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Continuous Degradation of Chitosan in a Convoluted Fibrous Bed Bioreactor with Immobilized *Trichoderma reesei**

WU Mianbin(吴绵斌), XIA Liming(夏黎明) and CEN Peilin(岑沛霖)**
Department of Chemical and Biochemical Engineering, Zhejiang University, Hangzhou 310027, China

Abstract Continuous hydrolysis of chitosan was performed in a convoluted fibrous bed bioreactor (CFBB) with immobilized *T. reesei*. At dilution rate of 0.4 d^{-1} and substrate concentration of 2%(mass *vs.* volume), the average degree of polymerization of hydrolysate can be kept at 1.25—1.35, which can be easily regulated by changing dilution rate or inlet chitosan concentration.

Keywords continuous degradation, chitosanase production, chitosan degradation, fibrous bed bioreactor, *Trichoderma reesei*

1 INTRODUCTION

It is well known that the functional oligosaccharides can promote the growth of bifidobacteria *in vivo*. As a kind of important functional oligosaccharides, low-molecular-weight chitosan oligosaccharides have received a growing attention because of their various biological activities such as antitumor[1,2], immunostimulants[3], antibacterial[4,5] and antifungal[6,7]. Chitosan-oligosaccharides can be obtained with either acidic or enzymatic hydrolysis of chitosan. Hydrolysis by concentrated HCl[8] has been used as a conventional method which is low in yield and complex in product fractionation. An enzymatic hydrolysis procedure[9,10], however, is mild in operation conditions and high in product yield.

The conventional enzymatic hydrolysis process is indeed a two-step process. The first one is to produce chitosanase by fermentation and the second is to apply the chitosanase in chitosan hydrolysis. The enzyme must be partially purified and the procedure is relatively complicated. It will be certainly beneficial if two steps can be put together and take place in the same bioreactor. There are several successful examples of combined processes, such as the simultaneous saccharification and ethanol fermentation.

The immobilized-cell fermentation allows cell reuse and continuous operation. Compared to free cell culture, better results, including greatly improved the reactor productivity, separation of cells from products, and operation at high dilution rates without washout, may be observed. However, immobilized bioreactor often suffers from poor long-term stability due to reactor bed clogging, membrane fouling, cell degeneration, or contamination. Yang *et al.*[11–13] developed a fibrous-bed, immobilized cell bioreactor for continuous fermentation, in which, cells are immobilized in a porous convoluted fibrous support. The structured fibrous bed allows for good multiphase flows and provides renewable surfaces for cell immobilization, resulting in a high cell density, high reactor productivity, and good long-term stability. This bioreactor system has been effectively used in the immobilization of bacterium and yeast, but has not been used in the immobilization of filamentous fungus.

In this study, the fibrous bed bioreactor system will be applied to immobilized *T. reesei* fermentation for continuous production of chitosanase and hydrolysis of chitosan. The long-term stability of the bioreactor will be evaluated.

2 MATERIALS AND METHODS

2.1 Strain

Trichoderma reesei ATCC56764 used in this study was maintained on a potato dextrose agar slant.

2.2 Chitosan

Chitosan is a product of Yuhuan Biochemical Co. Ltd., the average molecular weight is about 30,000, and the degree of deacetylation is 93.5%.

2.3 Medium

Growth medium($g \cdot L^{-1}$): glucose 50, polypeptone 5.0, $(NH_4)_2SO_4$ 1.4, urea 0.3, KH_2PO_4 2.0, $CaCl_2 \cdot 2H_2O$ 0.4, $MgSO_4 \cdot 7H_2O$ 0.3, Tween80 0.5 and trace element solution 1 ml.

Trace element solution ($g \cdot L^{-1}$): $FeSO_4 \cdot 7H_2O$ 5.0, $MnSO_4 \cdot H_2O$ 1.6, $ZnSO_4 \cdot 7H_2O$ 1.4, $CoCl_2 \cdot 6H_2O$ 3.7.

Degradation medium($g \cdot L^{-1}$, unless indicated otherwise): Soluble chitosan[5] 10, KH_2PO_4 2.0, $CaCl_2 \cdot 2H_2O$ 0.4, $MgSO_4 \cdot 7H_2O$ 0.3, Tween80 1.0 and trace element solution 1 ml.

