 2%.

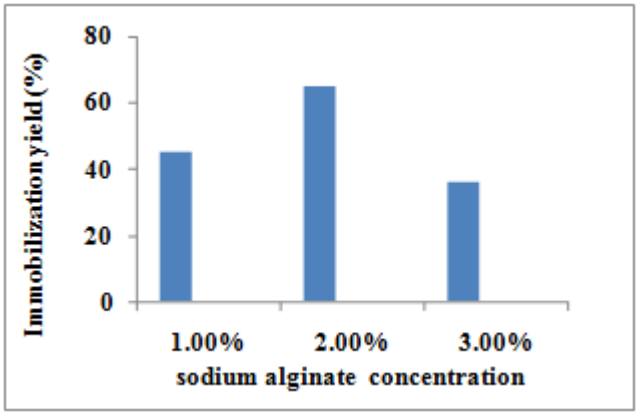

Figure 1: Effect of concentration of sodium alginate on immobilization yield.

4.1 Effect of pH on the activity of immobilized α-amylase

The effect of pH on the activity of α-amylase immobilized in calcium alginate beads was compared with that of free α-amylase at different pH values (2-9). Figure 2 shows that the optimum pH for free and calcium alginate entrapped α-amylase was 5. It was observed that entrapment of α-amylase was not affected by pH.

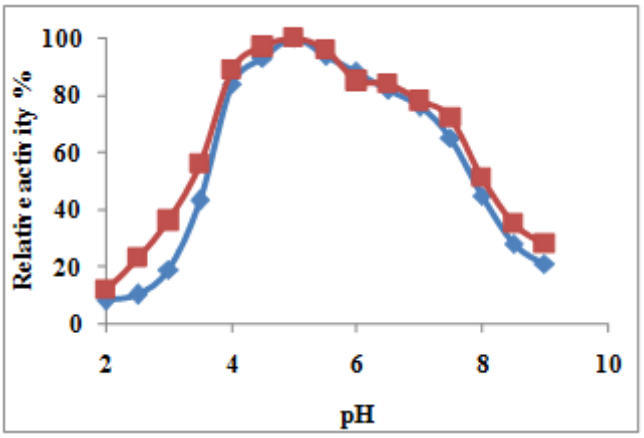

Figure 2: Effect of pH on activity of (♦) free and (■) immobilized α-amylase.

4.2 Effect of temperature on the activity of immobilized α-amylase

The activity of α-amylase immobilized in calcium alginate beads was compared with that of free α-amylase at temperatures (20-80°C). The optimum temperature for free enzyme was 50°C. The optimal reaction temperature of the free amylase shifted by 10 ±1°C for the enzyme immobilized in calcium alginate beads, showing a shift from 50 to 60°C. There is a significant increase in the optimum temperature on immobilization by entrapment in calcium beads (Fig. 3).

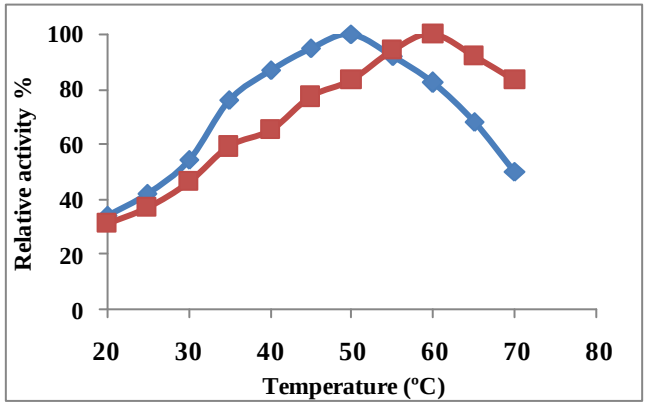

Figure 3: Effect of temperature on activity of (♦) free and (■) immobilized α-amylase.

4.3 Effect of substrate concentration on immobilized α-amylase

K_m and V_{max} for the free and immobilized α-amylase were calculated using Lineweaver-Burk plot with starch as substrate. The calculated K_m value of the immobilized enzyme were higher than that of the free enzyme. Alpha amylase entrapped in alginate beads showed an apparent K_m of 2.8×10^{-3}g/ml (Fig. 4). The K_m of amylase entrapped in alginate beads is approximately 1.7 times higher than that of the free enzyme. V_{max} on immobilization was decreased from 1388 (units/min/mg) for free enzyme to 1295 units/min/mg for immobilized enzyme. The increase in K_m values when compared with that of the free enzyme may be due to the lower accessibility of the substrate to the active site of the immobilized enzyme.

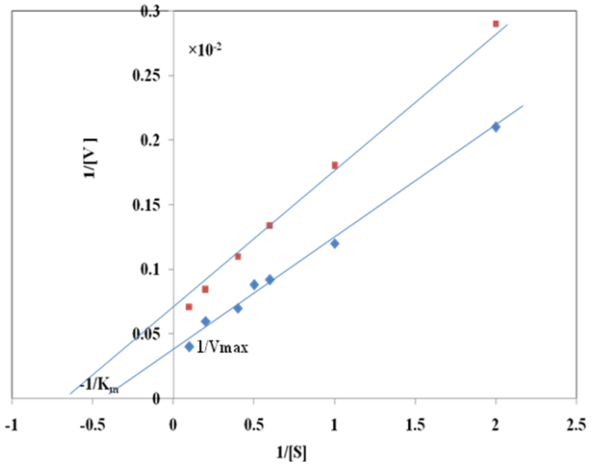

Figure 4: Determination of K_m for ■free and ■immobilized α-amylase by Lineweaver-Burk plot method.

