Advanced synthesis and application of Nano SiC@ β-glucosidase@ Fe₃O₄ composite

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Abstract. An improved method for immobilizing β-glucosidase was studied in present work. The immobilization included the following steps: preparing a nano silicon carbide carrier, mixing the carrier, β-glucosidase and a cross-linking agent to obtain immobilized β-glucosidase. Then, the immobilized β-glucosidase was mixed with Fe₃O₄ nanoparticles and self-assembled to obtain nano-silicon carbide@β-glucosidase@ Fe₃O₄ composite. This method improved the activity of immobilized β-glucosidase, and solved the technical problems of low β-glucosidase reuse rate and low enzyme activity retention on rate.

1. Introduction
β-Glucosidase (β-D-Glucosidase, EC3.2.1.21), also known as β-D-glucosidase, which belongs to the cellulase class and is an important component of the cellulolytic enzyme system. β-Glucosidase acts mainly on β-(1, 4) glycosidic bonds, hydrolyzes the non-reducing β-D-glucose bonds bound to the terminal and releases β-D-glucose and the corresponding ligands. In food development, β-glucosidase is used as a special flavor enzyme in the preparation of fruit juice, tea juice, fruit wine, which plays a better flavor enhancement effect [1, 2].

Nano-sized silicon carbide is composed of diamond grains of tiny size, within (1-100) nanometers (Shown in Figure1). Nano-silicon carbide retains the excellent mechanical properties of diamond, and at the same time, the large specific surface area and high surface activity brought by the nano-scale [3]. Besides, nano-silicon carbide has high surface activity, low loose density, and extremely with good mechanical, thermal, electrical and chemical properties. Nano-sized silicon carbide has a good development prospect in biological immobilized materials [4].
Magnetic nanomaterials have excellent properties in terms of light, electricity, heat, magnetism, and sensitivity. They have been widely used in fields such as magnetic fluids, catalysts, bioengineering, biomedicine, and environmental protection [5]. Among them, Fe₃O₄ magnetic nanoparticles have excellent characteristics such as superpara-magnetism, small size effect, surface effect, quantum tunneling effect, and so on. At the same time, Fe₃O₄ magnetic materials have small particle size and narrow distribution, excellent magnetic properties, stable surface properties and good biocompatibility [6].

The properties of immobilized β-glucosidase mainly depend on the immobilized carrier material and immobilization method [7]. In present study, nano-silicon carbide and Fe₃O₄ magnetic nano-magnetic materials are used as enzyme immobilization materials, and the immobilization method of β-glucosidase is optimized.

2. Experiment

2.1. Chemicals and biochemical reagents
α-pNPG, glucose-6-phosphate dehydrogenase, hexokinase and cellobiose were purchased from Sigma-Aldrich. Glutaraldehyde, salicin, silicon carbide, sodium acetate were purchased from Xi’an Hexin chemical reagent instrument co. LTD, China. All reagents were analytical-reagent grade.

2.2. Preparation of nano silicon carbide carrier
0.5g nano silicon carbide powder (average particle size 40nm) was added into 80mLhydrogen peroxide (30%), magnetic stirring, and 105℃ reacted for 4h. 9000rpm centrifuged for 10min then repeated washing with distilled water to neutrality. After vacuum drying at 80℃ for 12 hours, hydroxylated nano silicon carbide powder was obtained. Dissolved 0.5g of hydroxylated nano silicon carbide powder in sodium acetate buffer (pH 4.8). Dispersed the powder by ultrasonic for 60 minutes. To connect glutaraldehyde (mass fraction of 5%), shaked for 5 hours, centrifuged at 9000 rpm for 10 minutes, and then washed. After the precipitation, nano silicon carbide carrier was vacuum dried.

2.3. Immobilized β-glucosidase
12 mg of the nano-silicon carbide carrier was dissolved in sodium acetate buffer (pH 4.8). After ultrasonic dispersion, 6 mg β-glucosidase was added, shake for 30 minutes, then 2% glutaraldehyde was mixed. The mixture was shaken at room temperature for 5 hours, to immobilized β-glucosidase was obtained after centrifugation and wash.
2.4. Self-assembly of SiC@ β-glucosidase@ Fe3O4 composite
16.56mg immobilized β-glucosidase and 8mg of Fe3O4 nanoparticles (particle size of 20nm) were mixed and shake at room temperature for 50 minutes to complete the self-assembly of SiC@ β-glucosidase@ Fe3O4 composite, then washed and magnetically separated.