The pH value of the medium was adjusted with

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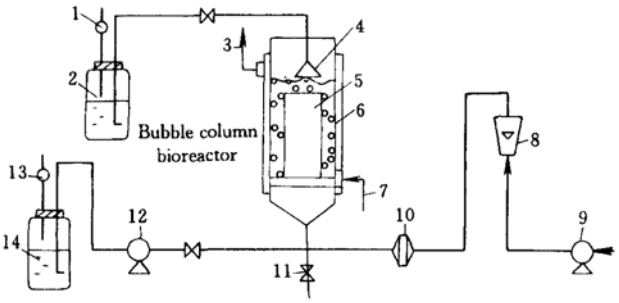
** To whom correspondence should be addressed.

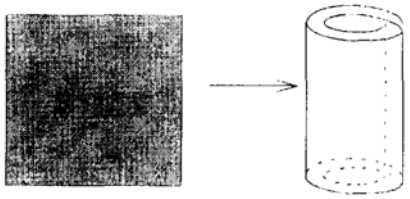
acetate buffer to pH 5.0.

2.4 Bioreactor

The immobilized cell bioreactor is a glass column ($\phi 105 \times 660$ mm) with a water jacket, in which a roll of porous polyurethane foam(PPF) sheet($\sim$ 6 mm in thickness) fixed on a stainless steel screen is inserted for cell immobilization. The void volume of PPF support is more than 90%. The working volume of the bioreactor is about 1.5 L. Sterilized air is supplied through a distributor in the bottom of the bioreactor. Fig. 1 shows the schematic diagram of the bioreactor system.



(a) Reactor system
1—air outlet; 2—product reservoir; 3—water outlet; 4—screen(9 mesh); 5—fixed PPF support; 6—water jacket; 7—water inlet; 8—flowmeter; 9—air compressor; 10—air filter; 11—sample connection; 12—pump; 13—air filter; 14—medium reservoir



(b) Construction of Spiral wound fibrous support

Figure 1 Experimental apparatus of immobilized-cell bioreactor system for chitosan degradation

2.5 Mycelia immobilization and chitosan degradation

150 ml of conidia suspension of *T. reesei* was inoculated into 1.5 L of growth medium, resulted in 10^6 conidia per milliliter. After incubated at 30℃ and 1.5 $m^3 \cdot m^{-3} \cdot min^{-1}$ air-flow rate, the spores were firstly adsorbed on the PPF sheet and then the mycelia were formed and would cover the PPF sheet after 3 d incubation. The mycelia were washed by 1.5 L 0.05 $mol \cdot L^{-1}$ acetate buffer for 3 times and ready for chitosan degradation. After washing, 1.5 L of degradation medium was added into the bioreactor and the substrate was pumped into the bioreactor continuously to start the continuous chitosan hydrolysis. The average degree of polymerization(ADP) here means the number of glucosamine which the molecules of chitosan or its hydrolyzates are composed of, it can be calculated by the following equation

$$ADP = \frac{c_s}{c_r} \times \frac{179.17}{161.11}$$

2.6 Assay methods

Chitosanase activity was determined by measuring the released amino sugars as D-glucosamine produced from chitosan. 1 ml enzyme solution was mixed with 1 ml 1% soluble chitosan dissolved in 0.05 $mol \cdot L^{-1}$ pH5.0 acetate buffer and the mixture was reacted for 10 min at 50℃. Adding 50 μl of 10 $mol \cdot L^{-1}$ NaOH to stop the reaction, and then removing the undigested chitosan. After cooling and centrifugation, amino sugars as D-glucosamine in supernatant were measured by Enrich method[14]. One unit of activity was defined as the 1 μmol of amino sugars produced from the substrate per minute with D-glucosamine as standard.

Reducing sugar taking as D-glucosamine was analyzed by DNS method[15].

The average degree of polymerization(ADP) of chitosan was determined by measurement of the viscosity by the method suggested by Maghami *et al.*[16]

Dissolved oxygen(DO) concentration was detected by Ingold DO electrode(Ingold Co. Ltd.).

D-glucosamine and chito-oligomer were analyzed by Waters 510 HPLC. The HPLC conditions used were as follows: column, Shodex SC1011 (Showa Denko Co.Lt.,Japan) at 80℃; effluent, distilled water at 0.5 $ml \cdot min^{-1}$; detector, differential refractometer(ERC-7510; Erma Optical Works Ltd., Japan).