4.4 Operational stability of immobilized α-amylase

The operational stability of immobilized enzymes is one of the most important factors affecting the utilization of an immobilized enzyme system. The α-amylase activity in calcium alginate beads remained unchanged during the first three to four cycles. Immobilized α-amylase retained 72.5% activity upto seven cycles (Fig. 5). After the seventh cycle, a decrease in the enzyme activity may be due to enzyme denaturation or may also be due to physical loss of enzyme from the carrier.

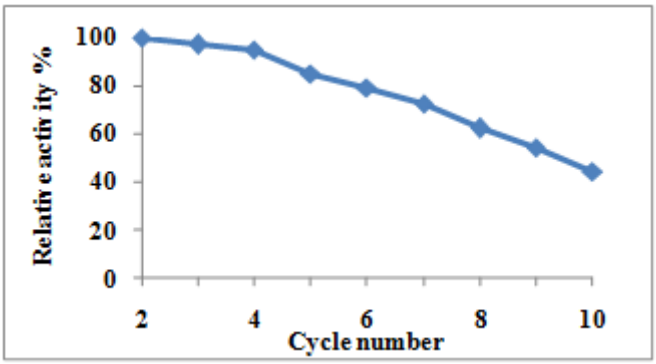

Figure 5: Reusability immobilized α-amylase.

5. Discussion

The immobilization yield is the key parameter, since it represents the general output of the efficiency of the immobilization process [22]. Immobilization of little millet α-amylase by entrapment with 2% sodium alginate gave an immobilization yield of 65%. Immobilization of *Bacillus acidocaldarius* α-amylase by entrapment with 2% sodium alginate gave an immobilization yield of 61.4% [23]. Dey *et al.* (2003) [24] reported that sodium alginate 4% gave the highest immobilization yield of 75% for *B. circulans* α-amylase. Immobilization of soybean urease by entrapment in sodium alginate gave an yield of 54% [25] and pigeon pea urease immobilized on 5% agar yielded an optimum immobilization of 51.7% [26].

The pH optima of little millet α-amylase did not alter on entrapment in calcium alginate beads. Arpana and Kayastha, 2011[16] reported a shift of pH towards the basic side for soybean α-amylase. Similar results were reported for mung bean α-amylase immobilized on chitosan and amberlite [17]. A shift towards acidic region has been observed when α-amylase was immobilized on zirconium dynamic membrane and poly (methylacrylate-acrylic acid) microspheres [27, 28]. Abdel-Naby *et al.* [29] reported the optimum pH of the immobilized enzymes was shifted to acidic range (optimum pH 5.25-5.75) in comparison to the free enzyme (optimum pH 6.0). The optimum pH of reaction was not affected by immobilization process (in case of covalent binding and ionic binding) [30]. El-Batal *et al.* (2005) [31] suggested that the α-amylase immobilized by ionic binding had the same pH optima as that of the free enzyme.

The temperature optima of little millet α-amylase showed a shift of 10°C on entrapment in calcium alginate beads. The higher temperature profile on entrapment in calcium alginate beads may be due to some conformational effects on enzyme entrapment, which protects the enzyme against heat denaturation. Free α-amylase from soybean reported an optimum temperature of 70°C [32], whereas α-amylase immobilized on amberlite showed an optimum temperature of 75°C [16]. Alpha amylase from *Bacillus circulans* immobilized in calcium alginate beads was reported to show an increase in operating temperature [24]. Temperature

optima of immobilized *Bacillus acidocaldarius* α-amylase activity shifted toward higher temperature from 50°C to 60°C [30].

Immobilized little millet α-amylase exhibited K_m values higher than the free enzyme. The maximum rate of the reaction catalyzed by the immobilized enzyme was lower than the free enzyme. An increase in K_m, clearly indicates an apparent low affinity of the enzyme towards its substrate compared to the soluble enzyme. Increased K_m values of amylases after the immobilization process were similarly reported [16, 17, 30, 33, 34, 35]. Decrease in V_{max} value of α-amylase after immobilization covalently on plastic supports was reported [36]. Kumar *et al*. (2006) [37] reported that K_m for both free and entrapped *Aspergillus oryzae* α-amylase enzymes remained the same. In this case alginate entrapment did not have any effect on the binding of substrate to the enzyme. However, V_{max} of the entrapped enzyme was decreased.

The operational stability of the immobilized little millet α-amylase was evaluated in repeated process. Calcium alginate immobilized α-amylase retained 72.5% activity upto seven cycles. With repeated use, the strength of binding between the matrix and enzyme is weakened, leading to leaching of enzyme from the matrix and loss in activity. *Bacillus circulans* α-amylase immobilized by entrapment in calcium alginate beads retained 83% of the initial activity after 7 cycles [24]. The immobilized *Bacillus acidocaldarius* α-amylase on a cation exchange resin and glass beads retained 70 and 73.4% from the initial activity after upto 6 cycles [30]. Soybean α-amylase immobilized onto chitosan and amberlite beads showed a residual activity of 38 and 58% respectively, after 10 reuses [16]. It was reported that the mung bean α-amylase immobilized onto amberlite beads and chitosan exhibited a residual activity of 43 and 27% respectively, after 10 cycles [17].

6. Conclusion

In this study *Panicum sumatrense* α-amylase was successfully immobilized by entrapment in calcium alginate beads. The immobilized enzyme showed a high operational stability by retaining utmost initial activity after several reuses. The immobilized enzymes showed enhanced thermal stability than the free enzyme. The easy accessibility of *Panicum sumatrense* α-amylase, the ease of its immobilization on low-cost matrices, increased stability upon immobilization make it a suitable product for future applications.

7. Acknowledgements

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