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Short communication

Immobilized β-glucosidase on magnetic chitosan microspheres for hydrolysis of straw cellulose

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ABSTRACT

β-Glucosidase immobilized on magnetic chitosan microspheres for potential recycling usage in hydrolysis of cellulosic biomass was investigated. The immobilized enzyme had an activity of 6.4 U/g support under optimized condition when using cellobiose as substrate. Immobilization resulted in less increase of the apparent K_m, low drift of the optimal pH, as well as improved stability relative to the free enzyme. The immobilized β-glucosidase was applied to enzymatic hydrolysis of corn straw to produce 60.2 g/l reducing sugar with a conversion rate of 78.2% over the course of a 32-h reaction. This conversion rate was maintained above 76.5% after recycling the enzyme for use in eight batches (total 256 h), showing favorable operational stability of the immobilized enzyme.

1. Introduction

The enzyme β-glucosidase (EC 3.2.1.21), which is commonly found in bacteria, animals and plants, cleaves β-glucosidic linkages in disaccharide or glucose-substituted molecules [1]. It has been widely used to produce aromatic compounds [2], stabilize juices and beverages [3], and improve the organoleptic properties of food and feed products [4]. In addition, β-glucosidase also plays an important role in cellulose degradation. Efficient hydrolysis of cellulosic biomass requires the synergistic activities of three types of cellulases—endoglucanase, cellobiohydrolase, and β-glucosidase [5]. Insufficient β-glucosidase activity could decrease rates of cellulose hydrolysis and cause the accumulation of cellobiose, causing the activities of both endoglucanase and cellobiohydrolase to be severely inhibited [6]. Accordingly, when the cellulases produced by *Trichoderma reesei* are used for the enzymatic hydrolysis of cellulose, supplementation with β-glucosidase from *Aspergillus niger* is essential to complement the deficiency of β-glucosidase in *T. reesei* extracts [7]. Given that the high cost of enzymes remains an obstacle to full-scale bioconversion of cellulosic biomass, ways to improve the efficiency of enzymatic hydrolysis have become a focus of current research efforts. Recycled usage of enzymes through their immobilization has been considered to offer a potentially significant cost saving during the hydrolysis of cellulosic biomass.

Various techniques have been developed for β-glucosidase immobilization. These include adsorption on Amberlite DP-1 [8], adsorption on different soil colloids from paddy soil [9], adsorption on chitosan following crosslinking mediated by glutaraldehyde [10], binding to an amine agarose gel [11], covalent binding and adsorption on silica gel and kaolin carriers [12], cross-linking on a chitosan-clay composite [13], entrapment in alginate gel and polyacrylamide gels [14], immobilization on Eupergit C [15], immobilization on an agarose matrix with polyethyleneimine and amine-epoxy [16], immobilization in sol-gel and Lentikats [17], and aggregate coating on polymer and magnetic nanofibers [18].

Immobilized β-glucosidase was reported to promote degradation of cellulosic biomass [19]. But there are few instances directly using immobilized β-glucosidase in hydrolysis of straw cellulose. An attractive and important feature of using immobilized enzyme to hydrolyze lignocellulosic materials is that the enzyme can be easily recovered for recycling from solid suspensions. Das et al. immobilized cellobiase on sol–gel routed mesoporous silica particles, and used a two-step hydrolytic procedure that involved first breaking the biomass down to cellobiose by cellulase activity, and then separating the liquid phase for the hydrolysis of cellobiose to glucose using immobilized cellobiase in order to recovery the immobilized cellobiase. Nonetheless, two-step enzymatic hydrolysis weakened

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the synergistic effect of cellobiase [20]. Dekker first reported the use of a magnetic immobilized β-glucosidase preparation for the saccharification of steam-exploded lignocellulose residues. The enzyme, which was immobilized onto polyethyleneimine-glutaraldehyde activated magnetite (PAM) or TiO_2-coated magnetite (TAM), could easily be recovered by applying a magnetic field, despite some loss of the immobilized enzyme during recovery [21].

