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Immobilization of Amylase by Entrapment Method in Different Natural Matrix

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ABSTRACT

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. It is a very effective alternative for gripping the problems of instability, repetitive use and reduction in the cost of enzyme. The aim of this research was to obtain the optimum condition of the making of immobilized amylase beads using a different natural matrix. The result obtain that amylase producing isolate i.e. isolated from hot water spring and was precipitate with ammonium sulphate with specific activity of 45.29 IU/mg and 1.43 fold purification. The purified amylase was immobilized by entrapment method with 3% concentration of sodium alginate and agar with immobilization yield of 72.18% and 34.89% for isolate MW2 and 0.25% chitosan concentration gave 66.45% immobilization yield. Hence, we propose that, this method can be used to produce immobilized amylase which can be used in various areas such as diagnostics, food, medicine and cosmetics.

Introduction

Among the enzymes, amylase are most widely used industrial enzyme that exhibit great significance having approximately 65% of the world enzyme market (Ali *et al.,* 2017) Amylases have attracted global enzyme market due to their vast applications in starch processing, detergent, alcohol, textile, food, paper and pharmaceutical industries (Mageswari *et al.,* 2012 and Couto and Sanroman, 2006). The α- amylase (EC 3.2.1.1) are extracellular enzymes which catalyzes random cleavage of the α-1,4 glycosidic bonds between adjacent glucose molecules inside the linear amylose chain of starch. The amylases can be obtained from various natural resources such as plants, animals and microorganisms (Saranraj and Stella, 2013). The microbial production of amylase is more effective than the other sources as the technique is easy, cost effective, consistent and fast which can be modified to obtain enzymes of desired

characteristics (Tanyildizi *et al.,* 2005). The major concern in an enzymatic process is the instability of the enzyme under repetitive use and inhibition by high substrate and product concentration. Immobilization is a very effective alternative in overcoming problems of instability and repetitive use of enzymes. The term 'immobilized enzymes' refers to 'enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities which can be used repeatedly (Tosa,1966). Immobilization provides a separation of enzyme from the product which minimizes the protein contamination (Saifuddin *et al.,* 2013). It also remarkably reduces the cost of enzyme and enzymatic products. Immobilization of enzyme by attachment to a matrix imparts rapid arrest of the reaction by removal of the enzyme from the reaction solution and improvement of enzyme stability against temperature, solvents, pH, contaminants and impurities (Tian *et al.,* 2009). It also helps in efficient recovery and reuse of expensive enzymes (Sheldon, 2007).

Generally, enzymes are immobilized by physical adsorption, ionic binding, covalent binding, cross linking, encapsulation and entrapment methods (Hassan *et al.,* 2016). Entrapment is defined as an irreversible method of enzyme immobilization where enzymes are entrapped in a support or inside fibers, either in lattice structure of a material or polymer membranes that allow the substrate and products to pass through but retains the enzyme (Klotzbach *et al.,* 2008). The nature of the solid support or matrix plays an important role in retaining the actual confirmation and activity of enzyme in the processes that utilize immobilized biocatalysts (Riaz *et al.,* 2009). Mostly, natural polymers such as alginate, chitosan and agar are widely used in enzyme immobilization because the gel formation with these polymers occurs at mild conditions, with low cost. (Devi *et al.,* 2012).Typically, entrapment can improve mechanical stability and minimize enzyme leaching. The enzyme does not chemically interact with the polymer. Therefore, denaturation is usually avoided (Shen *et al.,* 2011).

Therefore, the present study was mainly focused on amylase produced from MW2 isolated from hot water spring and immobilization by entrapment method in different natural matrix.

Materials and Methods

Amylase producing bacterial isolate i.e. MW2 was isolated from hot water spring of Manikaran, Kullu, Himachal Pradesh.

Production and Partial Purification of Amylase

The culture was inoculated in standardized enzyme production media. The flasks were incubated at 45±2°C for 72 h. The culture contents of the MW2 were centrifuged at 10,000 rpm for 10 min at 4°C. Cell free supernatant as crude enzyme extract thus obtained was collected. The cell free crude extracts of the enzyme was subjected to sequential ammonium sulphate saturations and dialysis, concentrated enzyme was kept at 4ºC for further application.

Immobilization of partial purified amylase

Immobilization by using sodium alginate (Rajagopalan and Krishnan, 2008)

The immobilization of enzyme was done by using sodium alginate. In this method 1, 2, 3 and 4% solution of sodium alginate was prepared in 0.1 M phosphate buffer (pH 7). After cooling down to room temperature, 1ml of enzyme stock solution was mixed with 9

ml of sodium alginate solution. The mixture was then suspended drop wise into pre-chilled 0.1, 0.2, 0.3, 0.4 or 0.5 M 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. These beads were then stored in 0.1 M phosphate buffer (pH 7.0) at 4°C. The filtered calcium chloride solution was collected for enzyme activity determination.