3 RESULTS AND DISCUSSION

3.1 Immobilization of mycelia of *Trichoderma reesei* on PPF sheet

When the conidia suspension was inoculated into the convoluted fibrous bed bioreactor, the spores of *T. reesei* were adsorbed into the PPF matrix immediately. After 12 h incubation, the mycelia began to grow on the surface of PPF sheet. The typical time course of immobilization was shown in Fig. 2, and the maximum dried mycelia reached 32.02 $g \cdot L^{-1}$, corresponding to 2.39 $g \cdot g^{-1}$ (based on the sheet) after 72 h incubation. The free mycelia concentrations in the broth during the cultivation with immobilized mycelia could not be detected during the culture course, and so the leakage of hyphae was not serious through the cultivation.

As shown in Fig. 2, the amount of mycelium with immobilized cell culture in the conroluted fibrous bed

bioreactor(CFBB) was about 15% higher than that of free cell culture under the same conditions in stirred tank reactor at the peak level. The immobilized living mycelia were grown not only on the surface of the PPF sheet, but also within the matrix, which was beneficial for increasing the interfacial area and reaction rate. Compared with free cell culture in stirred tank reactor, the volumetric glucose consumption rate ($g \cdot L^{-1} \cdot h^{-1}$) of immobilized cell in the CFBB was faster and the lag phase of culture was shortened. Furthermore, the mycelia present in PPF sheet was protected from shear damages by gas bubbling, which was favorable for keeping the activity of hyphae.

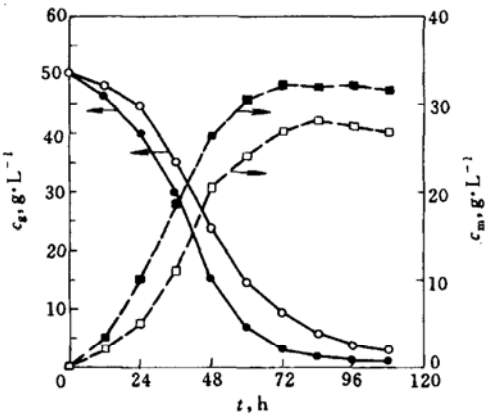


Figure 2 The immobilization course of *Trichoderma reesei* mycelia

■, ● immobilized cell culture; □, ○ free cell culture

The dissolved oxygen(DO) concentration in the CFBB during the immobilizing-course of *T. reesei* cells was also studied. Compared with free cell culture in the stirred tank reactor(200 $r \cdot min^{-1}$ of stirring rate), the minimum concentration of DO in the CFBB appeared about 12 h earlier(Fig. 3). The DO concentration in the bioreactor systems is related to the cell growth phase and growth rate. The earlier appearance of the lowest DO level means the earlier exponential phase of cell growth and the faster DO consumption means the faster growth rate of hyphae in the CFBB process.

3.2 Continuous production of chitosanase and hydrolysis of chitosan with immobilized hyphae of *T.reesei*

The CFBB was used to perform the continuous production of chitosanase and hydrolysis of chitosan with immobilized hyphae of *T. reesei*. The results were shown in Fig. 4. The dilution rate was 0.4 d^{-1} and the chitosan concentration in the inlet was maintained at 2%. Fig. 4 showed that in continuous operation for 15 d, the chitosanase activity was maintained around 200 $U \cdot L^{-1}$, the *ADP* of product(chito-oligomer) was from 1.25 to 1.35 and the dry mass of mycelia immobilized on PPF support was about 2.3 $g \cdot g^{-1}$. From the results of HPLC analysis, the fractions contained monomer, dimer and a small amount of trimer of glucosamine throughout the continuous operation of the bioreactor (Fig. 5).

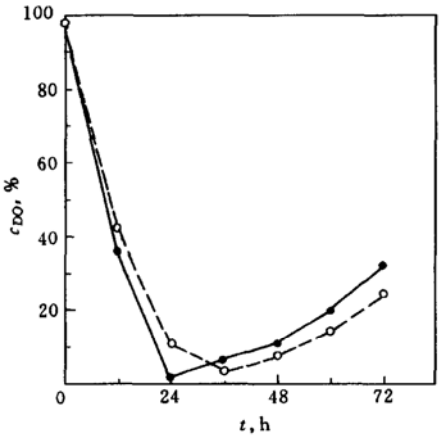


Figure 3 Dissolve oxygen concentration varied with time

● DO in CFBB; ○ DO in stirred tank reactor

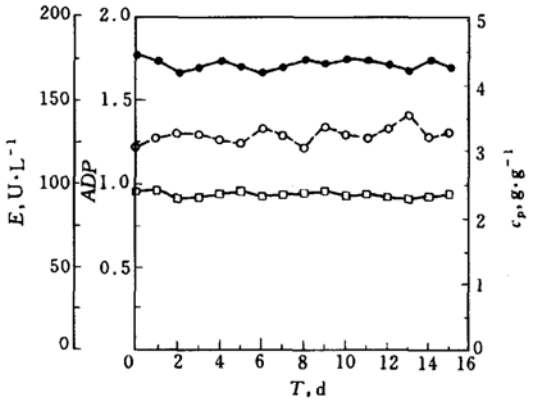


Figure 4 Time course of continuous hydrolysis of chitosan with immobilized hyphae of *T. reesei*