Owing to the presence of free amine groups, which could form a Schiff's base in the presence of glutaraldehyde, chitosan is a common and ideal support material for enzyme immobilization. Among its characteristic advantages are its remarkable affinity for proteins, capacity for gel formation, good mechanical strength, resistance to chemical degradation, and anti-bacterial properties [22,23]. In addition, chitosan can be used as a basic material for magnetic carriers, which can be quickly separated from the reaction medium and effectively controlled by applying a magnetic field [24]. Moreover, chitosan is economically attractive, given that it is second only to cellulose as the most abundant natural polymer. Magnetic chitosan microspheres have been reported to carry immobilized laccase [25], and lipase [26]. Here we describe the modification of magnetic chitosan microspheres with immobilized β-glucosidase, and the application of these microspheres to the enzymatic hydrolysis of corn straw.

2. Material and methods

2.1. Material

β-Glucosidase (*A. niger*, Novozym 188, liquid ≥250 U/g (cellobiose)) was purchased from Sigma–Aldrich (Shanghai), and cellulase (*T. reesei*, 240 FPU/ml (the filter paper activity)) was provided by the KDN Biotech Group (Qingdao, China). Chitosan, cellobiose and other chemicals of analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai).

2.2. Preparation of magnetic chitosan microsphere

Chitosan powder was dissolved in 50 ml of propionic acid solution. Then, polyethylene glycol (PEG-400) and Fe_3O_4 were added into the solution. The resulting mixture was stirred at room temperature to remove air bubbles, and subsequently dropped into 2 M NaOH solution to form microspheres. After immersion in NaOH solution for 10 h and washing with deionized water to pH 7, the formed microspheres were collected by filtration and treated with glutaraldehyde solution at 25 °C for 2 h. Finally, the magnetic chitosan microspheres were recovered by washing away the redundant glutaraldehyde. The optimal concentrations (v/v) of chitosan (1.0%, 2.0%, 3.0% and 4.0%), PEG-400 (0.04%, 0.06%, 0.08% and 0.1%), propionic acid (1.0%, 1.5%, 2.0% and 2.5%), and glutaraldehyde (0.5%, 1.0%, 1.5% and 2.0%) were determined using an OA_{16} matrix, which is an orthogonal array of four variables and four levels. The ratios of chitosan/Fe_3O_4 tested were 1:1, 1:2, or 1:3.

2.3. Immobilization of β-glucosidase

Ten ml of diluted β-glucosidase solution (0.3–1.5 U/ml) was mixed with 1.0 g of magnetic chitosan microspheres. The mixture was stirred at 25 °C and 150 rpm for 15 h, and the immobilized enzyme was separated by removing the supernatant and washed several times with 0.05 M citrate buffer (pH 4.8), stored at 4 °C.

$$\text{Enzyme activity yield}\,(\%) = \frac{\text{Activity units of enzyme bound to the support}}{\text{Total activity units of added free enzyme}} \times 100$$

$$\text{Immobilization efficiency}\,(\%) = (\text{Total activity units of added free enzyme} - \text{Activity units of enzyme remaining in the supernatant})/(\text{Total activity units of added free enzyme}) \times 100$$

2.4. Determination of immobilization parameters

The activities of the free and immobilized enzyme were measured using concentrations of the substrate cellobiose that ranged from 0.2 to 30 mM, (0.05 M citrate buffer, pH 4.8) and either 1 ml of diluted free β-glucosidase or 0.1 g of immobilized β-glucosidase, which was add to the pre-equilibrated substrate and buffer in a total volume of 2 ml. The K_m and V_{max} values were determined using the Hanes-Woolf linearization.

The optimal pH was determined by measuring β-glucosidase activity in the pH range 3.0–7.0 at 50 °C. The temperature optimum was determined by assaying the enzyme activities at temperatures 30–80 °C. The stability of the enzyme was determined by incubating the enzyme in citrate buffer (0.05 M, pH 4.8) at 50 °C for up to 15 days (360 h).

