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Immobilization of β-glucosidase on a magnetic nanoparticle improves thermostability: Application in cellobiose hydrolysis ☆

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HIGHLIGHTS

- Immobilization of β-glucosidase on a functionalized magnetic nanoparticles was achieved.
- Enhancement in β-glucosidase thermostability was observed upon immobilization to nanomaterials.
- Nanomaterial immobilized enzyme was used for cellobiose hydrolysis.
- Nanoparticle–enzyme conjugate retained more than 50% enzyme activity up to the 16 cycles of substrate hydrolysis.

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ABSTRACT

The objective of the present work was to develop a thermostable β-glucosidase through immobilization on a nanoscale carrier for potential application in biofuel production. β-Glucosidase (BGL) from *Aspergillus niger* was immobilized to functionalized magnetic nanoparticles by covalent binding. Immobilized nanoparticles showed 93% immobilization binding. Immobilized and free BGL were characterized using Transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR) techniques. Free and immobilized enzyme exhibited different pH-optima at pH 4.0 and 6.0, respectively, but had the same temperature optima at 60 °C. Michaelis constant (K_M) was 3.5 and 4.3 mM for free and immobilized BGL. Thermal stability of the immobilized enzyme was enhanced at 70 °C. The immobilized nanoparticle–enzyme conjugate retained more than 50% enzyme activity up to the 16th cycle. Maximum glucose synthesis from cellobiose hydrolysis by immobilized BGL was achieved at 16 h.

1. Introduction

Recently lignocellulosic ethanol has received attention as an alternative to transportation fuel to reduce global dependence on the limited fossil energy resources (Cho et al., 2012). Lignocellulosic biomass contains large amounts of cellulosic polymers (Sorensen et al., 2011) and the hydrolysis product of cellulosic polymers, glucose, can be readily fermented into ethanol or high-value biochemicals. First generation biofuel is based mainly on sugar and oil that are derived from food crops. The production of biofuels from first generation feedstock has potential to compete with food production if not implemented carefully. Second generation biofuel technology uses non-food feedstock, including lignocellulose biomass, such as the waste material from agricultural and forestry production, as well as urban organic waste and thus is environmentally more sustainable (Puri et al., 2012). Despite the sustainable availability and low cost of lignocellulosic biomasses, production processing costs are high making the current methods cost prohibitive.

During the hydrolysis of cellulose, increasing cellobiose concentration has an inhibitory effect on the activity of endoglucanases and cellobiohydrolase, thereby decreasing the rate of fermentable sugar production. The addition of extra β-glucosidase (BGL) reduces the cellobiose concentration and in turn helps maintain the activity of endoglucanases and cellobiohydrolase. However, this approach is not economical in the downstream process (Xue and Woodley, 2012). In order to make the biofuel production process more economical, improvement of the enzyme assisted technology is necessary. Enzymatic hydrolysis can be improved economically by increasing thermal stability, efficiency or recyclability of enzymes. Immobilizing the enzyme on/in a solid matrix

☆ Supplementary information (SI) available: Free and immobilized BGL catalytic activity as a function of pH and temperature.

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support can achieve one of more of these factors (Jung et al., 2011). Different immobilization supports have been evaluated for BGL immobilization (Jung et al., 2011). Recently, new novel nanomaterials have high surface areas and are attractive for increased enzyme loading compared to conventional bulk supports (Verma et al., 2013). Enzymes for bioenergy production were immobilized on various types of nanomaterials (Cho et al., 2012). Among binding methods, covalent immobilization provides the most stable bonding between amino groups of enzyme and carbonyl groups of the glutaraldehyde activated nanomaterials supports (Wang et al., 2009; Puri et al., 2013).

In this present work, iron oxide magnetic nanoparticles were synthesized and enzyme immobilization onto the activated nanoparticle supports was carried out using a covalent binding method. The activity, thermal stability and recyclability of free and immobilized enzyme were characterized with respect to cellobiose hydrolysis.

2. Methods

2.1. Materials

β-Glucosidase (EC 3.2.1.21) from *Aspergillus niger* (60 U/g), a purified enzyme in powder form, and a glucose oxidase (GOD-POD) assay kit, were procured from Sigma. A protein assay kit (Bio-Rad protein dye reagent concentrate) was procured from Bio-Rad. Sodium acetate, citric acid, sodium carbonate, p-nitrophenol, para-nitrophenyl-β-D-glucopyranoside, cellobiose, sodium chloride, potassium phosphate monobasic and dibasic, were from Sigma. Ferric chloride, ferrous chloride and sodium hydroxide were purchased from Ajax Fine Chemicals. All other reagents were of analytical grade.

