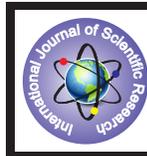


Development of the Optimal Conditions for Alpha-Amylase Immobilization



CHEMISTRY

KEYWORDS : Entrapment, immobilization, α -amylase, starch

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ABSTRACT

α -Amylase immobilization was carried out by entrapment in calcium alginate beads. The conditions of entrapment such as concentration of sodium alginate, calcium chloride and curing time were optimized for maximum apparent activity. 4% (w/v) Sodium alginate, 1M calcium chloride and 2 h curing time were used and 85% immobilization yield of α -amylase was obtained. The suitability of the support and technique for the immobilization of amylase was evaluated by estimating the enzyme activity, immobilization efficiency, stability at various temperatures and pH. The Michaelis constant, was estimated for both immobilized and free enzymes. Immobilized alginate-amylase had a higher K_m (2.54 mM) than that of the free enzyme (1.12mM). It was evident that after immobilization, substrate affinity of α -amylase was decreased.

INTRODUCTION

Amylases are one of the main enzymes used in industry. They can be obtained from several sources such as plants animals and microorganisms. α -Amylases are widely used in various industrial applications such as production of ethanol, starch liquefaction, detergents, desizing of textile, modified starches, paper recycling [1]. α -Amylases (EC 3.2.1.1) are metalloenzymes that catalyses the hydrolysis of internal α -1,4-glycosidic linkages in starch to glucose, maltose and maltotriose units [2-4]. Previously, starch was hydrolyzed into glucose by acid hydrolysis. But this method has drawbacks like the operating conditions are of highly acidic nature and high temperatures. These limitations are overcome by enzyme hydrolysis of starch to yield high fructose syrup. Immobilization allows for easy separation of the enzyme from the starch hydrolysis products which can save the enzyme, labor, and overhead cost [5]. Calcium alginate beads are widely used in enzyme immobilization because the gel formation occurs at mild conditions and poses no risk of harm to humans. Several efforts have been taken to immobilize α -amylase by binding it to solid carriers [6-17]. Entrapment is taken as the most preferable method because it prevents excessive loss of enzyme activity after immobilization, increases enzyme stability in microenvironment of matrix, protects enzyme from microbial contamination [18]. Physical entrapment of alpha-amylase in calcium alginate beads has shown to be a relatively easy, rapid and safe technique [19] in comparison with other immobilization methods. Entrapment requires that the immobilized enzyme should be a large enough molecule to be kept inside the gel matrix but the substrate and product should be small enough to pass through the pores of the gel.

The present study deals with the immobilization of α -amylase (Diastase) by entrapment in calcium alginate gel beads. The conditions of entrapment such as concentration of sodium alginate, calcium chloride and curing time were optimized for maximum apparent activity. Immobilized α -amylase was characterized in terms of optimum temperature, pH and K_m and compared with those of free α -amylase.

MATERIALS AND METHODS

α -Amylase (Diastase), sodium alginate, calcium chloride, starch and DNS (3,5-Dinitrosalicylic acid) were procured from Himedia laboratories (Mumbai). All the other chemicals used were of analytical grade.

Preparation of enzyme solution

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 further studies.

Immobilization of amylase

Sodium alginate of varying concentrations (1.0-5.0% w/v) were used for preparing beads and the most appropriate concen-

tration was taken for subsequent studies. The stock solution of alginate in 0.05M 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 9 ml of sodium alginate solution (the total volume of matrix and enzyme mixture being 10 ml). The mixture was taken into a syringe, and beads were formed by dropping the polymer solution from a height of approximate 20 cm into an excess (100 ml) of stirred 1M CaCl_2 at 4°C for 2 h. As soon as the drop falls the free enzyme is entrapped in a cage of calcium alginate in the shape of a bead. Beads of calcium alginate with entrapped enzyme were thoroughly washed with distilled water and were stored in suspended buffer at 4 ° C for further studies. These were subsequently used for the starch hydrolysis reaction. The filtered calcium chloride solution was collected for enzyme activity determination.

Amylase assay

The α -amylase activity was determined using starch as a substrate. The amount of reducing sugar (estimated as maltose) released was measured using 3, 5-dinitrosalicylic acid [20]. In this study, the reaction mixture containing 2.5 mL of starch (1.5% w/v) solution in phosphate buffer (0.1M 6.5 pH) and 0.5 mL of enzyme solution were incubated at 37 °C. Enzymatic reaction was stopped with 0.5 mL of DNS reagent after 10 minutes incubation and absorbance was measured at 540 nm spectrophotometrically. 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.

