

Optimization of immobilization of α -amylase in alginate gel and its comparative biochemical studies with free α -amylase

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Abstract

The α -amylase was immobilized by entrapment in calcium alginate beads. The effect of concentration of sodium alginate, calcium chloride and curing time on immobilization yield of α -amylase in calcium alginate beads were investigated and immobilized α -amylase was characterized. Three percent (w/v) sodium alginate, 1M calcium chloride and 2 h curing time were used and 90% immobilization yield of α -amylase was achieved with enhanced thermal and acidic condition stabilities. Significant changes in optimum pH and temperature values of the enzyme were recorded after immobilization. The activity of immobilized enzyme was affected by the size of the bead and bead size of 2.4mm was found to be most effective for starch hydrolysis. From the enzyme kinetic study, decrease in substrate affinity and velocity of enzyme reaction were observed after immobilization of enzyme. Immobilized α -amylase retained 35% activity after 10 reuses with 30 min of each reaction time.

Keywords: calcium alginate, enzyme immobilization, entrapment, α -amylase, starch hydrolysis

INTRODUCTION

The α -amylase (EC 3.2.1.1) enzyme which hydrolyzes starch to maltooligosaccharide is of great importance in present day biotechnology with applications ranging from food, baking, brewing, fermentation, detergent applications, textile desizing, paper industries, etc. [1, 2]. This starch degrading enzyme has received a great deal of attention because of its perceived technological significance and economic benefits. The industrial application of enzymes is often hampered by a lack of availability, high price and limited stability under operational conditions. The use of enzymes in a free form is very uneconomical because the enzymes generally cannot be recovered at the end of the reaction. These drawbacks can be overcome by immobilization of the enzyme thereby rendering it more stable and easy to recover and recycle [3, 4]. Immobilized enzymes pave the way to industrial development of continuous enzyme reactors. This procedure prevents enzyme losses due to washout and at the same time maintains enzymes at high concentrations in order to reduce the cost of the enzymes [5]. The above features would be important in the development of an economically feasible continuous bioreactor for the starch hydrolysis industry. Thus immobilizing α -amylase would be of great significance.

Several efforts have been taken to immobilize α -amylase by binding it to solid carriers [6-16]. However, these covalent binding techniques involve chemical modification of the enzyme. It is preferable that the method employed for immobilization of enzyme should cause as little disturbance to the enzyme as possible.

Entrapment fulfills this criterion. Entrapment technology has been designed to entrap materials within a semi-permeable polymeric membrane and/or a gel matrix [17]. Enzyme immobilization by entrapment produces the particle structure which allows contact between the substrate and enzyme to be achieved and, additionally, it is possible to immobilize several enzymes at the same time [18]. Among the many matrices available, one of the most frequently used is entrapment within porous matrices, such as alginate often in the form of beads [19]. This sort of system is reasonably safe, simple, cheap and offering good mechanical strength, high porosity for substrate and product diffusion and above all the simple procedural requirements for immobilization [20].

Thus, in the present study, α -amylase (Diastase) was immobilized in calcium alginate gel beads. The conditions of entrapment like concentration of sodium alginate, calcium chloride and bead size were optimized for highest apparent activity. The entrapped α -amylase was characterized in terms of optimum temperature and pH, kinetic parameters and compared with those of free α -amylase.

MATERIALS AND METHODS

Materials

Sodium alginate, calcium chloride, α -amylase (Diastase), starch and DNSA (3, 5-Dinitrosalicylic acid) were purchased from Himedia (Mumbai). All the other chemicals used were of analytical grade.

Preparation of enzyme solution

Freeze-dried α -amylase (Diastase) was added to 0.1 M sodium phosphate buffer (pH 7.0) to the concentration of 1 mg/ml. This enzyme stock solution was stored at 4°C for future tests.

Received: Oct 17, 2011; Revised: Jan 12, 2012; Accepted: Feb 14.

