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Improving the stability of cellulase by immobilization on modified polyvinyl alcohol coated chitosan beads

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Abstract

The application of cellulases in various industries demands highly stable enzymes, able to perform at extreme pH values and temperatures. In this study, improving the stability of the acid cellulase in the neutral pH range was aimed. For this purpose, modification planned to be done on polyvinyl alcohol (PVA) by maleic anhydride in contrast to many studies in which enzyme was modified. The chitosan beads were coated with polyanionic modified PVA and cellulase was immobilized on the modified PVA coated chitosan beads. As a result of this modification, the pH optimum of enzyme shifted from pH 4.0 to 7.0 and the immobilized cellulase beads showed better pH stability than free enzyme at neutral pH range. The activity yield of the immobilized cellulase was found to be as 87%, and it was found no change of the optimum temperature after immobilization.
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Keywords: Cellulase; Polyvinyl alcohol; Maleic anhydride; Epichlorohydrin; Chitosan

1. Introduction

Cellulases are enzymes that hydrolyze the β-(1–4) linkages in cellulose. They are produced as a multicomponent enzyme system comprised usually of three enzymes that act synergistically in the hydrolysis of cellulose: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and cellobiase (β-glucosidase, EC 3.2.1.21). The first two enzymes act directly on cellulose, yielding mainly cellobiose and glucose as the reaction products. The cellobiose is then hydrolyzed into glucose by cellobiase. Endoglucanases and cellobiohydrolases degrade soluble cellodextrins and amorphous cellulose. However, it is the cellobiohydrolases that degrade crystalline cellulose most efficiently [1].

Cellulases are commonly used in various industries, including the food, brewery and wine, agriculture, textile, detergent, animal feed, pulp and paper, as well as in research development [2,3]. The application of these enzymes in detergent, leather and paper industries demand identification of highly stable enzymes, able to perform at extreme pH values and temperatures [4].

Biotechnology of cellulases began in early 1980s first in animal feed followed food applications. During the last two decades, the use of cellulases has increased considerably, especially in textile, pulp and paper industries [5–7]. Cellulases can be used to give denim a worn, abraded look. This is a major application for industrial enzymes in the textile industry today [8]. Acid cellulases are still popular for finishing denim, especially from a cost perspective. But acid cellulases are aggressive, and although it produces high levels of abrasion, they also reduces the fabric's tensile strength and results in a high amount of redeposition, or back-staining. Neutral cellulases with a typical pH of 6.5 do not reduce the fabrics tensile strength or back-stain as severely as acid cellulases does. However, neutral cellulases are not aggressive as acid cellulases and typically commands a higher market price [9].

Chitosan is a copolymer of β-(1–4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose. This polycationic biopolymer is generally obtained by alkaline deacetylation from chitin, which is the main component of the exoskeleton of crustaceans, such as shrimps [10]. Chitosan's availability and its ability to be made into a variety of useful forms (i.e. films, fibers and beads as well as powders and solutions) and its unique chemical and biological properties make it a very attractive biomaterial for enzyme immobilization

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[11–13]. Polyvinyl alcohol (PVA) is a cheap synthetic polymer and non-toxic to microorganisms, has been used for cell and enzyme immobilization [14–16].

Tripolyphosphate (TPP) is a non-toxic polyanion which can interact with chitosan via electrostatic forces to form ionic cross-linked networks. TPP can be used for the preparation of chitosan beads and micro spheres because of its quickly gelling ability [17].

Epichlorohydrin (ECH) was selected as a convenient base catalyzed cross-linking agent. The advantage of using ECH as a cross-linking agent is that it does not eliminate the cationic amine function of chitosan, most notably, the cross-linking with ECH can considerably improve the wet strength of the chitosan films [18]. The aim of this work was to improve the stability of the enzyme in the neutral pH range by means of immobilization on maleic anhydride modified PVA coated chitosan beads. In this study, the chitosan beads were used as form maker and cellulase was immobilized on chitosan beads which are coated with maleic anhydride modified PVA membrane.

