Immobilization of protease in biopolymers (mixture of alginate-chitosan)

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ABSTRACT

The Protease enzyme breaks down protein into amino acids as its constructors. Protease immobilization onto appropriate support materials plays an essential role in various fields of technology including the food and detergent industries. Accordingly, improvement of protease immobilization has been highly regarded due to its applications in bio catalysis. Alginate and chitosan are natural polysaccharides that have been studied so extensively in enzyme immobilization.

In this research, the physical immobilization of proteases in alginate-chitosan beads showed satisfactory activity and stability. These beads were prepared by adding protease-alginate dropping into Chitosan and Calcium chloride solution. Then proteases enzyme encapsulated in alginate-chitosan beads. In the end, the different conditions such as temperature, pH and stability of the enzyme were studied.

The immobilized protease was optimized in temperature of 47°C and at pH 8.5. The results demonstrated that the protease enzyme immobilized in alginate-chitosan beads exhibits reasonable stability and good activity.

Keywords: Protease enzyme; Biopolymer; Alginate; Chitosan; Immobilization; Stability.

INTRODUCTION

Enzymes are biological catalysts that facilitate the conversion of substrates into products by providing favorable conditions that lower the activation energy of the reaction. An enzyme may be a protein or a glycoprotein and consists of at least one polypeptide moiety [1]. Serine alkaline proteases (SAP) are one of the most important groups of industrial enzymes that are widely used in detergent, leather and meat industries. They account for approximately 35% of the microbial enzyme sales [2]. Immobilization is a general term describing a wide variety of the cell or particle attachment or entrapment in spherical beads [3]. It can be applied to basically all types of biocatalysts including enzymes, cellular organelles , animal and plant cells. Currently, different kinds of immobilization techniques have wide applications not only in the field of biotechnology, but also in pharmaceutical, environmental, food and biosensor industries [4]. Their use as detergent additives still represents the largest application of industrial enzymes, both in terms of volume and value[5]. All detergents contain similar ingredients and are based on similar detergency mechanisms. To improve detergency, modern types of heavy-duty powder detergents and automatic dishwasher detergents usually contain one or more enzymes [6]. Immobilization is generally necessary for optimum performance in non-aqueous media. In the traditional method of using enzymes as lyophilized (freeze-dried) powders, many of the enzyme molecules are not readily accessible to substrate molecules [7]. Immobilization can also help to enable the use of enzymes in different conditions such as chemical solvents, pH, temperature and exceptionally high substrate concentrations [8]. Covalent binding is a conventional method for immobilization; it can be achieved by direct attachment with the enzyme and the material through the covalent linkage. Covalent method of immobilization is mainly used when a reaction process does not require enzyme in the product, this is the criteria to choose covalent immobilization method [9]. Adsorption involves the physical binding of enzymes on the surface of an inert support [10].

Alginate is a natural polysaccharide that is synthesized by brown seaweeds and by soil

bacteria [11]. It is widely employed in the food processing industry [12]. Sodium alginate is the most commonly used alginate form in the industry, since it is the first byproduct of algal purification. Sodium alginate consists of α-L-guluronic acid residues (G blocks) and ß-D-mannuronic acid residues (M blocks), as well as segments of alternating guluronic and mannuronic acids (GM blocks) [13].

The calcium alginate matrix formed is usually very permeable and little or no drug release can actually be controlled in the case of soluble drugs [14]. Chitosan is a natural based-polymer obtained by alkaline deacetylation of chitin. This biopolymer is nontoxic, biocompatible, and biodegradable. These properties make chitosan a good candidate for the development of conventional and novel drug delivery systems. Chitosan has been found to be used as a support material for gene delivery, cell culture, and tissue engineering. However, practical use of chitosan has been mainly confined to the unmodified forms [15].

The present research was based on the entrapment of *Bacillus subtilisin calsberg* in calcium alginate-chitosan- mixture and the characteristics of immobilized enzyme such as activity and stability were assessed.

MATERIALS AND METHODS

Protease assay

Protease was purchased from sigma. Activity of protease enzyme was determined by Anson method [16]. In this method, 5.4 mg of enzyme was added in 1ml Tris-HCL buffer (pH=8.1) and mixed with 0.5 ml of casein solution.

The solution was incubated in a water bath at 37°C for 20-30 minutes. After that TCA (110mM) was added to stop the reaction. Finally the solution was mixed with 5ml of sodium carbonate and 0.5 ml of Folin's reagent for 25 minutes and the absorbance value was determined in 660nm.

Preparation of Alginic acid-Protease Enzyme Solution

First of all 200 mg of alginate was dissolved in 10 ml Tris-HCl buffer (0.1M pH 8.0) by heating at 30°C-40°C for 30 minutes. When the dissolving of all alginate particles completed, the liquid protease enzyme (0.5 mg/ml) was slowly added by stirring into the alginate solution. This mixture was stirred for 1 hour for complete homogenate of enzyme and alginate.

