Optimization of Co-immobilization of Cellulase and \( \beta \)-glucosidase

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Abstract: Cellulase can hydrolyze cellulose to produce reducing sugars such as cellobiose and cellotriose. \( \beta \)-glucosidas can further hydrolyze cellobiose and cellobiose produced by cellulase to produce glucose. According to the hydrolysis mechanisms of cellulase and \( \beta \)-glucosidas, we selected two enzymes, cellulase and \( \beta \)-glucosidas to study optimization of co-immobilization of cellulase and \( \beta \)-glucosidas. Meanwhile, we selected two materials, activated carbon and sodium alginate to co-immobilize cellulase and \( \beta \)-glucosidas by the immobilization method of embedding-adsorption. The immobilization conditions, alginate, CaCl\(_2\) and activated carbon was optimized. The results showed that the optimum concentrations of sodium alginate and CaCl\(_2\) were 2% and 2%, respectively, and the quality of activated carbon is 0.15 g. The optimal ratio of cellulase to \( \beta \)-glucosidas was 1:1.5. The results indicated that cellulase and \( \beta \)-glucosidas had a synergistic effect and that their compound degradation of cellulose was better than the separate effects of the two enzymes acting independently.

1. Introduction

Enzyme has strong stability, high catalysis and selectivity to substrate, so it is widely used in industrial production and scientific research. However, due to the instability of free enzyme, poor recoverability and easy to be affected by external factors, so the use of free enzyme is limited.

In order to make the enzyme economic and efficient in industrial production, it needs to be fixed with immobilized carrier to reuse [1,2]. So immobilized enzymes are mainly prepared by chemical methods, physical methods and entrapment. Due to simple and inexpensive immobilization within sodium alginate, alginate is widely used in experimental research and industrial processes [3,4]. Compared to a single immobilized enzyme, the co-immobilization technique is to place different enzymes and enzymes, cells and cells or cells and enzymes on the same carrier at the same time, and immobilize them by some methods to make them a system [5].

Due to the application of co-immobilization technology, multiple enzymes are immobilized in the carrier, which have become a hot research topic. In addition, co-immobilization of multiple enzymes can make up for the deficiency of single enzyme catalysis.

There have been many studies on the mechanism of multi enzymes immobilization. In living cells, one enzyme may be the substrate of another enzyme, and the intermediate can form the complex of enzyme, which enables the enzyme to effectively transfer from one site to another, relying on the adjacent enzyme active site for catalytic reaction [6]. In an in vitro system, the reaction conditions are
easier to control than in vivo methods. However, it is still a huge challenge that designing an efficient enzyme cascade reaction. If the enzyme is treated in an appropriate way, it can effectively limit the mass transport of products-structure. So, it is necessary to transfer reactants directly from one active center to another without affecting the diffusion of the receptor, so as to reduce the effects of the competitive reactions and to prevent the formation of toxic intermediates [7] [8]. In order to increase the catalytic efficiency of the cascade reaction, many approaches have been proposed. For example, gene coding techniques are used to facilitate substrate channelization by minimizing the distance from one active site to another [9] [10]. Affinity markers are used to selectively immobilize proteins to achieve specific co-immobilization on support [10]. However, due to chemical modification or denaturation generally residues, lead to structural changes in the enzyme activity loss [11]. The connection of enzymes to carriers or the construction of nanostructures with various enzymes have been widely studied. However, little attention has been paid to the design of carriers itself [12].

Therefore, we designed a composite immobilization carrier. Activated carbon and sodium alginate were prepared as immobilized carriers, and cellulase and β-glucosidase were co-immobilized. After co-immobilization of cellulase and β-glucosidase, the catalytic efficiency and stability of the enzymes were significantly improved compared with those of equimolar mixed free enzymes. Also, cellulase and β-glucosidase had a synergistic effect and their compound degradation of cellulose was better than the separate effects of the two enzymes acting independently.