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Enzyme assay

The α -amylase activity was determined using starch (1%, w/v) as a substrate [21]. The amount of reducing sugar (estimated as maltose) released was measured using 3, 5-dinitrosalicylic acid [22]. One unit (U) of enzyme activity is defined as the amount of enzyme required to release 1 μ mol of reducing sugar per minute at 37°C.

Enzyme immobilization

A 3% (w/v) sodium alginate solution in 50mM sodium phosphate buffer (pH 7.0) was prepared by warming at 50°C. After cooling down to room temperature, 1ml of enzyme stock solution was mixed with 9ml of sodium alginate solution (the total volume of matrix and enzyme mixture being 10ml). The mixture was taken into a syringe, and beads were formed by dropping the solution into 1M calcium chloride solution with gentle stirring at 4°C for 2 h. The formed beads were recovered by filtration and thoroughly washed with distilled water. The beads were dried using filter paper (Whatman no. 1) followed by exposure to the open air for 1 h before use [23, 24]. The filtered calcium chloride solution was collected for enzyme activity determination.

Determination of enzyme immobilization yield

The immobilization yield was defined here as the yield for enzyme which was immobilized in the calcium alginate beads and expressed by the following equation.

$$\text{Immobilization yield (\%)} = (\text{Activity of immobilized enzyme/A-B}) \times 100$$

Where A is the activity of free enzyme added, and B is the activity of remaining enzyme in washed water and filtered calcium chloride solution. Both A and B were evaluated from the amount of reducing sugars produced enzymatically in the corresponding solutions.

Optimization of parameters for immobilization in alginate gel

Different sodium alginate concentrations (1-4% w/v), calcium chloride concentrations (0.1-5M) and curing time (30-180 min) were used during immobilization of α -amylase to achieve 100% immobilization yield.

Effect of pH and Temperature on α -amylase activity

The effect of pH on enzyme activity was determined in the pH range of 3-9 using 0.1M buffers (pH 3-5, sodium citrate buffer; pH 6-8, sodium phosphate buffer; pH 9, NaOH/glycine buffer). To determine the optimum temperature of free and immobilized α -amylase, enzyme activities were measured in the temperature range of 30-90°C.

Effect of bead size on rate of starch hydrolysis

Alginate gel beads were prepared by dropping 3% (w/v) sodium alginate solution with α -amylase (the total volume of matrix and enzyme mixture being 10ml) through three gauges of hypodermic needles (18, 22, 26 gauge) into 1M Calcium chloride solution. The resulting beads were of 2.8, 2.6, and 2.4 mm diameter respectively. One gram of each of the bead sizes was added to 20

ml of 1% (w/v) starch solution in 0.1M sodium citrate buffer pH 5.5 and incubated at 60°C in a water bath shaker. Aliquots were withdrawn after every 60 min and checked for amount of maltose produced.

Kinetic Analysis

Kinetic parameters of free and immobilized α -amylase were estimated by measuring initial reaction rates using different starch concentrations in the range of 0-20mg/ml at predetermined optimum pH and temperature of each form. K_m , V_{max} values of free and immobilized α -amylase were calculated from nonlinear regression fitting of the initial reaction rates corresponding to different starch concentrations with GraphPad Prism software.

Reusability of immobilized α -amylase

To investigate the effect of immobilization on reusability of α -amylase, the immobilized α -amylase after each cycle of 30 min reaction time at 60°C was washed with buffer and then suspended again in a fresh reaction mixture to measure enzyme activity. The residual activity was calculated by taking the enzyme activity of the first cycle as 100%.