2.5. Hydrolysis of cellulose via cooperation of cellulase and β-glucosidase

Pretreatment and hydrolysis experiments that involved corn straw were performed as described by Zheng [27]. The 80 g/l pretreated corn straw (PCS) was soaked in citrate buffer (0.05 M, pH 4.8) with the addition of cellulase at 20 FPU per gram PCS in a total working volume of 50 ml, and hydrolyzed at 50 °C and 80 rpm for 32 h. At the same time, free or immobilized β-glucosidase (10 U per gram PCS) was added to the mixture to assist the hydrolysis. All experiments were carried out in triplicate. Samples were taken from the reaction mixture periodically during incubation, and boiled for 10 min to terminate the reaction for sugar analysis. The total reducing sugar was estimated using the 3,5-dinitrosalicylic acid colorimetric method [28]. The enzymatic conversion was calculated as follows: enzyme conversion (%) = (reducing sugar × 0.9/carbohydrate in substrate) × 100.

The content of carbohydrate (cellulose and hemicellulose) in PCS ranged from 82 to 87% in various batches, when assayed according to the method of Van Soest [29].

2.6. Recycling usage of immobilized β-glucosidase in batch hydrolyses of pretreated corn straw

Batch hydrolysis of PCS was performed under the same conditions described above, except that only the immobilized β-glucosidase was used to assist hydrolysis of cellulose. After each cycle, the magnetic microspheres that carried immobilized β-glucosidase were harvested under an external magnetic field generated with amagnetic stirring apparatus JB-1A (Shanghai Scientific instrument Co.) by decanting the nonmagnetic solids and then washing the magnetic particles once with buffer (0.05 M, pH 4.8) for re-use in the next run.

2.7. Assay of β-glucosidase activity

Activity of β-glucosidase was measured as described by Ghose [30]. The reaction mixture with total volume of 2 ml, which comprised 15 mM cellobiose in citrate buffer (0.05 M, pH 4.8) and 1 ml of diluted free enzyme or 0.1 g immobilized enzyme, was incubated at 50 °C for 30 min, and then boiled for 10 min to terminate the reaction prior to glucose analysis using an SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, China). One unit (U) of enzyme activity was defined as the amount of enzyme that released 2 μmol of glucose per min.

3. Results and discussion

3.1. Preparation of magnetic chitosan microspheres

To enable simpler preparation of better chitosan microspheres than those generated from chitosan solution and acetic acid by shaping microspheres in a dispersion that comprises mineral oil-petroleum ether and an emulsifier [24–26], chitosan powder was dissolved in propionic acid solution, and NaOH solution was replaced above dispersion medium to generate the microspheres. Orthogonal experiments (our unpublished data) revealed the optimum formula for chitosan microsphere preparation to involve 1.0% (v/v) chitosan, 2% (v/v) propionic acid, 0.08% (v/v) PEG-400 and 0.5% (v/v) glutaraldehyde. The impact of different factors on the activity of immobilized enzyme was determined to be chitosan > glutaraldehyde > PEG400 > propionic acid, with the concentration of chitosan showing the largest effect and the content of propionic acid to have almost no effect.

In order to obtain magnetic chitosan microspheres, the magnetic material Fe_3O_4 (analytical grade) was added during the process used to generate chitosan microspheres. The chitosan/Fe_3O_4 ratio was reported to be the most effective parameter for the magnetic quality of the microspheres [24]. Both absorption capacity and activity of immobilized enzyme were tested on the magnetic chitosan microspheres with various chitosan/Fe_3O_4 (w/w) ratios (1:1, 1:2 and 1:3). The activity of immobilized enzyme, as well as the enzyme activity yield, both increased gradually with increases in Fe_3O_4 concentrations. This suggested that Fe_3O_4 might promote the immobilization of the enzyme on chitosan in addition to rendering the carrier magnetic. Analysis of the observed structures using