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Improving the performance of immobilized β-glucosidase using a microreactor

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Here, we have presented a technically simple and efficient method for preparing a continuous flow microreactor by employing immobilized β-glucosidase in a silica quartz capillary tube. Developing an immobilized enzyme layer on the inner wall of the capillary tube involved the modification of the inner wall using bifunctional crosslinking agents 3-aminopropyltriethoxysilane and glutaraldehyde before attaching β-glucosidase. The microreactor afforded unique reaction capacities compared with conventional batch operational configurations. These included enhanced pH and thermal stability during storage tests, increased conversion rates of cellobiose, and reduced product inhibition. The maximum conversion rate of soluble substrate cellobiose digestion in the microreactor was 76% at 50°C and pH 4.8 when the microreactor was operated continually over 10 h at a flow rate of 7 μL/min. This was markedly contrasting to the observed conversion rate of 56% when cellobiose was digested in a conventional batch mode under the same pH and temperature conditions. Reaction inhibition by glucose was significantly reduced in the microreactor. We postulate that the increased capacity of glucose to diffuse into the continual flowing media above the immobilized enzyme layer prevents glucose from reaching inhibitory concentrations at the substrate–enzyme interface.

[**Key words:** β-Glucosidase; Immobilization; Modification; Continuous-flow; Microreactor]

There remains a considerable scope for the development and commercial application of microreactor technologies using immobilized enzymes. Immobilized enzyme microreactors (IEMs) are cost-effective, small-volume and technically simple microfluidic devices that may be operated in batch or continuous flow configurations using microliter to milliliter reaction volumes (1). The operation of the IEMs presents some significant advantages over large scale solution-based bioreactor operations, most notably in terms of cost and energy savings associated with bioreactor design, construction and operation (2). IEMs application involves continuous and/or intermittent media flow rates through the vessel without potential loss of enzymes (washout) from the reactor. The near laminar media flow characteristics within the IEMs, when operated in a continuous- or intermittent-flow formats, reduce potential foam formation and turbulence or shear force aberrations that often affect large scale solution bioreactors (3,4). Immobilization of the enzyme increases its resistance against denaturation and enhances enzyme stability. A micro-scale reaction volume within the bioreactor vessel also allows for a more efficient heat transfer to occur within the vessel and rapid stabilization of reaction temperatures. Large interfacial areas and high specific activities of bound enzyme are features of IEMs. These features enable relatively rapid reaction times to be achieved consistently (5,6). Schwarz et al. (7) prepared a microstructured immobilized enzyme reactor based on the thermo-stable β-glycosidase CelB from *Pyrococcus furiosus* for the production of β-glucogallin (βGG). Substrate conversion measured as release of *o*-nitrophenol (*o*-NP) was rapid, leading to the complete depletion of the glucosyl donor within only 6–10 s in all experiments (7). IEMs have been successfully applied in various fields, from analysis and medical diagnostics, industrial production of drugs, chemicals, and fuels, and to facilitate greater process design innovation.

Surface materials can be modified by physical or chemical methods to obtain an infinite variety of surface properties conducive to immobilized enzyme microreactor development. There are numerous methods for enzyme immobilization such as surface adsorption on microfluidic channels or on beads (8–10). Kecskemeti and Gaspar (11) prepared a microchip-immobilized enzymatic reactor on which trypsin was covalently immobilized by carbodiimide activation. Barsan et al. (12) prepared an immobilized enzyme microreactor using the layer-by-layer (LbL) technique, for constructing a new enzyme biosensor.

β-Glucosidase (EC3.2.1.21, cellulases) is widely found in many plants, insects, yeasts, molds and bacteria in nature. β-Glucosidase participates in the metabolism of carbohydrate and plays an important role in maintaining normal physiological function of the organism. Thomsen et al. (13) prepared a coated-wall microreactor for continuous biocatalytic transformations using immobilized thermophilic β-glycosidase CelB. Song et al. (14) selected immobilized β-glucosidase obtained from *Aspergillus niger* for dyeing textiles with indigo. Here, we report a technically simple and efficient method for preparing a continuous flow microreactor by employing the immobilized β-glucosidase in a silica quartz capillary tube. Developing an immobilized enzyme layer on the inner wall of the capillary tube involves the modification of the inner wall with the

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bifunctional crosslinking agents 3-aminopropyltriethoxysilane and glutaraldehyde before the attachment of β-glucosidase. The performance of the microreactor was evaluated using substrate cellobiose under different operational conditions. Moreover, the microreactor was applied for continuous hydrolysis of cellobiose under conditions of certain product inhibition.

