International Journal of Biotechnology Research Vol. 3(5), pp. 081-086, September 2015
Available online at http://academeresearchjournals.org/journal/ijbr
ISSN 2328-3505 ©2015 Academe Research Journals

Full Length Research Paper

Immobilization of α-amylase in operationally stable calcium-alginate beads: A cost effective technique for enzyme aided industrial processes

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Accepted 24 September, 2015

Enzyme immobilization is the limitation of enzyme mobility to a confined area in order to increase the operational stability of enzyme. Among various techniques of immobilization, entrapment is the one that results in the retention of active enzyme confirmation with high immobilization efficiency. In the present work, α-amylase produced from *Bacillus licheniformis AR-1* was immobilized in calcium-alginate beads with the aim of reducing the cost of enzyme aided industrial processes. Kinetics of calcium-alginate entrapped α-amylase was investigated and compared with that of the soluble enzyme. It was observed that following immobilization, time course for maximum enzyme-substrate reaction was increased by 5 min as compared to soluble enzyme. Substrate requirement of immobilized α-amylase was also increased and 2 gm% starch was found to be the optimum substrate concentration for entrapped α-amylase activity. Immobilized α-amylase showed no change in optimum pH as compared to free enzyme, whereas optimum temperature of α-amylase activity was shifted from 50 to 55°C. Immobilized α-amylase was also checked for its reusability and it was found that calcium-alginate entrapped enzyme could be reused for up to four repeated cycles.

Key words: α-amylase, calcium-alginate, *Bacillus licheniformis AR-1,* immobilization.

INTRODUCTION

Enzyme immobilization refers to the techniques used to restrict the mobility of enzyme in a defined area. It has become an exciting and enormous aspect of Biotechnology especially if the enzymes of industrial importance are expensive and difficult to produce (Ameel, 2012). For this purpose, three principle techniques are applied namely, matrix entrapment, adsorption on a solid support and ionic or covalent binding. Selection of technique is based on the type of process in which enzyme is to be used. Entrapment is the most preferable technique due to its simplicity and very low structural modification of enzyme (Nisha et al., 2012). In entrapment, the enzymes or cells are not directly attached to the support surface, but retained inside the polymer matrix. Entrapment is carried out by mixing the biocatalyst into a monomer solution, followed by polymerization initiated by a change in temperature or by a chemical reaction. Several natural and synthetic polymers like polyacrylamide, agar, cellulose acetate, calcium alginate or carrageenan, etc., are used as the matrices. Alginate has been used especially for immobilization of enzymes used in food industry due to its inertness towards the enzyme and its use in different food items (Hari et al., 1996). Immobilized enzyme has numerous benefits over free enzyme catalysis, especially the increased storage, thermal and operational stability of enzyme (Cakmakci et al., 2014; Basturk et al., 2013).

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α-amylase (E.C. 3.2.1.1.) is a hydrolytic enzyme that cleaves α-1,4 glycoside linkages in starch, yielding dextrin, oligosaccharide, maltose and glucose (Crabb and Mitchinson, 1997). It is one of the most extensively used enzymes in various industries like pharmaceutical, food, textile, paper, detergent and fuel ethanol production industry (Soleimani et al., 2012; Ashwini et al., 2011). The usage of immobilized α-amylase in these industries would positively affect their economy as the single batch of immobilized enzyme can be reused for several repeated cycles (Kayastha and Das, 1999) that will ultimately reduce the cost of the overall enzyme aided processes (Morais et al., 2013). Therefore in this study, we aimed to entrap α-amylase in calcium-alginate beads and to optimize and compare the kinetics of free and immobilized α-amylase.

MATERIALS AND METHODS

Materials

In this study, sodium alginate (3%), calcium chloride (0.2 M), soluble starch (2 gm %) from Sigma Chemicals, USA, and crude α-amylase were used.

Enzyme production

Bacillus licheniformis AR-1 used in this study was previously isolated in our laboratory. It was used to produce α-amylase in a medium containing (gm/L): 10.0 starch, 5.0 peptone, 2.0 yeast extract, 0.5 sodium chloride, 0.15 calcium chloride and 0.5 magnesium sulphate. The fermentation was carried out at 50°C for 24 h. After 24 h, the cell free filtrate (crude enzyme) was collected by centrifugation of fermented medium at 10,000 rpm for 10 min at 0°C.