RESULTS AND DISCUSSION

Optimization of parameters for immobilization in alginate gel

a) Effect of sodium alginate concentration

It has been reported that the immobilization yield of enzyme depends on concentration of sodium alginate [19], the porosity of the calcium alginate beads depend upon the alginate type and the gelling agent concentration [25]. Various concentrations of sodium alginate were used for preparation of calcium alginate beads in order to vary the relative degree of cross linking which would create different pore size. The immobilization yield was found to be highest (90%) for a final sodium alginate concentration of 3% (w/v) (Fig. 1). Decreasing immobilization yield with increase in sodium alginate concentration has been due to the decrease in the porosity of the gel beads, which caused diffusion limitation of the substrate. In practice reducing the porosity can reduce leakage but some initial leakage of the enzyme molecule is certain to occur [26]. So 100% immobilization yield could not be attained due to above mentioned reasons. The lower immobilization yield in case of lower concentration of sodium alginate solutions might be due to larger pore size and consequently greater leakage of the enzyme from matrix [19].

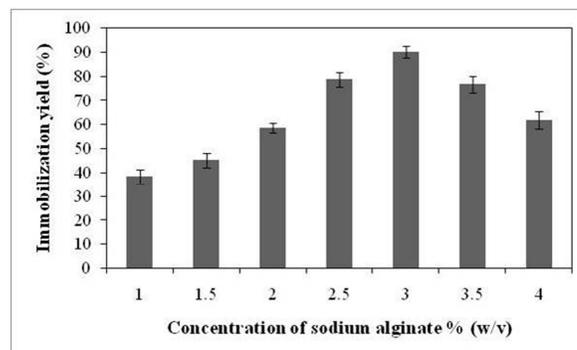


Fig1. Effect of concentration of sodium alginate on immobilization yield

b) Effect of concentration calcium chloride

Concentration of calcium chloride was also varied in order to acquire stable beads capable to secure maximum enzyme. It was found that 1M calcium chloride retained highest (89%) α -amylase and as the concentration of calcium chloride increased beyond 1M, immobilization yield of α -amylase was decreased (Fig.2). A decrease in the relative protease activity with increase in calcium chloride concentration has been reported [27, 28]. It might be due to change in pH of calcium chloride solution with change in calcium chloride concentration.

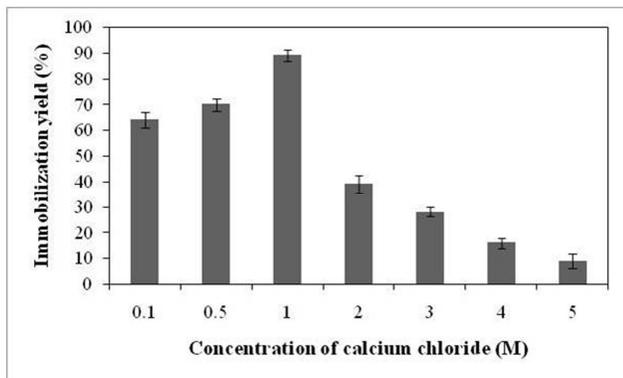


Fig 2. Effect of concentration of calcium chloride on immobilization yield

c) Effect of curing time of calcium alginate beads

Hardness of the calcium alginate beads depends upon time required for the gel to set [19]. It is an important parameter in immobilization as it produces stable calcium alginate beads which could reduce the enzyme leakage and increase the immobilization yield of enzyme. So the effect of curing time on the enzyme immobilization yield was evaluated. The treatment of the beads in a calcium chloride bath for 120min gave 90% immobilization yield (Fig.3). Prolonged curing of the beads with calcium chloride solution did not improve the immobilization yield. It might be due to constant hardness of the calcium alginate beads observed after 120min curing time [19]. This could have resulted in constant leakage of α -amylase above 120min curing time. At lower curing time, the beads were very soft and fragile resulting in more leakage and hence giving very low percent immobilization yield.

