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# *Penicillium* sp. ZD-Z1 Chitosanase Immobilized on DEAE Cellulose by Cross-linking Reaction

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**Abstract**–Chitosanase obtained from *Penicillium* sp.ZD-Z1 was immobilized on DEAE cellulose with glutaraldehyde by cross-linking reaction. The optimal conditions of immobilization were as follows: 0.1 g DEAE cellulose was treated with 5 ml 5% glutaraldehyde solution; then 2.3 mg chitosanase was immobilized on the carrier. The optimal temperature and pH was 60 °C and 4.0, and the $K_m$ value was 18.87 g/L. Under optimal conditions, the activity of immobilized enzyme is 1.5 U/g, and the recovery of enzyme activity is 81.3%. After immobilization, the optimal temperature and $K_m$ value increased (from 50 °C to 60 °C, from 2.49 g/L to 18.87 g/L), whereas the optimal pH was reduced (from 5.0 to 4.0). The enzyme activity loss was less than 20% after 10 times batch reaction; the immobilized enzyme showed good operation stability.

Key words: DEAE Cellulose, Immobilization, Chitosanase, Chitosan

## INTRODUCTION

Chitosan is one of the most abundant renewable polysaccharides prepared from chitin through chemical N-deacetylation. Chitosan has been developed into new physiological materials since it possesses antibacterial activity [Allan et al., 1979; Hadwiger et al., 1980; Walker-Simmons et al., 1983; Hirano et al., 1989; Uchida et al., 1989], hypocholesterolemic activity [Maezaki et al., 1993; Hirano et al., 1990; Sugano et al., 1980, 1992] and anti-hypertensive action [Okuda et al., 1997; Suzuki et al., 1986]. Increasing attention has recently been given to converting chitosan to their oligosaccharides. Chito-oligosaccharides possess additional functional properties such as antitumor activity [Suzuki et al., 1986, 1996; Tsukada et al., 1990], immuno-enhancing effects [Suzuki et al., 1992; Tokoro et al., 1988], enhancing protective effects against infection with some pathogens in mice [Yamada et al., 1993; Tokoro et al., 1989], antifungal activity [Hirano et al., 1989; Kendra et al., 1989], and anti-microbial activity [Hirano et al., 1989; Uchida et al., 1989]. Additionally, they have lower viscosity, low molecular weights, short chain lengths and are soluble in neutral aqueous solutions. Subsequently, they seem to be readily absorbed *in vivo* in contrast to chitosan and chitin. Chito-oligosaccharides can be prepared by chemical or enzymic depolymerization [Alla et al., 1999]. Enzymic hydrolysis is becoming a preferable method because of easy control, mild conditions of hydrolysis and low pollution to the environment.

In the previous study, a strain was screened to produce an extracellular chitosanase with high hydrolysis activity to chitosan in culture. In this work, this kind of chitosanase was immobilized on DEAE cellulose. The conditions of immobilization and characters of the immobilized enzyme were studied systematically.

## MATERIALS AND METHODS

### 1. Materials

Commercial chitosan with an average molecular of weight 290 kDa, 93% N-deacetylated was used (Zhejiang Yuhuan Aoxing Chitin Co. Ltd.). Chitosanase was prepared in our laboratory. All other chemical reagents were of analytic grade (Shanghai Chemical Reagent Co. Ltd.).

### 2. Preparation of Chitosanase

*Penicillium* sp. ZDZ1 was stored on agar slant at 4 °C. The microorganism was scraped off from the agar slant and washed out with sterilized water. Then 1 ml of microorganism solution was inoculated into a 250 ml flask containing 60 ml of the medium, and incubated on a rotary shaker at 180 rpm for 72 h at 30 °C. The composition of the medium (pH 4.5) was (w/v), chitosan 0.6%, amidoglucose 0.4%, $(NH_4)_2SO_4$ 0.1%, carbamide 0.1%, $KH_2PO_4$ 0.06%. The cells of the culture broth were removed from the medium by centrifugation at 2,500 rpm for 15 min at 4 °C and the supernatant was collected. The chitosanase was extracted by adding 20-70% ethanol into the collected supernatant, and the precipitates were collected by centrifugation at 5,000 g for 15 min. The precipitates were dissolved in an appropriate volume of distilled water and dialyzed against water overnight at 4 °C. Finally the partially purified enzyme was used for immobilization.