For assay of immobilized amylase, the appropriate amount of beads were incubated in 0.1M phosphate buffer (pH 6.5) containing 1.5% starch at 37 °C with intermittent shaking. Following incubation of 10 minutes an aliquot of 1 mL was withdrawn from the reaction mixture and assayed as described as above.

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 (1-5% w/v), calcium

chloride concentrations (0.5-3M) (and curing time (30-180 min) were used during immobilization of α -amylase to achieve maximum percentage of immobilization yield.

Steady-state kinetics

The effect of pH on enzyme activity (soluble as well as immobilized) was determined in the pH range of 4-9 using 0.1M buffers of sodium citrate (pH 3-5), sodium phosphate (pH 6- 8) and NaOH/glycine (pH 9). Optimal temperature was studied by measuring free and immobilized enzyme activities in the temperature range of 30-80°C. Effect of substrate concentration on amylase activity was investigated at 37 °C by varying the starch concentrations in the range of 5mM- 20mM at optimum pH for soluble and immobilized enzymes. The activity assay was performed as described earlier and K_m was determined from the Lineweaver-Burk plot.

RESULTS AND DISCUSSION

Optimization of conditions for immobilization in alginate gel:

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 and the gelling agent concentration [21]. Pore size of the beads should be such that substrate and product can easily diffuse in and out of the alginate gel matrix but the enzyme should retain in the micro environment of the beads. 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 maximum (85%) using the sodium alginate concentration of 4% (w/v) (Fig.1). Lower the concentration of sodium alginate solution, greater will be the pore size of the beads and consequently leakage of enzyme from the beads will increase [22]. Similarly, the pore size of beads will decrease with the increase in concentration of sodium alginate solution. Increased sodium alginate concentration interfered the entry of substrate into the bead; that led to the lower immobilization efficiency [23].

Effect of concentration of calcium chloride

In order to obtain stable beads capable to secure maximum enzyme, concentration of calcium chloride was also varied. It was found that 1M calcium chloride retained maximum (85%) 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 [24, 25]. It might be due to change in pH of calcium chloride solution with change in concentration of calcium chloride.

Effect of curing time of calcium alginate beads

Curing time 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. Hardness of the calcium alginate beads depends upon time required for the gel to set [19]. The treatment of the beads in a calcium chloride bath for 120 min gave 85% 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 120 min curing time [19]. This could have resulted in constant leakage of α -amylase above 120 min 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.

The optimum incubation period for immobilized alpha amylase enzyme was 10 minutes. The activity was determined at different incubation time with 1.5 % starch. Incubation time was con-

tinued upto 60 minutes. Ten minutes incubation time was sufficient for required enzyme activity.

Effect of pH and Temperature on α -amylase activity

Immobilization usually results in shift of optimum pH due to conformational changes in enzymes. Optimum pH values were 7 and 6 for free and immobilized α -amylase respectively. The activity of both free and immobilized α -amylase was determined at different pH (Fig.4) with 1.5 % (w/v) starch. 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 [26]. Similar shift in the optimum pH towards acidic direction had been observed [27] during α -amylase immobilization.

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 [28,19]. The improved stability of immobilized enzyme was supposed to be aided by the matrix, which absorbs a considerable amount of heat and protects the enzyme against denaturation.

Effect of substrate concentration on K_m

The amylase was immobilized in alginate gel by entrapment method and investigated for the effect of substrate concentration on amylase activity. It was observed that there was a significant increase in K_m for alginate-immobilized amylase (2.54mM) when compared with free amylase (1.12mM)(data not shown). Increased in K_m of immobilized enzyme is due to diffusional resistance offered by the gel. Consequently, substrate affinity of α -amylase was decreased after immobilization. It reveals that substrate takes more time to reach the site of catalysis and hence results an increase in K_m .