MATERIALS AND METHODS

Materials We used silica quartz capillary tubes (590 μm inner diameter) to construct enzyme bioreactors. The silica quartz capillary was obtained from the Nanjing Glass Tube Company.

Hydrofluoric acid (HF; 40%) was obtained from Shanghai Shenbo Chemical Co., Ltd. (Shanghai, China). Sodium hydroxide was purchased from West Long Chemical Co., Ltd. (Guangzhou, China). Hydrochloric acid was purchased from Shanghai Linfeng Chemical Reagent Co., Ltd. (Shanghai, China), and toluene was obtained from Shanghai JiuYi Chemical Co., Ltd. (Shanghai, China). 3-Aminopropyltriethoxysilane (APTES; ≥98%), cellobiose, and glutaraldehyde (25% aqueous solution) were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). β-Glucosidase (EC3.2.1.21) was obtained from Shanghai Source Biological Technology Co., Ltd. (Shanghai, China). APTES solution (10%) was prepared by mixing APTES and anhydrous toluene (1:10, v/v).

Preparation of β-glucosidase-immobilized microreactors The β-glucosidase-immobilized microreactor was prepared by adapting the procedure described by Gunda et al. (10). A schematic representation of the microreactor design is shown in Fig. 1. The design was based on constructing a larger area on the inner wall of the microtube using HF etching. The inner surface of the capillary was incubated with 1 mL of 20% (v/v) HF for 1 h at room temperature, and then rinsed with 100 mL of deionized water. The capillary was injected with a 1 mL solution of 25 mM hydrochloric acid and 25 mM sodium hydroxide using syringe pumps, followed by rinsing with deionized water until the pH of the eluent was neutral (15). This process increased the density of the silicon hydroxyl on the inner surface of the capillary tube.

The above capillary tube was perfused with 10% APTES for 12 h at room temperature. For removing unreacted silane, the capillary was repeatedly rinsed by anhydrous ethanol and deionized water. Then 2.5% glutaraldehyde was injected into the capillary.

The modified capillary was injected with 1 mg/mL β-glucosidase solution for approximately 14 h at room temperature, and subsequently rinsed with 20 mL of 5 mM sulfuric acid solution (pH was adjusted to 4.8 using ammonia water). The β-glucosidase-immobilized microreactor was filled with sulfuric acid solution (pH 4.8) and stored at 4°C until further used.

The amount of immobilized enzyme bound onto the inner surface of the capillary tube was quantified by measuring the protein concentration of the coupling solution before and after the immobilization procedure using the Bradford assay method (16). The amount of immobilized enzyme protein was calculated by the difference (17).

Continuous β-glucosidase activity assay The enzymatic activity of the microreactor was measured as the decomposition of 17 mM cellobiose under standard conditions (50°C, sulfuric acid solution pH 4.8) under continuous flow.

The microreactor was washed with sulfuric acid solution (pH 4.8) for 10 min. Then, cellobiose solution was injected at a flow rate of 7 μL/min for approximately 4 h. At steady-state conditions, samples were withdrawn from the outlet, and the amount of hydrolyzed cellobiose was measured by high performance liquid chromatography (HPLC). The enzymatic activity of each microreactor was determined as the hydrolysis concentration of substrate per residence time (μM/min).

HPLC analysis was conducted using a microbore HPLC system (Dionex, Surrey, UK) controlled by Chromeleon 7.0 software with a multi-well plate autosampler (Spark, Emmen, Holland). The HPLC system contained an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, Hertfordshire, UK), and analysis was conducted at 55°C using a mobile phase of 0.005 M H_2SO_4 at 0.6 mL/min. Detection was performed using a differential-refraction detector.