### 3. Immobilization of Chitosanase

DEAE cellulose was refined by using the method proposed by Mehta [Mehta et al., 1991]. 0.1 g refined DEAE cellulose was treated with 5 ml glutaraldehyde at a definite concentration and was stirred at room temperature for 24 h. The DEAE cellulose-glutaraldehyde compound was allowed to stand with occasional stirring for 24 h at 4 °C. The cellulose-glutaraldehyde compound was washed several times with distilled water until the washings were free of glutaraldehyde. Chitosanase was added to cellulose-glutaraldehyde complex and allowed to stand for 24 h at 4 °C occasional stirring. The supernatant was removed by centrifugation at 3,000 g. DEAE cellulose-chitosanase compound was washed with distilled water until protein in the washing solution could not be detected. The immo-

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bilized chitosanase was then collected and stored at 4 ℃.

**4. Measurement of Enzyme Activity**

4-1. The Activity of Free Chitosanase

The activity of free chitosanase was determined by a 3,5-dinitrosalicylic acid method [Sun et al., 1997].

4-2. The Activity of Immobilized Chitosanase

Immobilized enzyme 0.1 g was added into 5 ml 1% chitosan solution and incubated at 50 ℃ for 30 min. The reaction was stopped by adding 0.5 ml of 1 M NaOH solution. The mixture was centrifuged at 3,000 g for 10 min. The concentration of reducing sugar was determined by a 3,5-dinitrosalicylic acid method. One unit was defined as the amount of enzyme that could produce 1 mmol reducing sugar in 1 min.

## RESULTS AND DISCUSSION

**1. Immobilization of Chitosanase on DEAE Cellulose**

1-1. Effect of Concentration of Glutaraldehyde on Chitosanase Immobilization

0.1 g DEAE cellulose was treated with 5 ml different concentrations of glutaraldehyde ranging from 1 to 6%, and DEAE cellulose-glutaraldehyde was then treated with 2.76 mg chitosanase. As shown in Fig. 1; the optimal concentration of glutaraldehyde for immobilization was 5%. Enzyme activity increased with increasing concentration of glutaraldehyde. When the concentration of glutaraldehyde was higher than 5%, enzyme activity decreased due to the deactivation of the enzyme. Superfluous glutaraldehyde will result in the decrease of the enzymatic activity.

1-2. Effect of the Amount of Enzyme on Chitosanase Immobilization

Protein concentration is an important parameter which strongly affects the activity of the bound protein. 0.1 g DEAE cellulose-glutaraldehyde was treated with different amount of chitosanase, and the immobilized amount of enzyme ranged from 0.46 to 2.3 mg. The activity of immobilized enzyme was assayed and the results are shown in Fig. 2. An almost linear increase of enzymic activity (up to 2.3 mg) in the immobilizing mixture was accompanied with the increasing of added enzyme. Once the value of 1.4 U/g was achieved, enzymic activity tended to decrease. This was likely due to the formation of a dense protein layer which produced steric and/or diffusion barriers, thus limiting further access of the enzyme to the carrier.

