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Preparation and enzymatic activity of penicillin G acylase immobilized on core–shell porous glass beads

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ABSTRACT

In this work, core–shell porous glass beads were used to immobilize the PGA based on the theory that diffusion is the rate-determining step of a reaction with fast kinetics. To improve the loading amount of PGA, chitosan was grafted on the support to increase the interaction between PGA and the support. The effects of flow rate, chitosan concentration and initial concentration of Pen G on immobilized PGA were investigated. The core–shell structure of the support resulted in a higher efficiency of the diffusion of substrate and products, which improved the activity of immobilized PGA. The conversation rate of Pen G (26.9 mmol/L) reached 91.1% when the residential time was about 30 s in the fixed bed reactor. Meanwhile, the conversation rate of 100 mL of Pen G (134.3 mmol/L) reached 89.1% after 8 h in the reactor with recirculation. Moreover, 97.5% activity was retained after seven days of continuous reaction.

1. Introduction

Enzyme biocatalysts have been widely used in the fine chemicals industry, including the food and pharmaceutical industries [1]. Enzyme immobilization solves the common problems of free enzyme, including the lack of long-term stability, low tolerance to organic solvents, and difficult recovery and reuse [2–8]. Among all kinds of enzymes, penicillin G acylase (PGA, E.C. 3.5.1.11) is an important enzyme that catalyzes the hydrolysis of Penicillin G potassium (Pen G) to produce 6-aminopenicillanic acid (6-APA), which is the precursor for manufacturing β-lactum antibiotics [9]. Considering the hydrolysis of Pen G, which is a fast reaction, diffusions of substrate and products are the key factors that control the reaction. Therefore, the property of the support remarkably influences the diffusion rates of substrate and products, and then determines the catalytic performance of immobilized PGA.

Organic materials are widely used as the supports of immobilized PGA because of the presence of rich functional groups, which provide essential interactions with enzymes [1]. For instance, Eupergit C is commercially used as a support for immobilization. PGA is covalently attached to Eupergit C and used in industrial production [10]. However, the major shortcoming of Eupergit C is its diffusion limitations, the effects of which are more pronounced in kinetically controlled processes [11]. Researchers have also immobilized PGA onto other organic materials, including amine-functionalized PVC membranes and glutaraldehyde-activated chitosan [12,13]. Inorganic materials are applied to enzyme immobilization because of their special characteristics, including thermal and mechanical stability, high resistance against organic solvents, and nontoxicity. For example, PGA molecules were immobilized on hollow silica nanotubes [14], in which the thin shell structure of the immobilized enzyme effectively reduced mass transfer resistance that led to a high activity yield. Macro-mesoporous silica spheres were prepared with a micro channel and were used as the support for PGA immobilization [15]. The introduction of macropores increased the enzyme loading amount and decreased the internal mass transfer resistance. This condition resulted in a high apparent enzymatic activity. In addition, PGA was also immobilized on mesostructured cellular foams, macrocellular heterogeneous silica-based monoliths, aminoalkylated polyacrylic supports, and oxirane-modifiedmesoporous silicas [16–20]. These studies strongly illustrated the importance of diffusion rate to catalytic performance.

Materials with core–shell structure are also good choices for PGA immobilization. In our previous work, a one-step subcritical water treatment method was developed to prepare porous glass beads which have core–shell structure [21]. Ion exchange properties were further investigated [22], and chitosan was supported on this kind of inorganic material, which was used as a green adsorbent for heavy metal adsorption [23]. Porous glass beads have several advantages, which are as follows: (1) can effectively eliminate the influence of inner diffusion because the distance of mass diffusion is

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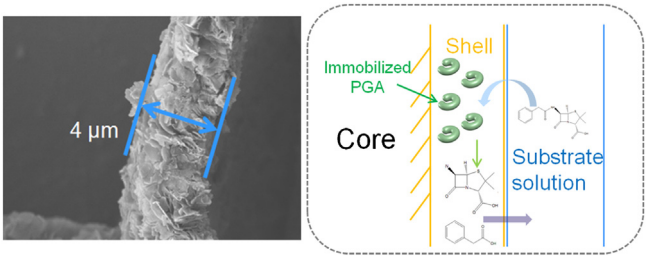

Fig. 1. The process of hydrolysis reaction catalyzed by PGA immobilized on the porous glass beads.

the thickness of the porous shell, (2) can be easily modified because of the numerous hydroxyl groups on the surface, (3) contain good chemical and thermal stabilities, and (4) can be easily filled and separated based on the appropriate particle size (75–150 μm). In this work, PGA immobilized on the chitosan-treated porous glass beads was investigated. As shown in Fig. 1, the thickness of the porous shell was about 4 μm. Thus, the process of hydrolysis reaction is considered the contact between the shell layer and the aqueous phase layer. The short distance of mass transfer improves the efficiency of the diffusion of substrate and products. PGA molecules are centralized in a small shell area, which means that the density of the enzyme is larger than those of any other uniform sphere supports. Hence, the contact probability of enzymes and substrates could be increased. To achieve continuous production, the obtained immobilized PGA was filled in a fixed bed reactor. This process could avoid the damage of mechanical stirring and the direct contact of enzyme and alkaline, which ensures good stability of the enzyme. The physical properties of the immobilized PGA were characterized, the effects of flow rate, chitosan concentration, initial concentration of Pen G, and the accumulation of 6-APA on immobilized PGA were studied.