○ *ADP*; ● **E**; □ c_p

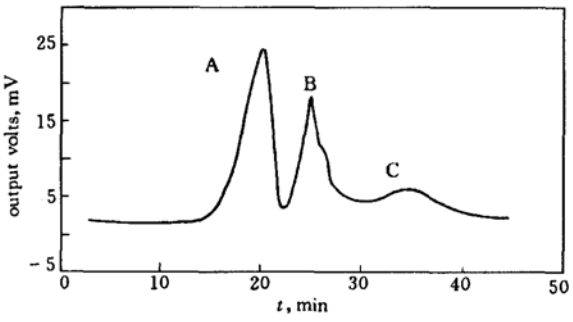
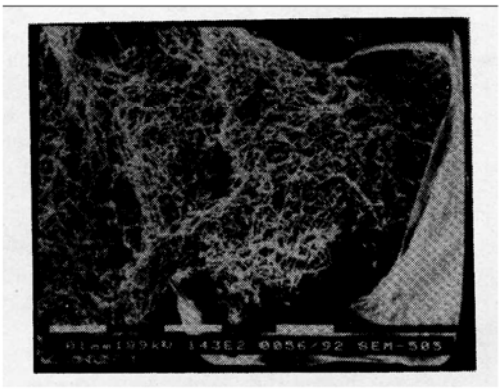


Figure 5 HPLC pattern of hydrolyzate derived from soluble chitosan

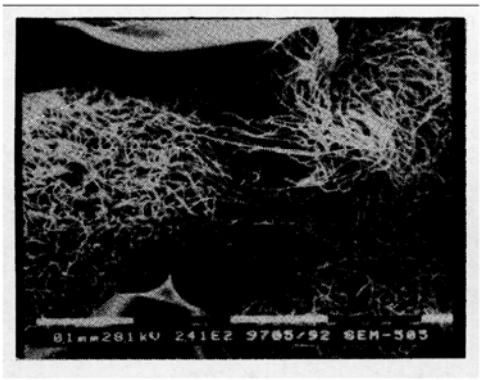
A—glucosamine; B—chitobiose; C—chitotriose

The continuous operation of the immobilized cell bioreactor was carried out without clogging or contamination problem. The hyphae of *T. reesei* grew

well during the continuous culture and adhered tightly to the PPF support as shown in Fig. 6. From the experiments, we observed that few of free mycelia were peered off from PPE support, which was beneficial for hyphae regeneration and for long-term operation stability.



(a) ×143



(b) ×241

Figure 6 SEM micrographs of immobilized cells of *T.reesei* after 15 days fermentation

3.3 Effects of operating conditions on *ADP* of product

In the continuous chitosanase production and chitosan hydrolysis process, the dilution rate and inlet chitosan concentration are two important operation conditions. The average degree of polymerization of product was directly related to the dilution rate and inlet concentration of chitosan as shown in Fig. 7. With the increase in either D or S_0, the *ADP* of product will increase too. Then, it is very simple to adjust the *ADP* of product according to the market requirement. At low dilution rate, such as less than $0.4\,d^{-1}$, the *ADP* of product was almost a constant and varied little with the increase of S_0. The reason is probably that the random attack of chitosanase on the β-1,4-glucoside bond. When *ADP* value was very close to 1, which means most product is glucosamine, the probability of interaction between enzyme and β-1,4-glucoside reduced greatly.