2.2. Synthesis and characterization of iron oxide nanoparticles

2.2.1. Synthesis of iron oxide nanoparticles

Magnetic iron oxide nanoparticles were synthesized using a hydrothermal technique. First, 60 ml of 0.125 M $FeCl_3{\cdot}6H_2O$ and 30 ml of 0.125 M $FeCl_2{\cdot}4H_2O$ aqueous solutions were mixed with 80 ml of deionized water. The aqueous solution was constantly stirred using a mechanical stirrer at 200 rpm. Then 30 ml of 1 M NaOH was added drop wise, resulting in the formation of black precipitates. The precipitated particles were autoclaved at 150 °C for 12 h. Subsequently, the washed nanoparticles were freeze dried at −80 °C for 24 h.

2.2.2. Characterization of magnetic nanoparticles

The crystal structure of the nanopowder was characterized by XRD using a PanAnalytical X'Pert pro X-ray diffractometer (40 Kv, 30 mA) with CuKα radiation at a step width of 0.02° and 2 s per step. The morphology of the synthesized particles was characterized by Transmission Electron Microscopy (TEM) using a JEOL 2100M microscope (Frenchs forest, NSW, Australia) with the electron beam energy of 200 kV. The particle size distributions of the synthesized nanoparticles were measured using a dynamic light scattering (DLS) method with a Malvern Zetasizer Nano instrument (Malvern instruments Ltd., Worcestershire, UK). The specific surface area of the iron oxide nanoparticles was analyzed by the Brunauer–Emmett–Teller (BET) gas absorption method using a micromeritics Tristar 3000 system (Micromeritics Instrument Corporation, Norcross, GA). Magnetizations versus applied magnetic field (*M–H* curves) measurements were conducted using a semiconductor quantum interference device (SQUID) magnetometer (Quantum Design Inc) at room temperature.

2.3. Immobilization of BGL on magnetic iron oxide nanoparticles

Magnetic nanoparticles were washed thrice with deionized water and then suspended in 1 M glutaraldehyde solution. Support activation was carried out at 37 °C in a shaker (250 rpm) for 1 h. The activated support (100 mg) was removed by a magnet and then washed at least three times with 30 ml of distilled water to remove the excess glutaraldehyde and subsequently washed with an enzyme assay buffer solution.

The purified enzyme (20 mg in 20 mM sodium acetate buffer, pH 4.0; 60 U/g) was incubated with the activated support in an enzyme assay buffer solution at 37 °C for 20 h. Non-covalently adsorbed protein was removed thereafter by thorough washing of the nanoparticles with deionized water and sodium acetate buffer solution. The supernatant was used for BGL protein analysis and residual enzyme activity. The immobilization efficiency and immobilization yield were calculated based on the earlier optimized method (Verma et al., 2012). Protein content of the enzyme solution before and after immobilization in the washing buffer solution was determined by the Bradford method (Bradford, 1976).

2.4. Biophysical characterization of immobilized nanoparticle

The enzyme-immobilized nanoparticles were characterized by TEM, and FTIR. The FTIR analysis was performed based on the earlier optimized conditions (Verma et al., 2012).

2.5. Determination of enzyme activity

The enzyme activity for free and immobilized BGL was measured as per the method of Takahashi et al. (2011). The reaction mixture contained 0.02 M sodium acetate buffer (2.9 ml) at pH 4.0, 10.0 mM p-nitrophenyl-β-D-glucopyranoside (0.09 ml), and enzyme solution (2 U) and was incubated at 60 °C for 10 min before being stopped by adding 0.5 M sodium carbonate (0.5 ml). The liberated 4-nitrophenol was measured by UV–Vis Spectrophotometer (Shimadzu, Japan) at 405 nm and its concentration calculated from a plot constructed for standard p-nitrophenol. One unit of enzyme activity is defined as 1 μmol of p-nitrophenol liberated per minute at pH 4.0, and a temperature of 60 °C. Activity of immobilized BGL (5 mg) was measured with pNPG (equal amounts corresponding to free enzyme) and cellobiose at 60 °C. The aliquots were removed at intervals of 4 h to measure the degree of cellobiose hydrolysis. All enzyme assays were performed in triplicate reported as mean values ± SD.

2.6. Biochemical characterization of the free and immobilized BGL

The optimum pH for immobilized and free BGL activity was determined using standard assay conditions in sodium acetate buffer at varying pH (pH 3.0–8.0, 20 mM). Relative activities of the free and immobilized BGL at varying pH values were quantified relative to the control activities at pH 4.0 and 6.0 for the free and immobilized BGL.

The effect of temperature on free and immobilized BGL activity at pH 4.0 and 6.0, was determined by varying the temperature in the 30–80 °C range. Other experimental conditions adhered to the standard assay protocol. The activity at 60 °C was taken as control for the calculation of relative residual activity for free and immobilized BGL.

2.7. Determination of kinetic parameters

Kinetic parameters of free and immobilized BGL at 60 °C were determined using different pNPG concentrations in the range of 2–10 mM in 0.02 M sodium acetate buffer at pH 4.0 and 6.0,

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