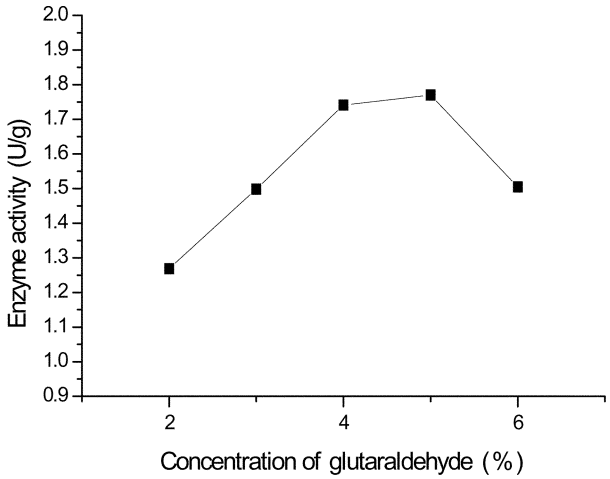


**Fig. 1. Effect of concentration of glutaraldehyde on chitosanase immobilization.**

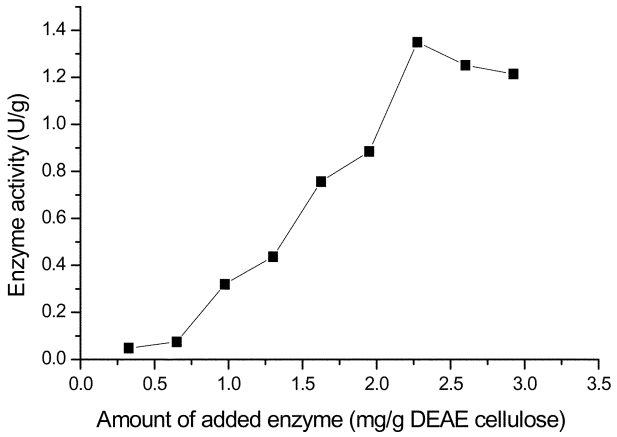


**Fig. 2. Effect of amount of added enzyme on chitosanase immobilization.**

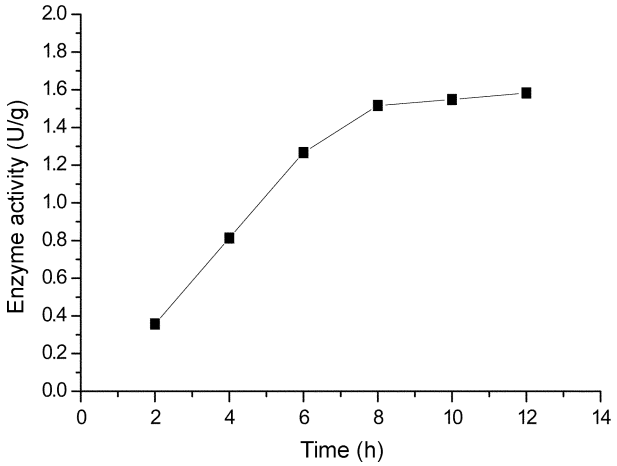


**Fig. 3. Effect of immobilized time on chitosanase immobilization.**

1-3. Effect of Immobilization Time on Chitosanase Immobilization

0.1 g DEAE cellulose was treated with 5 ml 5% glutaraldehyde. and DEAE cellulose-glutaraldehyde was then treated with 2.3 mg chitosanase at different time ranging from 2 to 12 hours. The activity of immobilized enzyme was tested and the results are shown in Fig. 3. Enzyme activity increased with increasing immobilized time. When the immobilized time was more than 8 h, enzyme activity did not increase further; therefore, 8 h was selected as the optimal immobilization time. The activity of immobilized enzyme was 1.5 U/g, and the recovery rate of enzyme activity was 81%.

1-4. SEM of Immobilized Enzyme

The immobilized enzyme and the free carrier were subjected to SEM. As shown in Fig. 4 and Fig. 5, there is a distinct change on the surface of DEAE cellulose after immobilization. The surface became more crinkly, and some granules of chitonase were immobilized on the matrix of the surface by crosslinking reactions.

**2. Characterization of Immobilized Chitosanase**

2-1. Effect of pH on the Activity of Immobilized Chitosanase

The activity of immobilized chitosanase was determined at various pH ranging from 3.0 to 5.5 at 50 ℃. As reported earlier by Gold-


**Fig. 4. SEM micrograph of free carrier.**


**Fig. 5. SEM micrograph of immobilized enzyme.**

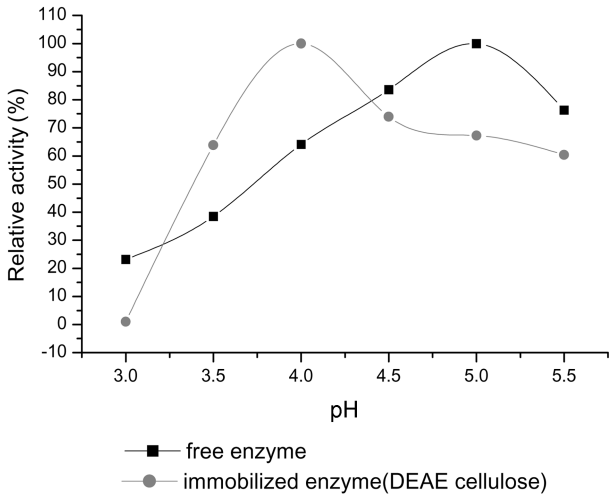


**Fig. 6. Effect of pH on the activity of immobilized chitosanase.**

stein et al. [1964], binding of enzyme to negatively charged supports would result in a basic shift in the pH optimum: the optimal pH decreased from 5.0 to 4.0 (as shown in Fig. 6). The pH of immobilized enzyme was influenced by two factors: the electrical property of carrier and the intraparticle diffusion resistance which occurred with the immobilization. DEAE cellulose is a positively charged carrier ($\Psi>0$), and the pH within the immobilized enzyme is higher than that in the bulk solution, so the curve of pH-activity ought to shift to the acidic side.