2. Materials and methods

2.1. Chemicals

Cellulase (from *Aspergillus niger*), chitosan (85% deacetylated), polyvinyl alcohol average molecular weight 30,000–70,000 cold water soluble, tripolyphosphate, epichlorohydrin were purchased from Sigma Chem. Co. (St. Louis, MO, USA); maleic anhydride, carboxymethyl cellulose (CMC) were obtained from Fluka Chemie AG (Switzerland). All other chemicals were obtained from local suppliers and were analytical grade or better.

2.2. Cellulase immobilization on modified polyvinyl alcohol coated chitosan beads

2.2.1. Preparation of cross-linked chitosan beads

Chitosan solution was prepared by dissolving 1.0 g chitosan flakes into 50 ml of 2% (v/v) acetic acid solution. The solution was dropped through a needle into a gently shaken tripolyphosphate solution. The TPP solution (2%, w/v) was prepared by dissolving sodium TPP (5 g) in 250 ml of deionized water and its pH value was adjusted to pH 8.2 by 1N HCl. The chitosan solution was dropped into the TPP solution and the gelled spheres formed instantaneously. After 4 h of hardening, the beads were separated from the tripolyphosphate solution by filtration.

Five grams of wet beads were put into a flask that contains 12.5 ml sodium hydroxide (pH 10.0) and cross-linking agent, epichlorohydrin (0.04 M) was added and then stirred at 50 °C for 6 h. The cross-linked chitosan beads were then washed intensively with distilled water to remove any unreacted epichlorohydrin.

2.2.2. Modification of polyvinyl alcohol with maleic anhydride

Two grams of polyvinyl alcohol was dissolved in 20 ml distilled water. The magnetic bar and the pH probe were put into the PVA solution flask placed in an ice bath to maintain the temperature at 4 °C. One gram of maleic anhydride was added slowly to the reaction mixture and pH was maintained at 9.0 by adding 1N NaOH. Final volume of the solution was recorded as 50 ml.

2.2.3. Preparation of modified polyvinyl alcohol coated cross-linked chitosan beads

Cross-linked chitosan beads were introduced in modified polyvinyl alcohol solution and incubated in water bath at 40 °C for 1 h. Then the beads were filtered and introduced in a 10 ml sodium hydroxide solution (pH 10.0) containing 0.015 M epichlorohydrin heated to 40 °C for 2 h. The cross-linked chitosan beads were then washed intensively with distilled water to remove any unreacted epichlorohydrin.

2.2.4. Immobilization of cellulase on modified polyvinyl alcohol coated chitosan beads

Modified polyvinyl alcohol coated chitosan beads were put into 2% (w/v) cellulase enzyme solution. The beads were incubated at 4 °C on the orbital shaker for 3 h. Then the beads were filtered and the volume of filtrate recorded. The filtrate was collected for loading efficiency determination. Then the beads were cross-linked with epichlorohydrin as stated above.

$$\text{Loading efficiency}\,(\%) = \frac{C_\text{i} V_\text{i} - C_\text{f} V_\text{f}}{C_\text{i} V_\text{i}} \times 100 \qquad (1)$$

where C_i is the initial protein concentration, V_i the initial volume of enzyme solution, C_f the protein concentration in the total filtrate and V_f the total volume of the filtrate.

2.3. Determination of cellulase activity and protein concentration

The hydrolytic activity of cellulase was measured by using a 1% (w/v) CMC as the substrate. Reaction mixture was incubated in a water bath at 50 °C for 5 min, the amount of generated glucose was measured by the spectrophotometer at 546 nm with DNS agent as a color indicator [19]. One unit of cellulase activity was defined by the amount of enzyme, which produced 1.0 μmol of reducing sugar from the substrate per minute. The amount of protein in the beads was determined from the loading efficiency. Immobilization yield was defined as follows:

$$\text{Immobilization yield}\,(\%) = \frac{a_\text{imm}}{a_\text{free}} \times 100 \qquad (2)$$

where a_imm is specific activity of immobilized enzyme (μmol/(min mg protein)) and a_free specific activity of free enzyme (μmol/(min mg protein)). Protein concentrations were measured with the Bradford method using standard protocol [20].