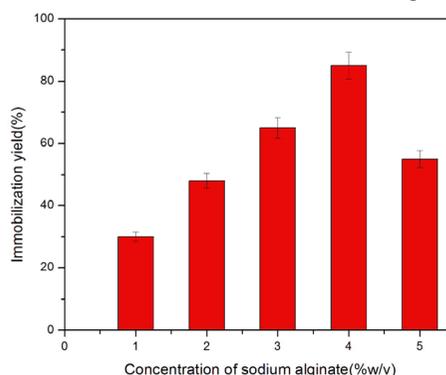
CONCLUSION

The α -amylase was immobilized by entrapment in calcium alginate beads with 85% enzyme activity. Immobilized enzyme has shown greater stability in high temperature i.e. 60°C, pH 6 and 10 minutes incubation time. The enzyme immobilization in alginate is simple, cheap and safe. Since the alginate is naturally occurring, nontoxic and biodegradable, the present investigations are useful in the development of a general approach for starch hydrolysis in food industry.

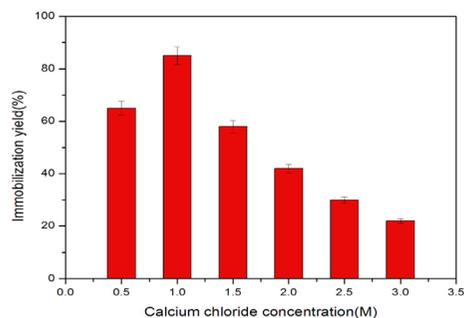
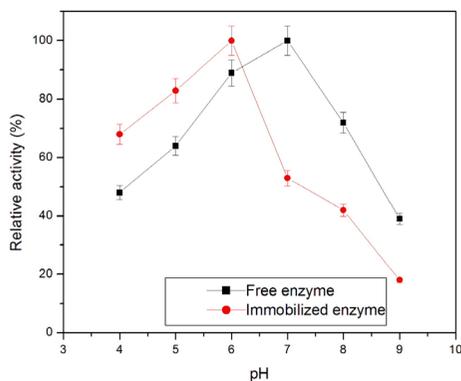
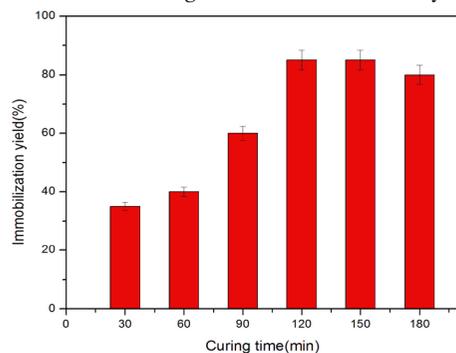
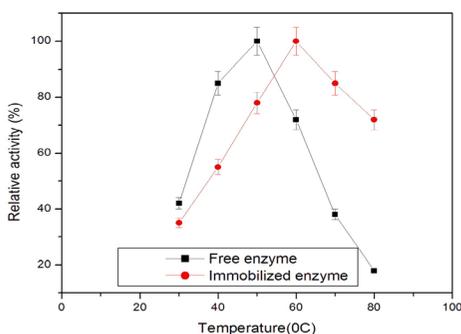
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Figure 1. Effect of concentration of sodium alginate on im-



mobilization yield

Figure 2. Effect of calcium chloride on Immobilization yield**Figure 3. Effect of curing time on immobilization yield****Figure 4. Effect of pH on activity of free and immobilized α -amylase****Figure 5. Effect of temperature on activity of free and immobilized α -amylase**