2. Materials and methods

2.1. Materials

Glass microbeads with diameters ranging from 75 μm to 150 μm were obtained from Hebei Chiye Corporation, with the composition of 59.7 wt% SiO_2, 25.1 wt% Na_2O, 9.8 wt% MgO, and 4.9 wt% CaO. Hydrochloric acid (HCl), acetic acid, and sodium hydroxide (NaOH) were produced by Beijing Chemical Company (Beijing, China). Chitosan (DD 85.1%) was purchased from Sinopharm Chemical Reagent Company (Beijing, China). Sodium dihydrogen phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), and glutaraldehyde (GA) were obtained from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). PGA (E.C. 3.5.1.11) was purchased from Shunfeng Haideer Co., Ltd. (Zhejiang, China). Pen G was purchased from Dalian Meilun Biology Technology Co, Ltd. (Dalian, China). 6-APA was obtained from Tokyo Chemical Industry Co., Ltd. (Japan). HPLC-grade methanol was produced by Anhui Fulltime Specialized Solvents & Reagents Co., Ltd. (Anhui, China). All chemicals were used as received without any further treatment.

2.2. Synthesis of porous glass beads grafted by chitosan

The porous glass beads were prepared via the subcritical water treatment method based on the reference [22]. In this method, 5.0 g of glass beads and 200 g of water were placed in a tank reactor. The temperature of the reactor was gradually increased to 300 °C, and the pressure was increased to 8 MPa. The subcritical state was maintained for 1 h, after which the reactor was cooled to room temperature. The porous glass beads were separated from water via filtration and were washed several times with water. To replace the metal ions contained in the shell part with hydrogen ions, the obtained porous glass beads were mixed with 500 mL of HCl solution with a concentration of 0.5 M. The mixture was shaken in the SHA-B Incubator Shaker at 160 rpm at 30 °C for 12 h. The obtained modified glass beads were filtered, washed with plenty of water, and dried at 80 °C for 24 h.

To obtain a larger specific surface area and a stronger connection with PGA molecules, the surface of the porous glass beads was coated with chitosan before the immobilization of PGA. Chitosan was dissolved in acetic acid (1 wt.%) to obtain the chitosan solution with a concentration that ranged from 0.5 wt.% to 3.0 wt.%. Up to 1 g of the modified glass beads was mixed with 30 g of chitosan solution, and the mixture was then shaken at 160 rpm at 30 °C for 8 h. After removing the supernatant, the porous glass beads that adsorbed chitosan was mixed with 15 g of 1.0 wt.% GA and was shaken for another 12 h at 160 rpm at 30 °C. After filtration, washing, and drying, porous glass beads coated with chitosan were obtained.

2.3. Immobilization of PGA

Up to 0.5 g of porous glass beads coated with chitosan were incubated with 30 mL of PGA solution (5.0 mg/ml), which was prepared by diluting the original PGA solution with phosphate buffer (pH 7.9, 0.05 M). The mixture was then shaken in the SHA-B Incubator Shaker at 160 rpm at 30 °C for 24 h. After that, the mixture was centrifuged to separate the PGA solution and the porous glass beads with the immobilized PGA. The protein concentration of the supernatant was measured to calculate the PGA loading amount.

The PGA loading amount (q_E (mg/g)), was calculated according to the following formula:

$$q_E(\text{mg/g}) = \frac{q_1 - q_2}{m}$$

where q_1 (mg) is the total amount of protein in the solution before immobilization, q_2 (mg) is the unimmobilized enzyme in the residual solution, and m (g) is the weight of the support.

2.4. Enzyme activity assays

To investigate the activity of immobilized PGA, the hydrolysis of Pen G was performed as follows: the immobilized PGA obtained in Section 2.3 was placed in a fixed bed reactor with a length of 50 mm and an inner diameter of 4 mm. Pen G solution in phosphate buffer (pH 7.9, 0.05 M) was allowed to flow through the fixed bed reactor in a water bath. The substrate and products of the hydrolysis reaction were analyzed via high-performance liquid chromatography (HP series 1200 HPLC system equipped with a wavelength-variable UV detector). An Agilent ZORBAX SB-C18 column with dimensions of 250 mm × 4.6 mm was used at 30 °C. The mobile phase was composed of 55% (v/v) potassium phosphate buffer (pH 3.5, 0.02 M) and 45% (v/v) HPLC-grade methanol. The flow rate of the mobile phase was 1.0 mL/min, and the detector was set at a wavelength of 225 nm.

The apparent activity (A, U/g), conversion rate of Pen G (X, %), and specific activity (SA, U/mg-enzyme) were calculated according to the following formulas:

$$A = \frac{C_{6-\text{APA}} \times F}{W}$$

$$X = \left(1 - \frac{C_{\text{Pen G,out}}}{C_{\text{Pen G,in}}}\right) \times 100\%$$

$$\text{SA}(\text{U/mg}) = \frac{A}{q_E}$$