2. Experimental materials

2.1 Selection of experimental materials

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\begin{align*}
\text{CaCl}_2 \cdot 2\text{H}_2\text{O}, \quad \text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}, \quad \text{C}_2\text{H}_3\text{NaO}_2, \quad \text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}, \quad \text{CH}_3\text{COOH}, \quad \text{C}_6\text{H}_5\text{NO}_3, \quad \text{Na}_2\text{CO}_3, \quad 3,5\text{-Dinitrosalicylic acid}, \quad \text{NaOH}, \quad \text{C}_4\text{H}_12\text{KNaO}_{10}, \quad \text{Na alginate(} \text{SA})\end{align*}
\]

were purchased from Sinopharm Chemical Reagent Co., Ltd.

2.2 Preparation of experimental materials

(1) Method for preparing immobilized enzyme

A certain quality of activated carbon and 100 mL of a certain mass fraction of sodium alginate solution are mixed and stirred. The mixture is cooled to normal temperature, and 10 mL of a certain concentration of enzyme solution is continuously added and stirred. After the mixture was allowed to stand for 3 min, it was pipetted with a No. 5 needle syringe, and dropped into a certain concentration of CaCl₂ solution at a constant rate. After hardening for 30 minutes, it was washed twice with distilled water to wash away the CaCl₂ solution remaining on the surface. The immobilized enzymes were filtered and stored in a refrigerator at 4°C.

(2) Orthogonal experiment of adsorption-embedding immobilization

The preparation conditions of the immobilized carrier were optimized by orthogonal experiment. The orthogonal experiment was carried out at three levels with three factors: activated carbon (g), sodium alginate (%), CaCl₂ (%). A certain amount of beads were placed in the methylene blue solution of 20 ml (20 mg/L) for adsorption. The change of the absorbance value was measured by spectrophotometer, and the methylene blue adsorption rate under each experimental condition was calculated. The adsorption performance and mass transfer effect of the beads were evaluated. Table 1 is the levels of orthogonal experimental factors [13].

| Level | Factor A | Factor B | Factor C |
|-------|----------|----------|----------|
|       | Activated carbon(g) | SA concentration(%) | CaCl₂ concentration(%) |
| 1     | 0.05     | 1.5      | 1.0      |
| 2     | 0.10     | 2.0      | 2.0      |
| 3     | 0.15     | 2.5      | 3.0      |

Table 1. Parameters and level-value of orthogonal experiment.
3. Experimental contents and methods

3.1 Measurement methods

(1) Measurement of the number of cellulase activity
Cellulase activity was measured using CMC (1% citric acetate buffer of pH 4.8) as a substrate [14]. For free enzyme mixture or immobilized cellulase, the amount of total reducing sugar were determined via 3, 5- dinitrosalicylic acid (DNS).

2 ml of CMC solution and 2 ml of the cellulase were incubated at 40 °C for 0.5 h. The reaction tube was put into the boiling water bath for 15 minutes to stop the reaction. The amount of reducing sugar was measured by adding 4 ml DNS as glucose reagent, and the glucose solution was used as the standard solution [15]. One unit of cellulase activity is defined as the amount of cellulase that produces 1 mol of glucose equivalent per minute at 40 °C and pH 4.8. The unit was calculated as follows: Eq (1)

$$\text{activity of cellulase (U)} = \frac{A \times n \times 1000}{T \times B \times 180 \times 60}$$

(1)

where A is the amount of reducing sugar(mg), n is the dilution multiple, T is the enzymatic hydrolysis time(min), B is Enzyme dosage(mL), respectively.

(2) Measurement of the number of β-glucosidase activity
β-glucosidase activity was determined in 40 mM citric acid/sodium hydroxide buffer (pH 5.0) at 50 °C using 4 mM cellobiose as substrate under gentle stirring.