2.4. Characterization of the cellulase immobilized on modified polyvinyl alcohol coated chitosan beads

2.4.1. Effect of pH and temperature on the activity of free and immobilized cellulase

The optimum temperature of free enzyme and immobilized cellulase beads were determined by incubating the enzymes in a water bath at various temperatures (from 4 to 65 °C) for 5 min. The temperature stability of each enzyme was determined by incubating enzymes to a given temperature for 1 h and assaying the residual activity.

The optimum pH of free and immobilized enzyme beads were determined by carrying out the enzyme assay at different pH values using the following buffers: 0.1 M potassium phosphate/citric acid (pH 3.0–7.0); 0.1 M monopotassium dihydrogen phosphate–dipotassium hydrogen phosphate (pH 7.0–8.0); 0.1 M Tris–HCl (pH 8.0–9.0). The pH stability of each enzyme was determined at 4 °C in the above buffers and incubating for 1 h. Upon completion of the incubation period, the residual activities were determined by DNS method.

2.4.2. Reusability and operational stability of cellulase in modified polyvinyl alcohol coated chitosan beads

In order to test the reusability of cellulase in modified polyvinyl alcohol coated chitosan beads, the beads were used several times for the hydrolysis reaction. Each run lasted 5 min after which the beads were separated and washed with 0.1 M phosphate buffer (pH 8.0). The reaction medium was then replaced with fresh medium. The activity of freshly prepared beads in the first run was defined as 100%. The operational stability of the immobilized enzyme beads were determined in a continuous stirred batch system by measurement of the activities of samples taken at regular time intervals in fresh reaction medium under optimum conditions. The relationship between operation time and decrease in the enzyme activity was determined and also the half life ($t_{1/2}$) of the biocatalyst was calculated according the formula given as $t_{1/2} = 0.693/k_D$, $k_D = 2.303 \log(a_0/a)/t$, where t is the operational time, k_D the decay constant, a_0 and a are the enzymatic activities at the beginning and at t time.

2.4.3. Storage stability

The stability of free enzyme and immobilized enzyme beads at storage were monitored during 11 days at 4 °C, using 1-day intervals. The residual activity was measured as stated above.

3. Results and discussion

Enzymes may be inactivated by a variety of conditions such as temperature, pH, ionic strength, denaturating agent, pressure or mechanical forces. They occur naturally in the folded form which an inactivation causes reversible unfolding of enzyme. Reversible unfolding occurs when the non-covalent forces responsible for the three-dimensional structure of the enzyme are disrupted. Chemical modification is done to overcome this problem [21]. Chemical modification of amino groups of the enzyme with acid anhydrides of dicarboxylic acids are a simple method and may provide a very valuable strategy for giving proteins some new and useful characteristics related to the stability and catalytic effect. In addition, the chemical modification may be used to change the physical property, substrate specificity, or even the type of reaction catalyzed by a particular enzyme [22]. In this study, modification planned to be done on polyvinyl alcohol by maleic anhydride instead of enzyme/protein. In the case after immobilization of the enzyme on modified PVA, cellulase showed better pH and thermal stability. This can be explained by the immobilization of the enzyme on modified polyvinyl alcohol coated chitosan beads which are supposed to preserve the tertiary structure of the enzyme from the conformational changes caused by the environment.

Chitosan beads have porous structure. However, immobilization of cellulase on the surface of chitosan beads rather than in beads is important for overcoming of diffusion limitations because cellulose which is the substrate of cellulase is a macromolecule. Cellulase was immobilized on the surface of the chitosan beads that coated with the maleic anhydride modified PVA. Chitosan beads were used as form maker and cellulase was immobilized on chitosan beads which are coated with maleic anhydride modified PVA membrane because it was hard to make beads with PVA membrane. PVA is used in many enzyme immobilization studies [23–26]. But the enzyme immobilization on the modified PVA coated chitosan beads is used first in this study. The aim of the coating beads with maleic anhydride modified PVA was to make the bead surfaces polyanionic and to shift the optimum pH value of the enzyme to a neutral range.