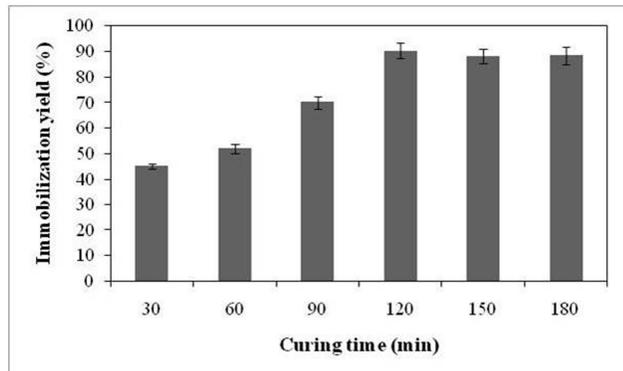


Fig 3. Effect of curing time on immobilization yield

Effect of pH and Temperature on α -amylase activity

The pH is one of the major parameters capable of shifting enzyme activities in reaction mixture. Immobilization usually results in shift of optimum pH due to conformational changes in enzymes. The effect of pH on activity of both free and immobilized α -amylase is given in Fig. 4. Optimum pH values were 7 and 5.5 for free and immobilized α -amylase respectively. This shift in optimum pH could be resulted from the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site [4]. During α -amylase immobilization, similar shift in the optimum pH towards acidic direction had been observed [29].

The activity of enzyme is also strongly dependent on temperature. The activity of free α -amylase increased with temperature and maximum activity was observed at 50°C (Fig.5). The optimum temperature of α -amylase was shifted to 60°C after immobilization in calcium alginate beads. As was evident from the data, α -amylase after immobilization in calcium alginate beads possessed temperature resistance than the free enzyme [30, 19].

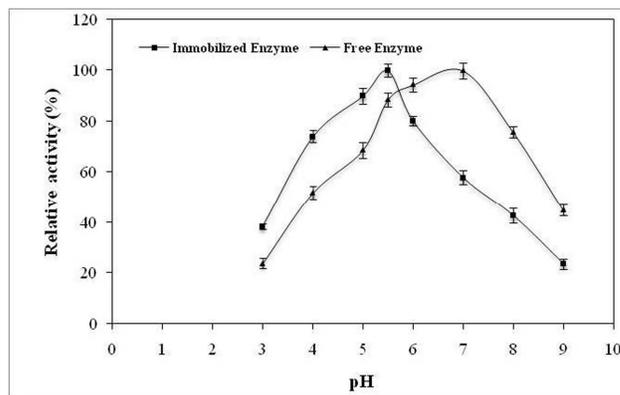


Fig 4. Effect of pH on activity of free and immobilized α -amylase

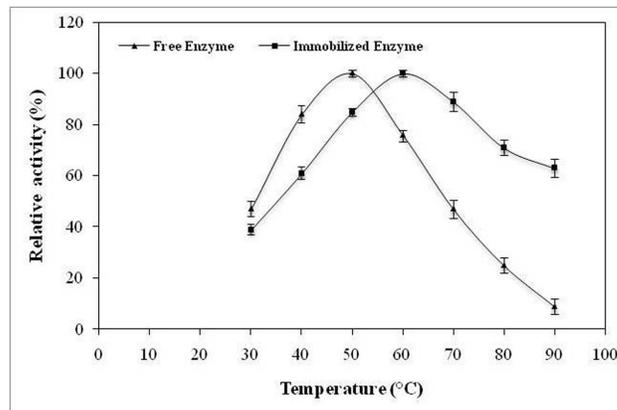


Fig 5. Effect of temperature on activity of free and immobilized α -amylase

Effect of bead size on rate of starch hydrolysis

In the system of enzyme immobilization by entrapment, substrate has to be transported from the bulk solution to the outer surface of the matrix and then to the inner part of matrix. So both the intraparticle diffusion and the external mass transfer should be taken into consideration. However it is assumed that greater contribution is from the intraparticle mass transfer. The intraparticle

mass transfer depends on size of the bead which has significant effect on the rate of starch hydrolysis [19]. As shown in Fig. 6, the highest rate of starch hydrolysis was observed with bead size of 2.4mm and the bigger sizes showed lower rate. From the above finding it may be concluded that the beads of 2.4mm offered lesser diffusion resistance compared to the larger beads.