2-2. Effect of Temperature on the Activity of Immobilized Chitosanase

The activity of immobilized chitosanase was determined at different temperatures ranging from 30 to 70 °C at pH 4.0. As was shown in Fig. 7, the optimal temperature for free enzyme was 50 °C, and that for immobilized enzyme was 60 °C. The immobilized enzyme maintained strong activity at temperatures ranging from 50 °C to 65 °C. The higher optimal reaction temperature and improved thermal stability of the immobilized enzyme indicated that covalent bind-

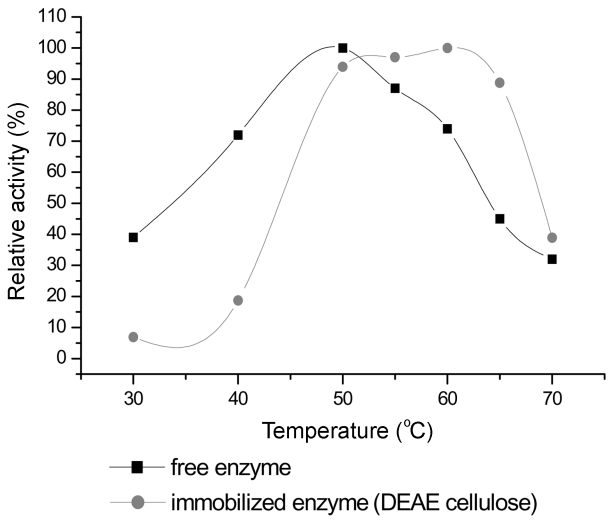


**Fig. 7. Effect of temperature on the activity of immobilized chitosanase.**

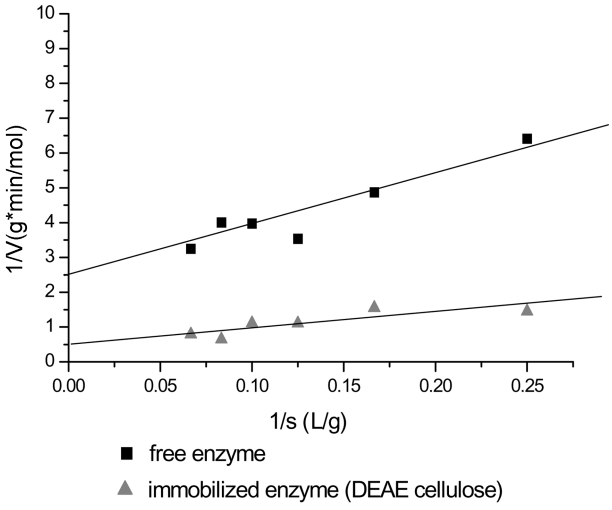


**Fig. 8. Determination of apparent $K_m$.**

ing enhanced thermal stability. Improved thermal stability could also be attributed to the restricted conformational change of the chitosanase upon immobilization, as suggested by Martinek et al. [1980] and Klibanov [1979].

2-3. Determination of Apparent $K_m$

The $K_m$ value of the immobilized enzyme was found to be 18.87 g/L by Lineweaver~Burk plots (Fig. 8). The $K_m$ value of the free enzyme was estimated to be 2.49 g/L. The $K_m$ value of the immobilized enzyme was higher than that of the free enzyme, which means a decrease in the flexibility of the enzyme molecule as described by Gottschalk and Jaenicke [Kusano et al., 1989]. The immobilized enzyme had less affinity for substrate. As we know, the Michaelis constant ($K_m$) of immobilized enzyme is affected by the electrostatic interaction between the carrier and substrate. When the carrier and substrate is oppositely charged, there will be an increased attracting force, which results in stronger affinity between immobilized enzyme and substrate, and the decreasing of the $K_m$ constant.

2-4. Operation Stability of Immobilized Chitosanase

2 g immobilized enzyme was added to 50 ml 1% chitosan solution in a water bath 50 °C for 6 h, and the reaction was repeated 10 times. After each reaction, the enzyme activity was determined. The immobilized enzyme showed good operation stability, and the enzyme activity loss was less than 20% after 10-time batch reaction.

## CONCLUSIONS

Chitosanase was immobilized on DEAE cellulose with glutaraldehyde by crossing-linking reaction. The immobilization conditions and characters of the immobilized enzyme were studied. The optimal conditions of immobilization were as follows: 0.1 g DEAE cellulose was treated with 5 ml 5% solution of glutaraldehyde, and then 2.3 mg chitosanase was added to immobilize on the carrier. Under optimal conditions, the activity of immobilized enzyme is 1.5 U/g, and the recovery rate of enzyme activity is 81%. After immobilization, the optimal temperature and kinetic parameter increased (from 50 °C to 60 °C, from 2.49 g/L to 18.87 g/L), whereas the optimal pH was reduced (from 5.0 to 4.0). The enzyme activity loss was less than 20% after 10 times of batch reaction. The immobilized enzyme showed good operation stability.

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