Immobilization by using agar (Matsunga *et al.,* 1980)

The immobilization of enzyme was done by using agar as material. In this method 0.5, 1, 2, 3 and 4% solution of agar solution was prepared in 0.1 M phosphate buffer (pH 7.0) by warming them at 50°C. After cooling down to room temperature, 1ml enzyme was mixed with 9ml agar solution (the total volume of matrix and enzyme mixture being 10 ml) and immediately casted on preassembled glass plates. After solidification at room temperature, the gel was cut into small beads of 5 x 5 mm size and washed several times before use to remove any enzyme attached to the gel surface the beads were stored in 0.1 M phosphate buffer (pH 7) and at 4°C. After immobilization, the retained activity and immobilization yield was calculated according to the following equation:

$$\text{Immobilized amylase activity (IU/g)} = \frac{\text{Enzyme activity of immobilized beads}}{\text{Quantity of beads}}$$

$$\text{Retained activity yield (\%)} = \frac{\text{Yield of immobilized enzyme}}{\text{Yield of free enzyme}} \times 100$$

$$\text{Immobilization yield (\%)} = \frac{\text{Total activity of enzyme in immobilized gel}}{A - B} \times 100$$

Where A is activity of free enzyme added and B is the activity of remaining enzyme in filtered calcium chloride solution.

Chitosan-covered beads (Zhau *et al.,* 2010)

After the beads were formed with above method, these beads was dipped in 0.25% solution of chitosan and kept under mild shaking for 30 min. The formed beads were recovered by filtration and thoroughly washed with distilled water. These beads were then stored in 0.1 M phosphate buffer (pH 7.0) at 4°C.

Results and Discussion

Amylase producing bacterial isolate i.e. MW2 was isolated from hot water spring of Manikaran, Kullu, Himachal Pradesh.

Production and partial purification of amylase

The isolate got precipitated with 0-80% ammonium sulphate with increased specific activity to 45.29 IU/mg. The fold purification was increased to 1.43 and with 76% purification for MW2 (Table 1). It was found that the dialysis further concentrated the amylase with specific activity of 58.62 IU/mg with 1.85 fold of purification.

Bukhari and Rehman (2015) purified *Bacillus subtilis* with ammonium sulphate precipitation (80%) and the purified amylase could be detected as a single band of 59 kDa by SDS polyacrylamide gel electrophoresis. Kohli *et al.,* (2016) also purified enzyme with 75 per cent ammonium sulphate with 21 fold

of purification. El-Kady *et al.,* (2017) isolated and purified thermophilic *Bacillus sp.* NRC12017. They purified alpha amylase by 60-80 per cent ammonium sulphate precipitation.

Immobilization of Partial Purified Amylase

Immobilization by using sodium alginate

Immobilization of amylase with 3% concentration of sodium alginate gave maximum activity of 87.78 IU/g with highest yield of 72.18% [Fig. 1(a) and Plate 1(a)]. With the increase in concentration of sodium alginate there was increase in the immobilization yield. However the reduction in yield was noticed beyond 3%. It was also found that at 1% sodium alginate no beads were formed. Several workers have used calcium alginate for immobilization of enzymes. Alginate is the one of supporting matrix that can be used for immobilization of enzyme. The main advantages of this matrix are non-toxic, high stability, high porosity, simple procedure for immobilization and relatively cheap (Anwar *et al.,* 2009). 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 beads (Riaz *et al.,* 2009).

Immobilization by using agar

Different concentration (0.5-4%) of agar for entrapment of partial purified amylase for immobilization in which 3% of agar concentration gave highest immobilization yield of 34.89% for MW2 [Fig. 1(b) and Plate 1(b)]. At lower concentration (0.5%) of agar no cubes were formed and subsequently yield increased with the increase in concentration of agar. However, at 4% concentration the agar started solidifying before the addition of enzyme and gave low activity and yield.

Agar is another natural polymer used as matrix for the immobilization of enzymes. It is acid stable and shows no reactivity with protein. It is less costly as compared to other materials (Prakash and Jaiswal, 2011). Sharma *et al.,* (2014) reported that at the lower concentration of agar, calcium chloride and shorter hardening time, beads get ruptured during decantation and washing of beads. They also reported that calcium agar beads prepared with 3% (w/v) agar and 75 mM calcium chloride and hardened for 20 min were physically stabile with entrapment efficiency (80%).

Chitosan-covered beads

The immobilization yield in alginate-chitosan [0.25% (w/v)] covered beads was 66.45% for MW2 (Plate 1(c)). It was evident from the data that the chitosan covered beads lowered the activity and immobilization yield for both the isolates. Chitosan is a linear polysaccharide which is used as the external layer of the immobilized beads. Alginate and chitosan which are polysaccharide biopolymers used in enzyme encapsulation (Zhou *et al.,* 2010). When calcium alginate is mixed with chitosan, a strong ionic interaction occurs between the amino groups of chitosan and carboxyl groups of alginate for the formation of a polyelectrolyte complex (PEC) which results in better mechanical properties of the support (Ngah and Fatinathan, 2008; Rodrigues, 2008; Shu and Zhu, 2002; Xu *et al.,* 2007). Oliveira *et al.,* (2018) also reported that lower yield with chitosan covered beads suggested that this may be due to the excessive amount of support causing lose accessibility of amylase to the substrate.