Preparation of Chitosan/CaCl2 Solution

500 mg of chitosan was added to acetic acid media (v/v, 2%) by heating at 50°C.

After gained a clear solution, $CaCl_2$(0.7 M) was added to the chitosan solution and was stirred at 50°C to 55°C.

Production of enzyme entrapped Alginate-Chitosan beads

In this study two phases were prepared. One of them is calcium chloride-chitosan and the other one is enzyme mixed with alginate solution. The protease-alginate mixture was added into chitosan-calcium chloride by means of a syringe.

Assay of immobilized protease enzyme

5.4 mg of immobilized enzyme was added in 1ml Tris-HCL buffer (pH=8.1) and mixed with 0.5 ml of casein solution. The solution was incubated in a water bath at 37°C for 30 minutes. After that TCA (110mM) was added to stop the reaction. The mixture was filtered by means of a filter paper. Finally the solution was mixed with 5ml of sodium carbonate and 0.5 ml of Folin's reagent for 25 minutes and the absorbance value was determined in 660nm.

Effect of different temperatures on immobilized protease enzyme

The immobilized protease activity was illustrated at different temperatures ranging from 20°C to 57°C in Anson method.

Scanning Electron microscope

The surface morphology of the immobilized enzyme in calcium alginate-chitosan beads are examined using a scanning electron microscope (LEO 44I).

Effect of pH on immobilized protease enzyme

The immobilized protease activity was considered by different pH values (7, 7.5, 8.5, 9 and 9.5) in Anson method.

RESULTS

In this project we studied the immobilized protease enzyme in various condition such as temperature, pH, stability.

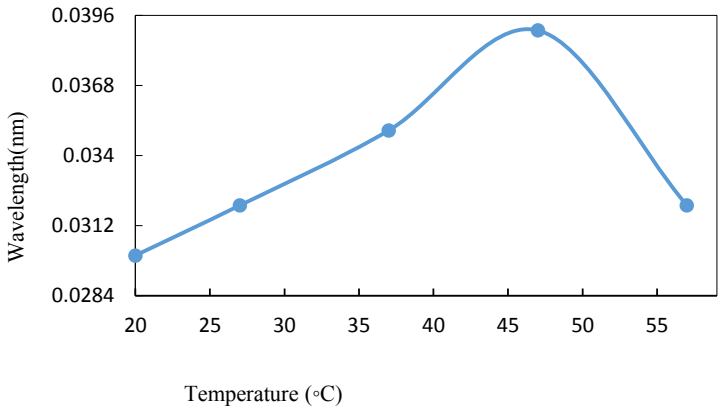

Figure 1. Effect of different temperatures on immobilized protease activity from *Bacillus subtilisin Carlsberg***.**

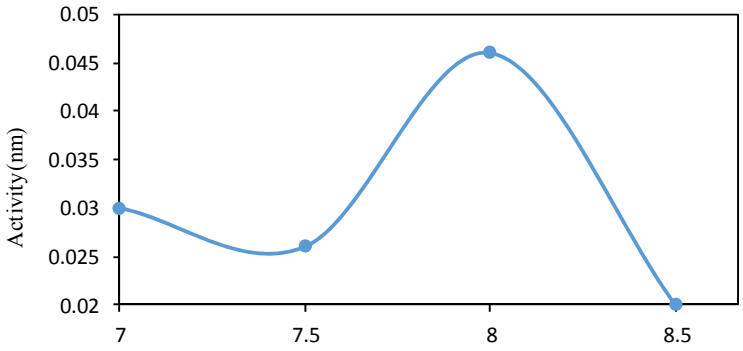

Figure 2. Effect of different pH on immobilized protease activity from *Bacillus Carlsberg.*

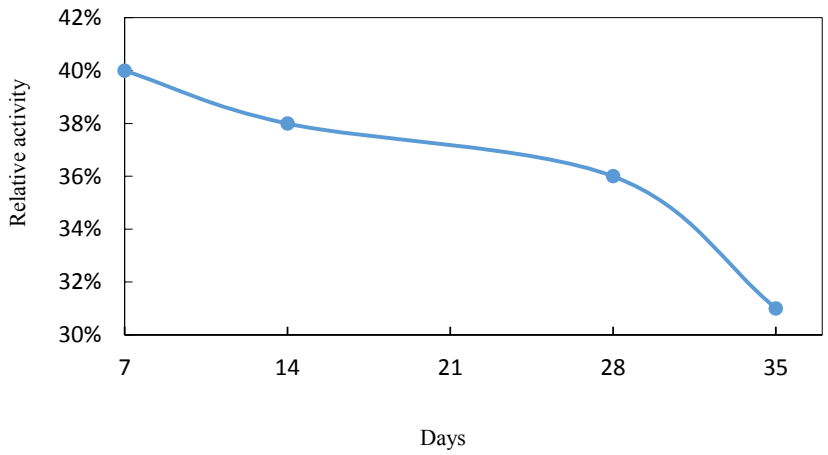

Figure 3. Decrease of immobilized protease activity from *Bacillus subtilisin calsberg* in 35 days.