Immobilization of α-amylase

Cell free filtrate was initially mixed with 3 gm % sodium alginate in the ratio of 1:1. The mixture was filled in burette. Chilled $CaCl_2$ solution was taken in a beaker that was placed in ice bath. The mixture was allowed to drop from the burette into a gently stirred $CaCl_2$ solution from a height of about 2 cm. As soon as the drops entered the $CaCl_2$ solution, spherical beads of enzyme-entrapped calcium-alginate were formed. The beads were left in $CaCl_2$ solution for 30 min and then filtered and washed with deionized water thrice. Beads were then allowed to dry and used for subsequent analysis (Riaz et al., 2009).

Immobilized enzyme assay

Activity of immobilized enzyme was determined by taking 0.5 gm enzyme entrapped beads in a test tube with 1 ml (2 gm% starch) substrate and incubated at 50°C for 10 min. The immobilized α-amylase activity was determined by estimating the reducing sugar released by hydrolysis of starch using DNS method (Miller, 1959).

An immobilized enzyme unit is defined as “the amount of enzyme that liberates 1 µ mol of reducing sugar from starch per gram of beads under the assay conditions”.

Immobilized enzyme kinetics

Effect of incubation time on immobilized enzyme activity was determined by varying the time of enzyme-substrate reaction from 5 - 20 min with 1 ml of 2 gm% soluble starch under the standard assay conditions.

Maximum substrate concentration for immobilized enzyme activity was measured by reacting 0.5 gm enzyme beads along with different concentrations of starch ranging from 1-4 gm%.

The effect of pH on the activity of immobilized enzyme was measured by using phosphate buffer (50 mM) of different pH values ranging from pH 5-9. The influence of temperature on immobilized α-amylase activity was determined by incubating 0.5 gm enzyme beads with substrate at different temperatures ranging from 45 - 70°C.

In order to find out the reusability or operational stability of entrapped enzyme, the beads were removed from the reaction mixture after first reaction and washed thoroughly with deionized water thrice and then reused for the next enzyme-substrate reaction. Same process was repeated till the entrapped α-amylase showed no activity.

The enzyme activity of the first cycle was considered as 100% for estimating the residual activity.

RESULTS

Effect of incubation time on immobilized enzyme substrate reaction

Immobilized α-amylase showed maximum activity when incubated with substrate for 10 min showing an increase by 5 min as compared to free enzyme. Beyond 10 min, immobilized α-amylase activity was observed to decrease as shown in Figure 1.

Effect of substrate concentration on immobilized α-amylase activity

Optimum substrate concentration for immobilized α-amylase was observed to increase as compared to free enzyme. Immobilized α-amylase exhibited maximum activity with 2.0 gm % starch (Figure 2). As the substrate concentration was increased beyond 2.0 gm %, the activity was declined.

Effect of temperature on immobilized α-amylase activity

In the present study, immobilized α-amylase activity was

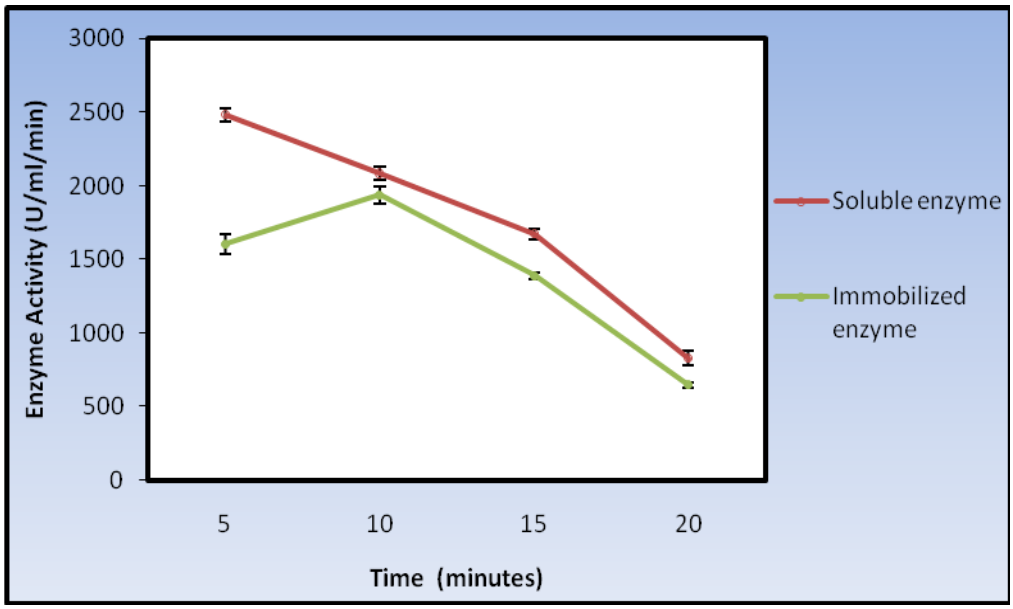

Figure 1. Time course for soluble and immobilized α-amylase activity. Values are plotted as mean of triplicate observations, while standard deviations are shown as standard bars.