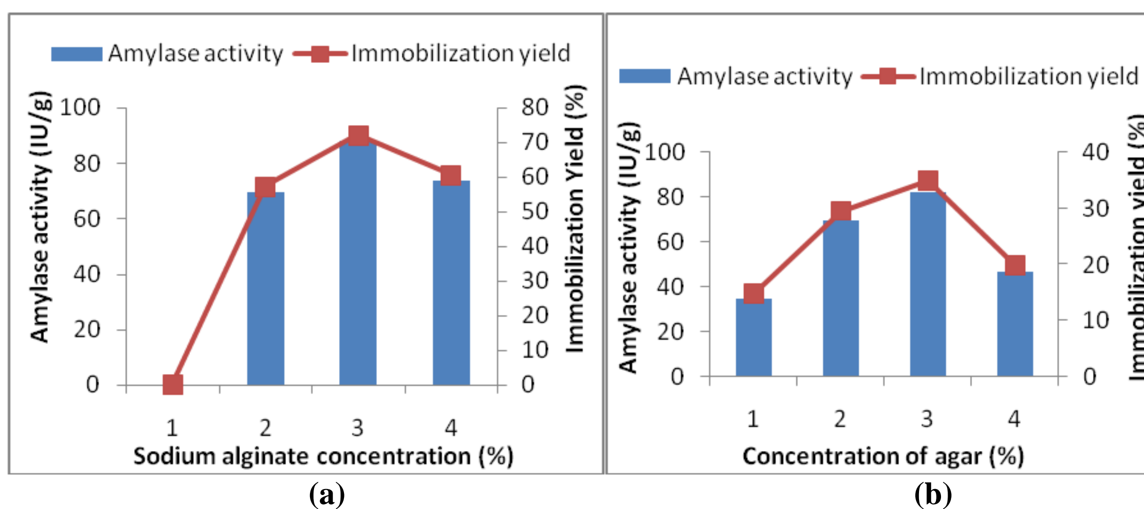
In conclusion, amylases are extensively used in industrial applications like starch modification and food processing. Amylase producing bacteria was isolated from hot water spring.

Table.1 Partial purification summary of amylase from bacterial isolate i.e. MW2.

Purification Step	Total Amylase activity (IU)	Specific Activity (IU/mg)	Fold Purification	Percent Purification (%)
Ammonium sulphate fractionation (0-80%)	263.16	45.29	1.43	76
Dialysis	235.69	58.62	1.85	68.07

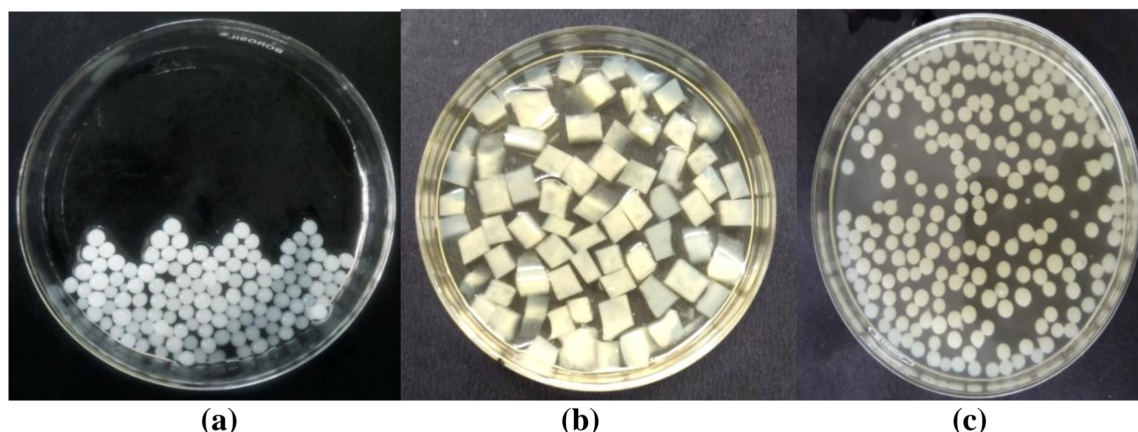
Total activity: Enzyme activity in given volume (IU)
Specific activity: Enzyme activity per unit protein concentration (IU/mg)
Purification fold: is increase in specific activity.
Percent purification: is remaining amylase activity as per cent of the initial amylase activity.

Fig.1 Effect of sodium alginate and agar concentration on amylase activity (IU/g) and immobilization yield (%)



(a) **(b)**

Plate.1 Beads formed by 3% sodium alginate (a), 3% agar (b) covering of 0.25% chitosan



(a) **(b)** **(c)**

The crude amylase was partially purified with ammonium sulphate fractionation with 68% recovery and immobilized by entrapment method on different matrices viz. alginate, chitosan and agar. Amylase immobilized in alginate matrix was found best matrix with maximum immobilization yield 72.18%. It can be concluded that immobilized enzyme has potential to be explored in various starch and food industries.

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