Cellulase was immobilized on modified PVA coated chitosan beads as it described in material and methods. Immobilization yield was determined by the difference between the amount of initial protein in the incubation media and amount of protein in the washings. It was found as 60%. Immobilized cellulase was retained 87% of its initial specific activity (Table 1). Wu et al. reported that the activity of cellulase immobilized by electrospinning was over 65% of that free enzyme and the loading efficiencies (%) with different enzyme amounts were changing between 2.5 and 10 [27].

3.1. Effect of pH and temperature on the activity of free and immobilized cellulase

The optimum pH and pH stability of free and immobilized enzyme beads were determined by carrying out the enzyme assay at different pH values using the following buffers: 0.1 M potassium phosphate/citric acid (pH 3.0–7.0); 0.1 M monopotassium dihydrogen phosphate–dipotassium hydrogen phosphate (pH 7.0–8.0); 0.1 M Tris–HCl (pH 8.0–9.0). The pH stability of

Table 1
Cellulase immobilization on modified polyvinyl alcohol coated chitosan beads

	Activity (U)	Protein (mg)	Specific activity (U/mg)
Free enzyme	18	9	2
Immobilized enzyme (10 beads)	0.25	0.144	1.74

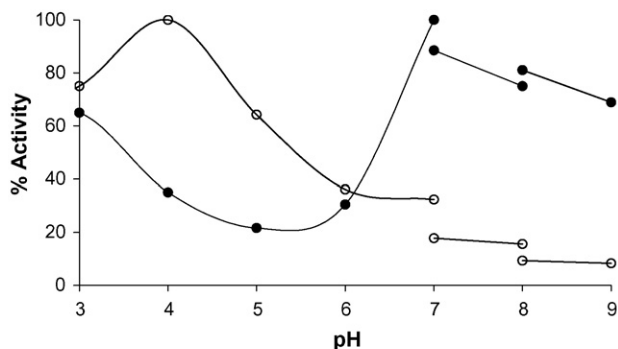

Fig. 1. Optimum pH of free (◯) and immobilized cellulase (●) 0.1 M potassium phosphate/citric acid (pH 3.0–7.0); 0.1 M monopotassium dihydrogen phosphate–dipotassium hydrogen phosphate (pH 7.0–8.0); 0.1 M Tris–HCl (pH 8.0–9.0).

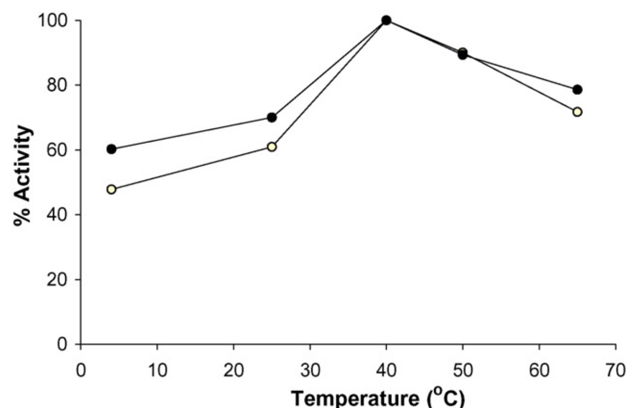

Fig. 3. Optimum temperature of free (◯) and immobilized cellulase (●).

each enzyme was determined at 4 °C in the above buffers and incubating for 1 h. The optimum pH curves for both native and modified cellulase were shown in Fig. 1. The native cellulase had a pH optimum at about 4.0 and the hydrolytic activity decreased sharply toward higher pH. The pH optima of the immobilized enzyme was shifted to neutral pH. This phenomenon might be caused by the polyanionic micro-environment surrounding the enzyme molecules [28]. The modified enzyme also showed a broaden pH optima compared with native enzyme. Immobilized cellulase displayed a greater stability at higher pH values Fig. 2.

The temperature dependence of the enzyme activity was studied in the temperature range of 4–65 °C. As shown in Fig. 3, optimum temperature of the free enzyme and the modified enzyme beads found to be 40 °C. Thermal stability of the free and immobilized cellulase were shown in Fig. 4. The modified enzyme beads showed better activity at higher temperatures than free enzyme after 1 h incubation.