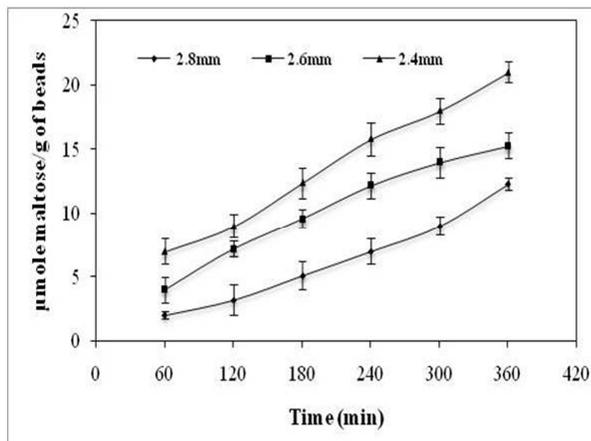


Fig 6. Effect of bead size on rate of starch hydrolysis

Kinetic Analysis

Kinetic parameters of both free and immobilized α -amylase were measured. For both forms of α -amylase, Michaelis-Menten type kinetic behavior was observed. The K_m and V_{max} values as determined from the GraphPad Prism Software were found to be 0.93 mg/ml and 2.30 μ mole/min (for free) and 1.12 mg/ml and 1.83 μ mole/min (for immobilized) respectively (Table 1). Therefore, for immobilized form of α -amylase V_{max} is decreased and K_m is increased as compared to free form. The K_m value shows the affinity of enzyme for its substrate. Lower the K_m value more is the affinity of enzyme for its substrate. So after immobilization, substrate affinity of α -amylase was decreased. This might be due to the lower accessibility of the substrate to the active site of the immobilized enzyme. This result is similar to that obtained in literature [31-33].

Table 1. Kinetic parameters of free and immobilized α -amylase

Enzyme	K_m (mg/ml)	V_{max} (μ mole/min)
Free α -amylase	0.93 \pm 0.014	2.30 \pm 0.045
Immobilized α -amylase	1.12 \pm 0.027	1.83 \pm 0.032

Reusability of immobilized α -amylase

The main advantage of enzyme immobilization is that it leads to a reusable enzyme preparation. Reusability of enzyme is a key factor for its cost-effective industrial use. Immobilized α -amylase in calcium alginate beads can be reused either by filtration or centrifugation. The reusability of immobilized α -amylase was studied up to 10 cycles. As shown in Fig.7, the activity of immobilized α -amylase decreased to approximately 35% of its original activity over ten cycles.

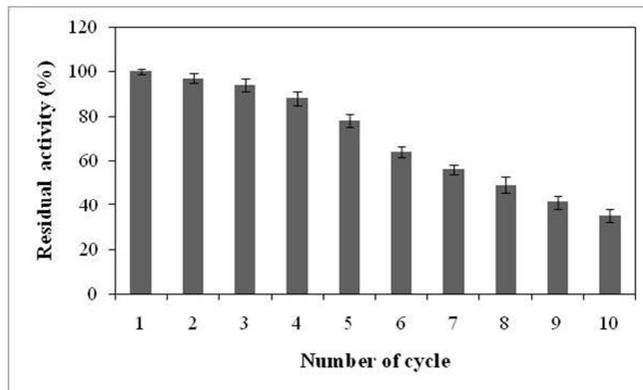


Fig 7. Reusability of immobilized α -amylase

CONCLUSION

The α -amylase was immobilized by entrapment in calcium alginate beads with 90% free enzyme activity recovery. It was demonstrated that the factors such as concentrations of sodium alginate, calcium chloride and curing time had significant effects on immobilization yield in alginate beads. Temperature and pH optima of α -amylase in alginate beads were shifted; this could increase the applicability of the enzyme. After immobilization, substrate affinity of α -amylase was decreased. The results of reusability experiment demonstrated that the immobilized α -amylase retained 35 % of activity after ten cycles of use. Hence, we conclude that properly optimized immobilization of α -amylase in calcium alginate is very useful for continuous starch hydrolysis.

ACKNOWLEDGMENTS

We thank to Kolhapur Institute of Technology's College of Engineering for financial support.

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