The activity of β-glucosidase was determined in 1 mL, 0.1 mol/ L citric acid / sodium hydroxide buffer (pH 4.8) at 50 °C with 0.1 mL, 10 mmol/ L p-NPG as substrate and under mild stirring. The amount of β-glucosidase was 0.3 mg/mL for both free and immobilized β-glucosidase. After 30 min reaction at 50 °C, 2 mL Na2CO3 (1 mol/ L) was quickly added to stop the reaction. The cooling was placed at 5 min, and the spectrophotometer was adjusted to 410 nm. The absorbance value of the spectrophotometer was measured at 410 nm [16]. The unit was calculated as follows: Eq(2)

$$\text{activity of β-glucosidase (U)} = \frac{c \times V}{T \times V_i} \times N$$

(2)

where is the p-NPG concentration (μmol/mL), V is the p-NPG content , T is the enzymatic reaction time(min), V_i is the Volume of supernatant(mL), N is the dilution multipliere spectively.

3.2 Determination of the best ratio of co-immobilized enzymes activity
According to the results of orthogonal experiment, the carrier with the highest enzyme activity after immobilization was selected. The mixture ratio of cellulase and β-glucosidase was 4:1, 3:1, 2:1, 1:1, 1:2, 1:3 and 1:4, respectively.

4. Experimental results and analysis

4.1 Results of immobilized orthogonal experiment

| Test number | Factor A | Factor B | Factor C | Methylene blue adsorption rate(%) | Sphericity |
|-------------|----------|----------|----------|----------------------------------|------------|
| 1           | 1        | 1        | 1        | 40.21                            | +^a        |
| 2           | 1        | 2        | 2        | 48.12                            | ++^a       |
According to table 2, the optimal immobilization is A3B2C2, which is 0.15 g activated carbon, 2.0% sodium alginate and 2.0% calcium chloride. This immobilization has the highest adsorption rate and best mass transfer. If the immobilized enzyme is prepared by embedding the free enzyme in the rubber ball, and then the rubber ball is placed in a certain concentration of enzyme solution for adsorption for a period of time, the substrate and the enzyme inside and outside the rubber ball are fully contacted, the catalytic reaction rate is higher, and the substrate is easier to get in and out of the rubber ball, that is, the mass transfer effect is better, and the enzyme activity is better. Therefore, this condition was used to prepare immobilized enzyme in this study.

### 4.2 Selection of the ratio of two enzyme activities

![Figure 1. Effect of co-immobilized double enzyme on enzyme activity](image)

| Parameter | Cellulase | β-glucosidase |
|-----------|-----------|---------------|
| 4:1       | 85        | 90            |
| 3:1       | 80        | 85            |
| 2:1       | 75        | 80            |
| 1:1       | 70        | 75            |
| 1:2       | 65        | 70            |
| 1:3       | 60        | 65            |
| 1:4       | 55        | 60            |

The quality of sphericity. The more number of the "+" is, the better the ball effect is.

Levels of each factor affecting the adsorption rate of methylene blue respectively.

The degree of influence of the above three factors on the adsorption rate of methylene blue.
It can be seen from the figure 1 that cellulase and β-glucosidase are fixed on the same carrier with different enzyme activity ratio. When the enzyme activity ratio of cellulase is higher than that of β-glucosidase, the enzyme activity of this enzyme is increased correspondingly, while β-glucosidase activity is relatively small. It is due to the synergistic effect of the two enzymes. When the mixture ratio of cellulase and β-glucosidase is 1:1.5 (enzyme activity ratio), both enzymes have good enzyme activity. Therefore, the best enzyme activity ratio of cellulase to β-glucosidase is 1:1.5.

5. Conclusions
In this paper, the co-immobilization of cellulase and β-glucosidase was carried out by the combination of adsorption and embedding, and the factors (CaCl₂ concentration, sodium alginate, and active carbon carrier) were optimized. The results show that the adsorption embedding method is better than the single embedding method. The adsorption embedding method provides a new idea for the study and design of multi enzyme co-immobilization. The enzyme co-immobilized with inorganic support has important application value in industrial process or bioremediation of polluted environment.

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