3.2. Reusability and operational stability of cellulase in modified polyvinyl alcohol coated chitosan beads

The operational and storage (non-operational) stabilities of immobilized catalyst are important parameters, which can determine the economic viability of any biosynthetic process [29]. The operational stability of enzymes is one of the most important factors affecting the success of the applications of an immobilized system [30]. The reusability of immobilized cellulase was examined by measuring the activity repeatedly. The relative activity of the immobilized cellulase compared to its initial value in percentage decreased along with the reusing times and immobilized enzyme retained over 52% of its initial activity after eight cycles reuse. In the study of immobilization of cellulase nanofibrous PVA membranes by electrospinnig, cellulase retained over 36% of its activity after six cycles of reuse [27]. The operational stability of immobilized cellulase was studied for 90 min at 50 °C. The activity of the immobilized catalyst lost about 21% of the initial activity after 90 min of continuous operation. The operational half life ($t_{1/2}$) of immobilized cellulase beads were established as 266.5 min.

3.3. Storage stability

The storage stability of an enzyme is of significant importance for scheduling its application in a particular reaction. The immobilized and free enzymes were stored in distilled water for 11 days at 4 °C. The residual activity of the enzyme was determined as a function of time. It was observed that the free enzyme lost its 28% activity, whereas the immobilized enzyme had no significant activity looses within 11 days. The shapes and the particle

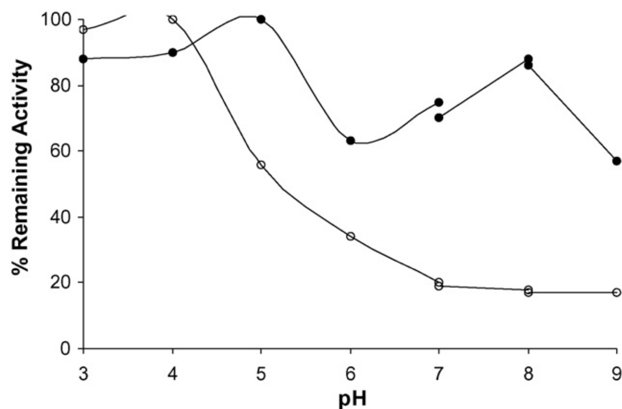

Fig. 2. pH stability of free (◯) and immobilized cellulase (●) 0.1 M potassium phosphate/citric acid (pH 3.0–7.0); 0.1 M monopotassium dihydrogen phosphate–dipotassium hydrogen phosphate (pH 7.0–8.0); 0.1 M Tris–HCl (pH 8.0–9.0).

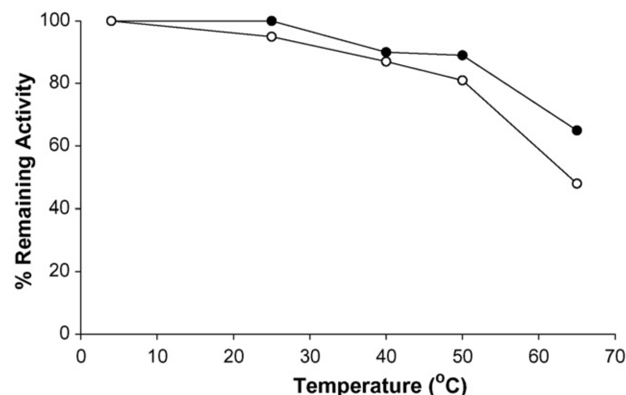

Fig. 4. Thermal stability of free (◯) and immobilized cellulase (●).

sizes of the beads were determined visually with a magnifying glass by measuring the increase in volume. The average diameter of cellulase immobilized modified PVA coated chitosan beads were found to be 0.82 mm. As compared to uncoated chitosan beads, they were 32% smaller, drop-like-spherical in shape and the mechanical strength was enhanced.

4. Conclusions

In the present paper, we described an effective immobilization method for cellulase. Chemically, with maleic anhydride modified polyvinyl alcohol was showed polyanionic character and used for chitosan beads coating. The cellulase immobilized on chitosan beads was showed increased pH stability when comparing to native enzyme. The pH range for catalytic activity was also increased after immobilization. The immobilized cellulase showed good storage and operational stability. After the immobilization acid cellulase became a neutral cellulase character.

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