2.5. Enzyme assays
The enzyme assays were performed at 30 °C, pH7, in 1 mL solution mixing 500μL of citrate potassium phosphate buffer. Reactions were carried out continuously by using the thermostated Agilent spectrophotometer. The α-glucosidase activity was detected by using α-p-nitrophenyl-D-glucopyranoside (α-pNPG) as substrate and monitoring the release of p-nitrophenyl (pNP) at 405 nm. Activity of other substrates was measured the release of glucose and the production of NADPH at 340 nm by using a coupled spectrophotometric assay, with the simultaneous use in the assay of hexokinase and glucose-6-phosphate dehydrogenase. The assay was performed by the addition of 20μL SiC@ β-glucosidase@ Fe3O4 composite to the 1 mL reaction mix containing 5 mM MgCl2, 1 mM ATP, 1 mM NADP, 5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase and substrate at required concentration. Velocities were obtained as the initial reaction rates (v), which were proportional to the amount of β-glucosidase.

3. Results and discussion

3.1. Immobilization efficiency and relative enzyme activity
The amount of hydroxylated nano-silicon carbide β-glucosidase, the enzymatic activity and the immobilization efficiency were all measured, as shown in Table 1. Among them, the immobilization efficiency was determined, that is, the ration of protein quality of the immobilized enzyme/the protein quality used for immobilization (measured by the BCA protein kit).

| SiC-OH (mg) | β-glucosidase (mg) | Glutaraldehyde (%) | Fe3O4 (mg) | Immobilization Efficiency (%) | Relative activity (%) |
|------------|------------------|-------------------|------------|-------------------------------|---------------------|
| 12         | 6                | 2                 | 8          | 92                            | 98                  |

Table 1. The test parameters of Immobilized β-glucosidase.

3.2. Determination of kinetic parameters
The immobilized β-glucosidase was reacted at 60°C, pH 5.5, and the substrate concentration was 0.5-50 mM, and the Michaelis constant Km and the maximum reaction rate Vmax were measured respectively. The hydrolysis reaction of salicin and cellobiose by immobilized β-glucosidase conformed to the Michaelis equation, and the kinetic parameters were obtained. Salicin was used as the substrate, Km was 7.85mM, and Vmax was 6.89mM/min. Cellobiose was used as the substrate, Km was 1.58mM and Vmax was 8.43mM/min. The Michaelis constant indicates of immobilized β-glucosidase had higher affinity for cellobiose than salicin.

3.3. Thermal stability determination
Free β-glucosidase and the immobilized β-glucosidase were reacted in a sodium acetate buffer at 70°C, pH=4.8, and the enzyme activity assay was performed every half hour. It showed from Figure 2 that the free enzyme almost lost all its activity within 1 hour, the relative enzyme activity of immobilized β-glucosidase was 78% for 2 hours, and 52% of the activity was still retained after 3 hours.
3.4. Determination for reusability

The immobilized β-glucosidase was reacted with α-pNPG in sodium acetate buffer at 60°C, pH=4.8 for 5 minutes, and the supernatant was removed after magnetic separation. After completing an enzymatic reaction, the isolated immobilized β-glucosidase is reacted again with newly α-pNPG. This process is repeated 6 times. The enzyme activity was measured (see Figure 3). The immobilized β-glucosidase retained 87% of the activity after 6 consecutive reactions (see Figure 5), keeping a high catalytic activity.

4. Conclusion

Nano SiC@ β-glucosidase@ Fe₃O₄ composite improved the immobilization efficiency and thermal stability of β-glucosidase, especially the catalytic activity. It also increased the service life of β-glucosidase, and initially reduces the use cost of β-glucosidase. This present work provides an advanced β-glucosidase coupling covalent method and cross-linking method by self-assembled magnetic material immobilization technology.
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