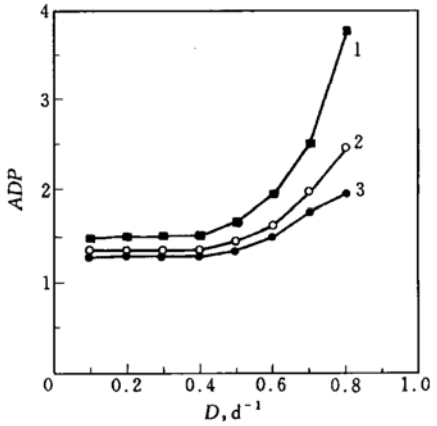


Figure 7 Effects of inlet substrate concentration and dilution rate on chitosan degradation in the fibrous bed bioreactor

substrate concentration (by mass), %: 1—1; 2—2; 3—3

3.4 Comparison between two-step process and coupled process

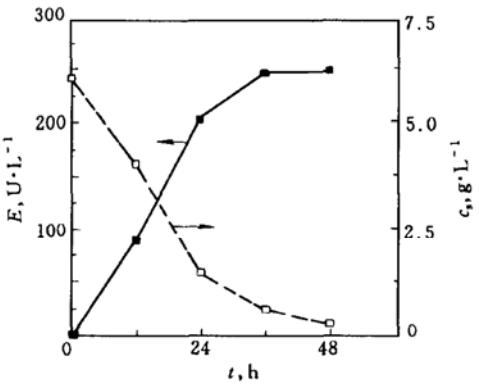
The conventional two-step hydrolysis process includs chitosanase production step and chitosan hydrolysis step, which are carried out respectively in different reactors and at different operation conditions. As shown in Fig. 8, a typical time-course of chitosan degradation was about 36 h for chitosanase production in the CFBB and 48 h for chitosan hydrolysis in the two batch processes, respectively. The total time was 84 h. The fermentation and hydrolysis must be carried out at 30℃ and 50℃, respectively. From Fig. 8(b), it was obvious that the chitosanase activity decreased fast because of high temperature.

In simultaneous chitosanase production and chitosan hydrolysis process, the two steps were carried out in the same reactor and at same conditions. The enzyme activity was kept in the same level without deactivation. Although the temperature was only 28℃, the residence time was only 60 h to reach the same *ADP* value(about 1.3). If the required *ADP* is 4 and the inlet chitosan concentration is 3%, the residence time will less than 30 h. The productivity of one-step process is much higher than that of two-step process, also more uniform in product quality.

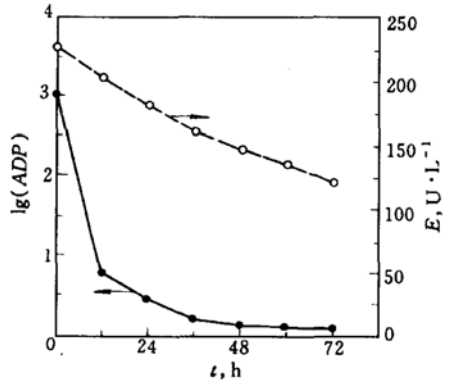
4 CONCLUSIONS

A novel process for continuous production of chitosanase and hydrolysis of chitosan with immobilized *T. reesei* ATCC56764 was evaluated. A CFBB, in which *T. reesei* hyphae was immobilized on a roll of polyurethane foam sheet, was developed. Experimental results showed that the system could be operated continuously without problem. The productivity of one-step process was higher than that of two-step process. And the *ADP* of produced chito-oligomer was

uniform and was able to be regulated easily, by changing dilution rate or inlet chitosan concentration.



(a)



(b)

Figure 8 (a)Time-course of chitosanase production with immobilized mycelium of *T. reesei* in CFBB (The medium was the same as degradation medium, except that chitosan was replaced by glucosamine)

(b) Time-course of the chitosan hydrolysis with chitosanase produced by *T. reesei* in the stirred tank reactor ($S_0 = 9.78\,mg\cdot ml^{-1}$, $ADP_0 = 1003$, $E_0 = 225\,U\cdot L^{-1}$, $T = 50℃$, pH=5.0)

NOMENCLATURE

ADP	average degree of polymerization of chito-oligosaccharides(number)
c_{DO}	dissolved oxygen concentration(by mass), %
c_g	glucose concentration, $g\cdot L^{-1}$
c_m	dry mass of mycelia, $g\cdot L^{-1}$
c_p	dry mass of mycelia immobilized on PPF support(g per gram dry support), $g\cdot g^{-1}$
c_r	concentration of reducing sugar, $g\cdot L^{-1}$
c_s	concentration of reducing sugar as D-glucosamine, $g\cdot L^{-1}$
D	dilution rate, d^{-1}
E	chitosanase activity, $U\cdot L^{-1}$
S	concentration of chitosan, $g\cdot L^{-1}$
T	reaction temperature, ℃
t	time, h

Subscripts

0	initial

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