REFERENCE

- Maldonado HG and Lopez OP, Amylolytic enzymes and Products derived from starch : a review, Critical Reviews in Food Science and Nutrition.1995; 35(5): 373–403 |
- Gupta R, Gigras P, Mohapatra, Goswami VK, Chauhan B. Microbial Amylases: a biotechnological perspective. Process Biochem. 2003; 38: 1599-1616 |
- Kandra L. Amylases of medical and industrial importance. Journal of molecular structure (Theochem) 2003; 666-667:487-498 |
- Rajagopalan G, Krishnan C. Alpha amylase production from catabolite derepressed Bacillus subtilis KCC103 utilizing sugar cane bagasse hydrolysate. Bioresour Technol. 2008; 99: 3044-3050 |
- Gerhartz, Wolfgang. (1990). Enzymes in Industry. Weinheim, FRD. |
- Pandey A, Soccol CR and Thomaz Soccol V. Biopotential of immobilized amylases. Indian Journal of Microbiology. 2000; 40: 1-4 |
- LincoYY,Saarinen P and Linco M, Starch conversion by soluble and immobilized alpha-amylase. | Biotechnol.Bioeng.1975; 17:153-159 |
- Dumitriu S and Popa M. Bioactive polymers 29 Immobilization of alpha -amylase on | Biozan-R,Br. Polym. J. 1985; 17: 56-59 |
- Zanin GM, Kambara LM, Calsavara LPV and De Moraes FF. Performance of fixed and fluidized bed reactors with immobilized enzyme. Appl. Biochem. Biotechnol,1994; 45/46: | 627-640 |
- Cong L, Kaul R, Dissing U and Mattiasson B. A moderm study on euorig and Poly ethyleneimine as soluble carriers of alpha - amylase for repeated hydrolysis of starch. | J.Biotechnol,1995; 42:75-84 |
- Kurakake M, Ueki M, Hashimoto S and Komaki T, Adsorption of alpha amylase on dextrin | immobilized on kieselguhr or chitin, Carbohydr. Polym. 1997; 34:54-59. |
- Aksoy S , Tumturk H and Hasirci N. Stability of alpha amylase immobilized on poly (methyl methacrylateacrylic acid) microspheres. J. Biotechnol.,1998; 60: 37-46. |
- Chen JP, Sun YM, and Chu DH, Immobilization of alpha-amylase to a composite tempera | ture-sensitive membrane for starch hydrolysis. Biotechnol. Prog. 1998; 14: 473-478. |
- Tanyolac D, Yuruksoy BI and Ozdural AR, Immobilization of a thermostable alpha-amylase, | TermamyI onto nitrocellulose membrane by cibacron blue F3GA dye binding. | Biochem. Engg.J. 1998; 2:179-186. |
- Tien CJ, and Chiang BH, Immobilization of alpha amylase on a zirconium dynamic | membrane , Process. Biochem , 1999, 35: 377-383. |
- Al-Ghaffar MA, and Hashem MS, Immobilization of alpha -amylase onto chitosan and its amino acid condensation adducts. J. Appl. Polymer Sci., 2009; 112: 805-814. |
- Turunc O, Kahraman MV, Akdemir ZS, Kayaman N -Apoohan and Gungo A, Immobilization | of alpha amylase onto cyclic carbonate bearing hybrid material, Food Chem, 200;112: | 992-997 |
- Kennedy JF, Enzyme technology In Biotechnology; Kennedy J.F,Cabral J.M S, Eds, | VCH Publ.-Verlagsgesellschaft mbH: Weinheim, Germany, 1987: 7a |
- Dey GB. Singh, R. Banerjee, Immobilization of alpha amylase produced by Bacillus | circulans GRS313, Braz.Arch. Biol.Techol. 2003; 46(2):167-176 |
- Miller GL, Use of dinitrosalicylic acid reagent for determination of reducing sugars. | 21. Longo MA, Novella IS, Garcia LA and Diaz M. Diffusion of proteases in calcium | alginate beads. Enzyme Microb. Technol.1992; 14: 586-590. |
- Zaborsky OR. (1973). Entrapment within crosslinked polymers. In: Immobilized enzymes. | CRC Press. pp. 83-91. |
- Dey G, Nigpal V, Banerjee R, Appl Biochem and Biotech. 2002; 103(1-3): 303-314. |
- Roig M.G., D.H. Rashid and J.F. Kennedy. High-alkaline protease from Bacillus | PB92 entrapped in calcium alginate gel: Physicochemical and microscopic studies. | Appl. Biochem. Biotechnol. 1995; 55: 95-121. |
- Anwar A, UI Qader SA, Rajj A, Iqbal S and Azhar A. Calcium Alginate: A Support | Material for Immobilization of Proteases from Newly Isolated Strain of Bacillus Subtilis | KIBGE-HAS. World Appl. Sci. J. 2009; 7 (10): 1281-1286. |
- Talekar S, Ghodake V, Kate A, Samant N, Kumar C and Gadagkar S. Preparation | and characterization of crosslinked enzyme aggregates of Saccharomyces cerevisia | invertase. Aust. J. Basic Appl. Sci. 2010; 4:4760-4765. |
- Prakash O. and Jaiswal N. Immobilization of a Thermostable "Amylase on Agarose | and Agar Matrices and its Application in Starch Stain Removal. World Appl. Sci. J., | 2011; 13 (3): 572-577 |
- Konsoula Z. and Kyriakides ML. Starch hydrolysis by the action of an entrapped in | alginate capsules á-amylase from Bacillus subtilis. Process Biochem. 2006; 41: 343-349. |