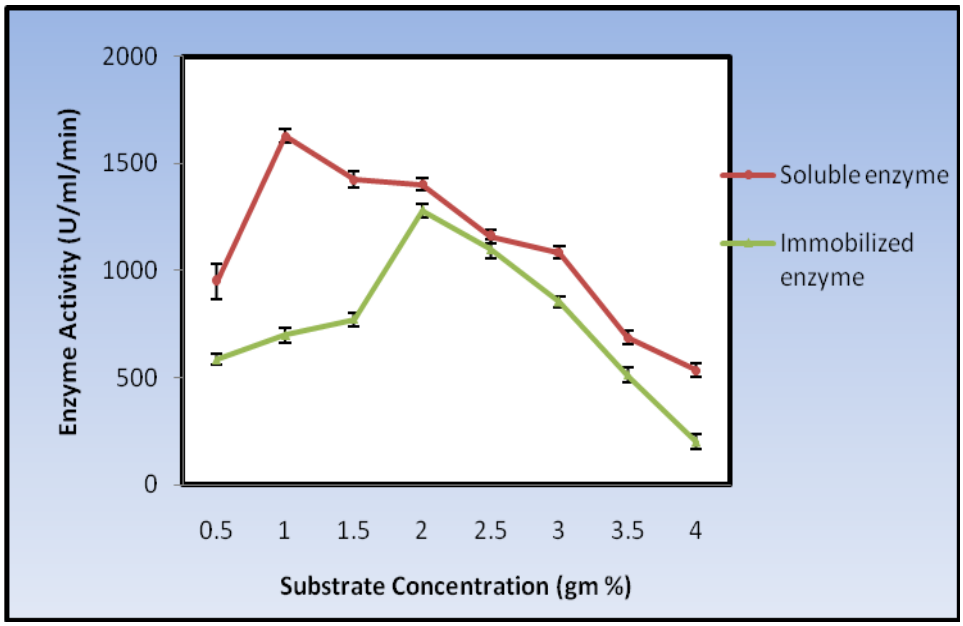

Figure 2. Effect of substrate concentration on soluble and immobilized α-amylase activity. Values are plotted as mean of triplicate observations, while standard deviations are shown as standard bars.

investigated at different temperatures and it was found that the optimum temperature for immobilized α-amylase in calcium-alginate beads was raised by 5°C and it exhibited maximum activity at 55°C (Figure 3) as compared to soluble enzyme which showed maximum activity at 50°C.

Effect of pH on immobilized α-amylase activity

pH is one of the most important factors that are capable of shifting enzyme activity in reaction mixture. It was observed that immobilization did not show remarkable effect on optimum pH of α-amylase. Figure 4 showed that