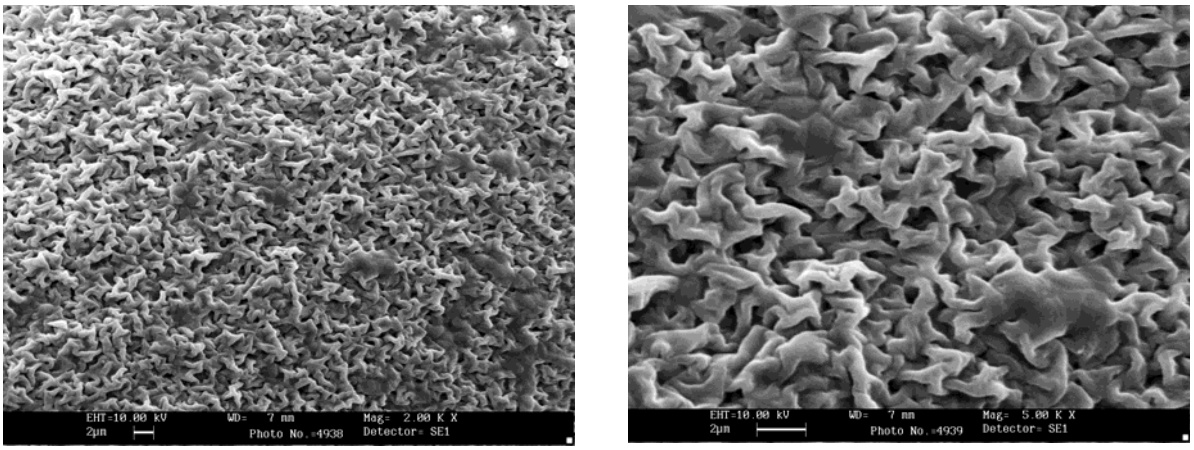

Figure 4. (a) Scanning electron with 2.00 kx mag. micrographs of calcium alginate-chitosan microspheres-Protease.**(b)** Scanning electron with 5.00 kx mag. micrographs of calcium alginate-chitosan microspheres-Protease.

Effect of temperature on stabilized protease enzyme

As it is shown in Figure 1 we reported that optimum temperature was 47°C.

Effect of pH on immobilized protease enzyme

Investigation of pH effect on immobilized enzyme activity was done at different pH values (7 , 7.5, 8.5, 9 and 9.5). The optimum pH was 8.5 (Figure 2).

Stability of encapsulated protease enzyme

The stabilization of immobilized enzyme has been studied in 5 weeks. In one week, the relative activity decreased up to 40%; then gradually, week by week, it mitigated up to 30% (in orderly, in the second week the relative activity reduced up to 36%. The week later, 31% and eventually, in the last week, the relative activity was observed 30%) (Figure 3).

The immobilized protease enzyme surface morphology

The immobilized enzymes morphology was studied by using SEM (Fig 4a, 4b). The results demonstrated that the protease enzyme has stabilized in calcium alginate-chitosan-mixture has a compact structure.

DISCUSSION

In general, high temperature enhances the rate of an enzyme's activity, because at high temperatures, molecules move around faster, so an enzyme is likely to come in contact with a substrate very quickly. In 2011, the effect of temperature on immobilized Glucose Oxidase in Alginate-Chitosan Microcapsules has been studied [17]. In 2009 some scientists surveyed immobilized protease enzyme in different temperatures [18]. They reported that optimum temperature was 50°C when enzyme entrapped in alginate beads alone (without chitosan). In this investigation, the absorbance value concerning immobilized enzyme activity was recorded in five temperatures from 20°C to 57 °C. It was concluded that the temperature 47°C was identified as the most preferable and appropriate temperature for immobilized enzyme. The activity of enzymes was strongly relied on various pH, and each enzyme met the best activity range at certain pH value. In this study, among of the effects of pH on immobilized enzyme activity, five of them have been analyzed (at pH7, 7.5, 8.5, 9 and 9.5); hence, the most primary pH recognized was at 8.5 (Figure 2); While in 2009, the best pH for protease stabilized on alginate (without chitosan) was reported at 10.5 [10].

In this study we investigated alginate-chitosan surface with SEM .Many large hollow pores or multiple small hollow pockets were observed in the alginate matrix [19].

The size of the beads was measured by Scanning Electron Microscope. The diameter of each bead was measured at three different angles and averaged. 7 beads were used to give an average bead size. The average bead size measured by an optical microscope was 2.6±0.2, 1.8±0.2. The activities of protease entrapped in the beads decreased as the bead size increased all samples were coated with gold prior to observation. The surface of the coated beads has looked like a mesh, and has very compact structures [20].

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