Biological catalysis properties of the microreactor The relative activities of free and immobilized β-glucosidase were assessed under varied pH (pH 3.5–7.5, 50°C) and temperatures (20–70°C, at pH 4.8) to determine the optimal reaction conditions. Relative activity is the percentage of enzymatic activity under the corresponding condition to the highest enzymatic activity.

For studying the stability of the microreactor, the residual enzyme activities of the free and immobilized β-glucosidase were investigated under varied pH (pH 2–8, 20°C) by incubating for 12 h and varied temperature (20–70°C, at pH 4.8) by incubating for 4 h. The storage stability of the microreactor was assessed by filling the reactor with sulfuric acid solution and maintaining it at 4°C. The residual enzyme activity is the percentage of the enzymatic activity under the corresponding condition to the initial enzyme activity.

Continuous-flow kinetics Enzymes used as biological catalysts exhibit increased production efficiencies; however, mass-transfer limits or environmental changes are not conducive to enzyme kinetic parameters (13,18–20). The conversion rate of the reactor is highly influenced by mass transfer. In continuous-flow kinetics, the variability in kinetic parameters [Km (app)] associated with media flow rate are investigated to identify conditions that are conducive to optimal reaction kinetics (20).

Enzyme kinetics in continuous-flow reaction systems can be evaluated using the Lilly–Hornby model (21), which is an adaptation of the standard Michaelis–Menten model for enzyme kinetics described by the following equation:

$$f \cdot [A]_0 = K_m \cdot \ln(1-f) + \frac{C}{Q} \quad (1)$$

Here, f is the fraction of the substrate converted to the final product during the reaction, Q is the flow rate of the substrate, $[A]_0$ is the initial substrate concentration, C is the reaction capacity of the microreactor, and K_m is the apparent Michael constant.

To investigate the impact of flow rate on kinetic constants, a kinetic study of continuous-flow experiments was conducted at 50°C. Flow rates ranged from 1 μL/min to 20 μL/min, and cellobiose concentration ranged from 4 mM to 70 mM in sulfuric acid solution (pH 4.8, pH value was adjusted with ammonia water).

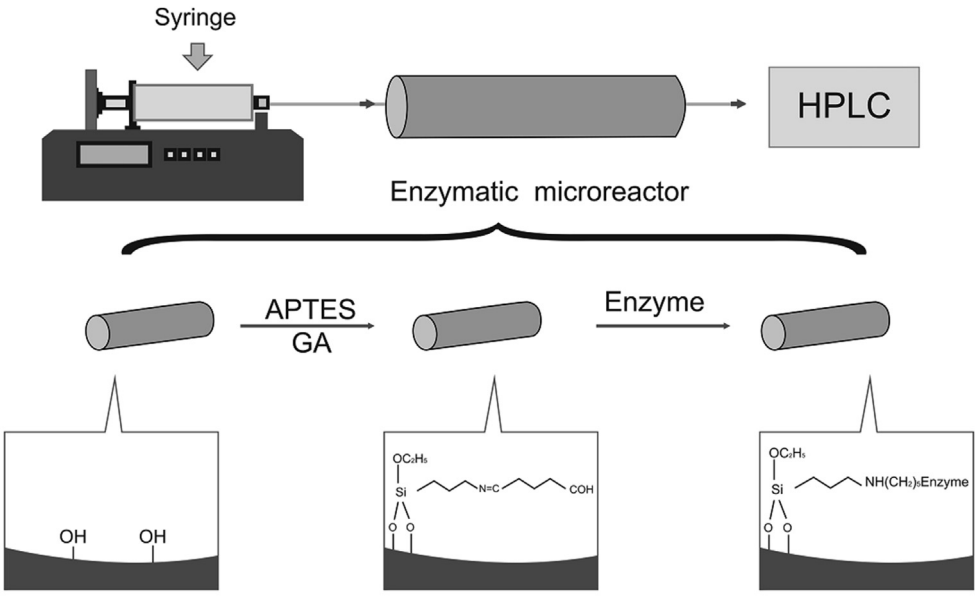

FIG. 1. Preparation of β-glucosidase-immobilized on the inner wall of a capillary microtube.