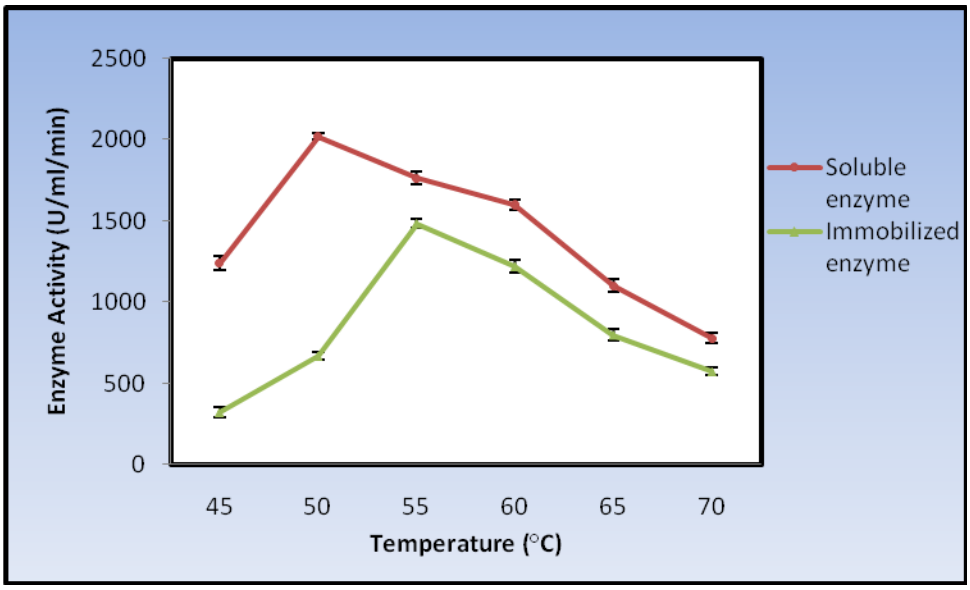

Figure 3. Effect of temperature on soluble and immobilized α-amylase activity. Values are plotted as mean of triplicate observations, while standard deviations are shown as standard bars.

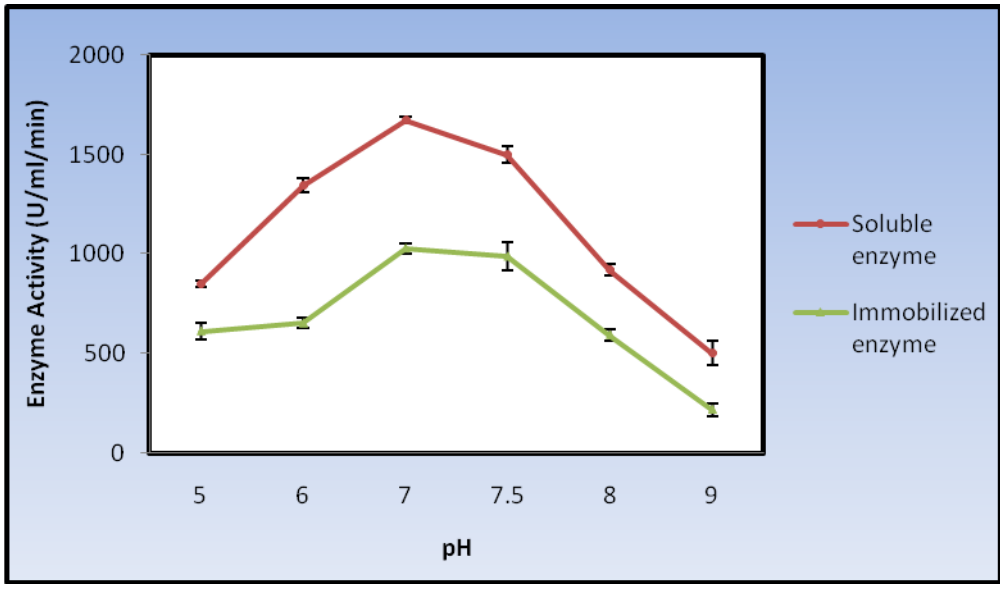

Figure 4. Effect of reaction pH on soluble and immobilized α-amylase activity. Values are plotted as mean of triplicate observations, while standard deviations are shown as standard bars.

the optimum pH for both soluble and immobilized α-amylase was 7.0.

Reusability or operational stability of immobilized α-amylase

In the present study, immobilized α-amylase showed 88% activity after first reuse and 54% activity after second reuse (Figure 5). Complete loss of activity was observed during the fifth repeated cycle.

DISCUSSION

Immobilization is the technique that is mainly applied to increase the thermal, storage and operational stability of industrial enzymes in order to reutilize the single batch of

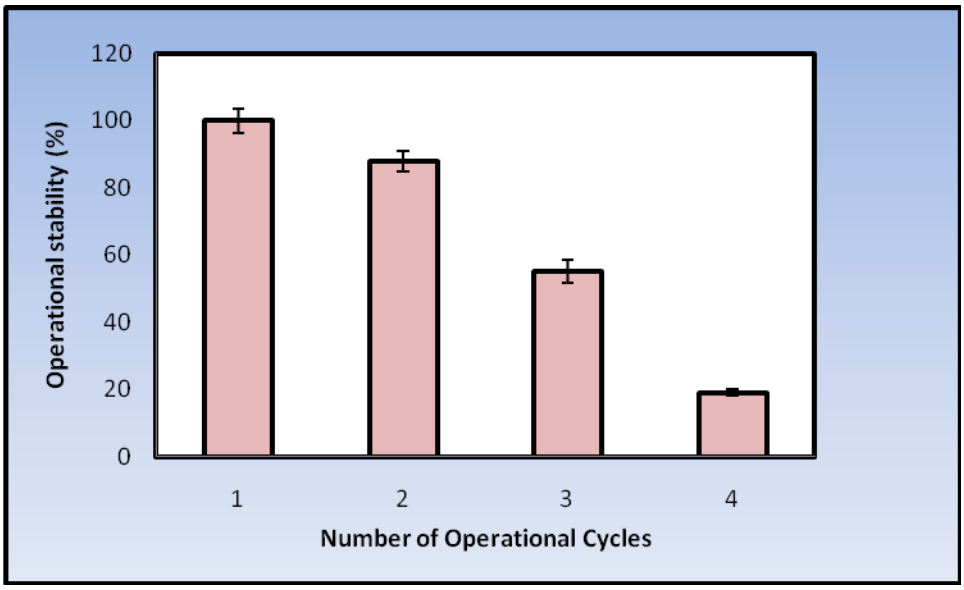

Figure 5. Operational stability of α-amylase following immobilization in calcium-alginate beads. Values are plotted as mean of triplicate observations, while standard deviations are shown as standard bars.

enzymes in high temperature processes (Sumitra et al., 2013). In the present study, α-amylase was produced from newly isolated strain of *Bacillus licheniformis AR-1*. The enzyme was then subjected to immobilization in calcium alginate beads and kinetics of free and immobilize α-amylase were estimated and compared.

Time course for maximum enzyme-substrate reaction was determined and noticed that immobilized α-amylase utilized 10 min for maximum reaction with starch molecules. That low activity of immobilized enzyme during the first 5 min might be due to the hindrance caused by the gel matrix with the diffusion of large starch molecules through the gel; therefore more time was required by the substrate molecules to reach the active site of enzyme (Konsoula and Liakopoulou-Kyriakides, 2005). Other investigators also reported the increased reaction time after immobilization (Qader et al., 2007; Mahajan et al., 2010).

Optimum substrate concentration for soluble enzyme activity was found to be 1 gm %. Following immobilization, substrate requirement was increased to 2 gm %. The increased requirement of substrate by immobilized enzyme might be due to the diffusion resistance faced by the large substrate molecules to enter inside the gel beads. Similar results were reported earlier (Dragomirescu et al., 2012; Anwar et al., 2009), whereas Hulya et al. (2008) reported that the Km value of α-amylase was decreased after entrapment in calcium-alginate beads.

To characterize the free and immobilized enzyme, the effects of temperature and pH were studied. The results showed increase in optimum temperature of immobilized α-amylase which might be due to high stability of gel and lower temperature inside the bead which resulted in a shift of optimum temperature towards the higher side (Kennedy and Kabral, 1987). Similar results were reported by Ichikawa et al. (2002) that optimum temperature of entrapped enzyme was raised from 60 to 80°C following immobilization. However pH of both free and immobilized α-amylase was found to be 7.0. The increase in optimum pH following immobilization has also been reported (Sharma and Tripathi, 2013; El-Banna, 2007) in contrast to those who reported no change in optimum pH before and after immobilization (Arya and Srivastava, 2006; Naganagouda and Mulimani, 2006). The shift in optimum pH could be due to the modification of ionization status of active site amino acids of enzyme protein (Taleker et al., 2010).

Reusability of immobilized enzyme is the most important factor that increases its applicability in industry as compared to free enzyme because the high cost for producing low amount of enzyme is the major problem faced by industrialists (Hulya et al., 2008). In the present study, entrapment in calcium alginate beads was proved to be the valuable method due to its simplicity and reusability of enzyme for four repeated cycles. Immobilized enzyme activity was lost during fifth cycle of repeated use which may be due to the enzyme denaturation or leakage from the beads. Operational stability of immobilized enzyme for four repeated cycles was also reported by Anwar et al. (2009) who immobilized proteases in calcium-alginate beads. Repeated use of entrapped amylase for 10 cycles was also reported by other investigators (Erten et al., 2007; Taleker and

Sandeep, 2012).

ACKNOWLEDGEMENT

The authors are thankful to Jinnah University for Women, Karachi, Pakistan for providing the funds for this research work.

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