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Dendrimer-like nanoparticles based β-galactosidase assembly for enhancing its selectivity toward transgalactosylation

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ABSTRACT

Functional nanomaterials have been pursued to assemble nanobiocatalysts since they can provide unique hierarchical nanostructures and localized nanoenvironments for enhancing enzyme specificity, stability and selectivity. Functionalized dendrimer-like hierarchically porous silica nanoparticles (HPSNs) was fabricated for assembling β-galactosidase nanobiocatalysts for bioconversion of lactose to galacto-oligosaccharides (GOS). The nanocarrier was functionalized with amino (NH_2) and carboxyl (COOH) groups to facilitate enzyme binding, benchmarking with non-functionalized HPSNs. Successful conjugation of the functional groups was confirmed by FTIR, TGA and zeta potential analysis. HPSNs-NH_2 showed 1.8-fold and 1.1-fold higher β-galactosidase adsorption than HPSNs-COOH and HPSNs carriers, respectively, with the highest enzyme adsorption capacity of 328 mg/g nanocarrier at an initial enzyme concentration of 8 mg/ml. The HPSNs-NH_2 and β-galactosidase assembly (HPSNs-NH_2-Gal) demonstrated to maintain the highest activity at all tested enzyme concentrations and exhibited activity up to 10 continuous cycles. Importantly, HPSNs-NH_2-Gal was simply recycled through centrifugation, overcoming the challenging problems of separating the nanocarrier from the reaction medium. HPSNs-NH_2-Gal had distinguished catalytic reaction profiles by favoring transgalactosylation, enhancing GOS production of up to 122 g/l in comparison with 56 g/l by free β-galactosidase. Furthermore, it generated up to 46 g/l GOS at a lower initial lactose concentration while the free counterpart had negligible GOS production as hydrolysis was overwhelmingly dominant in the reaction system. Our research findings show the amino-functionalized HPSNs can selectively promote the enzyme activity of β-galactosidase for transgalactosylation, which is beneficial for GOS production.

1. Introduction

The specificity and selectivity of enzyme open a new door for development of green and sustainable enzyme-catalyzed bioprocesses. Enzymatic reactions are environmentally friendly due to low chemical consumption without release of toxic by-products. Nevertheless, the biocatalysts constitute the majority of the operational cost when they are applied in large scale processes, and their low stability and reusability in reactor conditions impede their industrial applications [1]. The immobilization of enzyme on support materials is of immense scientific interest. Such technology is able to shelter and/or stabilize enzymes when they are exposed to chemical and environmental harsh conditions. The immobilization also allows the recovery and reuse of these biocatalysts, hence reducing the cost of the biocatalytic process [2,3]. Enzymes have been immobilized onto natural/synthetic polymers or inorganic materials [4]. Despite the fact that optimal immobilization protocols have been extensively studied, retaining the enzyme activity upon immobilization entails persistent investigation. Enzyme activity reduction on support materials remains a significant challenge due to the conformation change upon immobilization [5]. Hence, the development of appropriate enzyme carriers, which can preserve or enhance the enzyme catalytic function, presents a profound research interest.

Nanobiocatalysis, an emerging technology that assembles enzyme molecules onto nanomaterial carriers, synergistically integrates advanced nanotechnology with biotechnology [7]. The technology can increase the enzyme stability and enhance enzyme activity. To date, the enzyme has been immobilized on diverse support materials such as nanofibres [8], nanotubes

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[9], nanocomposites [10], nanogel [11] and nanoparticles [12]. Silica nanoparticles offer uniform nanopores and tuneable periodic nanostructures which harmonise the diffusion of guest biomolecules with different sizes [13]. It has been recognized that the pore size is vital for enzyme immobilization. Enzyme molecules are incapable to enter into the pore of nanoparticles when the pore is smaller than the molecular size of the enzyme, resulting in a low enzyme loading. In contrast, nanoparticles with pore sizes larger than the enzyme molecular size may reduce the enzyme activity, which may be ascribed to the enzyme leaching or reduction in the enzyme stability [14,15]. Anchoring of biomolecules onto the nanoparticles surface nay result in a strong binding that may decrease the biological activity of the enzyme [16]. In addition, chemical reagents in the immobilization protocol such as crosslinking agents (i.e., glutaraldehyde and epichlorohydrin) lead to a certain level of enzyme activity reduction [6]. Furthermore, enzyme molecules embedded inside the traditional enzyme supports could not directly contact substrates, thus the enzyme supports are unable to maximize the usage of the enzyme, and therefore some of the expensive enzyme is wasted. It is, therefore, constant efforts are being made into exploring robust and functional enzyme carriers.

Dendrimer-like silica nanoparticles with hierarchical pores (HPSNs) were recently developed with unique properties [17]. The carriers have been demonstrated as advanced delivery nanocarriers for siRNA and DNA molecules. The materials were constructed with specific pore structures to increase the surface area for a high loading of biological molecules. Each cavity in the nanostructure can act as a nanobioreactor for enzymatic reaction after the cavity is immobilized with the enzyme. Importantly, the nanocarrier possesses centre-radial open pores that are vital in coordinating diffusion of molecules with different sizes such as substrates and products, and as a result, it enhances the biomolecule interactions as well as reduces the associated mass transfer limitation. Another major issue associated with nanobiocatalysis is to recover nanobiocatalysts from the aqueous solution and reuse them due to their nano-meter size. Magnetic nanoparticles are often employed to realize the recovery by placing them in a magnetic field [18]. For instance, the immobilization of lipase was realized on magnetic chitosan microspheres [19] and Fe_3O_4/poly(styrene-methacrylic acid) magnetic microsphere [20] for enzymatic production of biodiesel from soybean oil. Such an operation poses a significant challenge for scaling up. HPSNs, however, offer easy separation and recovery through sedimentation or centrifugation, which can simplify the recycle process for HPSNs-based nanobiocatalysts.

In the present work, HPSNs were synthesized as nanocarriers to host β-galactosidase. The nanocarriers were functionalized with amino (NH_2) and carboxyl group (COOH) to promote covalent binding with the enzyme. Non-specific binding of the enzyme is expected to occur on the surface of non-functionalized HPSNs. The schematic representation of HPSNs functionalization and enzyme binding is illustrated in Scheme 1. The adsorption performance of three types of HPSNs was evaluated through bovine serum albumin (BSA) and β-galactosidase at different protein concentrations. The β-galactosidase activity was compared for the three HPSNs. HPSNs-NH_2 was then chosen to immobilize β-galactosidase for assessing its recyclability up to 10 continuous runs and its performance in converting lactose into galacto-oligosaccharides (GOS).

2. Materials and methods

2.1. Chemicals

Cetyl trimethylammonium bromide (CTAB), ammonia, ethyl ether, ethanol, tetraethoxysilane (TEOS), 3-aminopropyltriethoxysilane (APES), hydrochloric acid (HCl), *N*,*N*-dimethylformamide (DMF), succinic anhydride, triethylamine (TEA), ammonium hydroxide, sodium carbonate and phosphoric acid were of analytical grade without further purification. Bovine serum albumin (BSA), Kluyveromyces lactis β-galactosidase, *o*-nitrophenyl-β-D-glactopyranoside (ONPG) and coomassie brilliant blue G-250 were obtained from Sigma–Aldrich. Potassium phosphate buffer (PBS) (pH 7.2) was procured from Gibson.

2.2. Preparation and functionalization of nanoparticles

The preparation and functionalization of HPSNs-NH_2 were conducted using the methods developed by Du et al. [17]. A total of 0.5 g of CTAB was dissolved in an emulsion system composed of 70 ml of H_2O, 0.8 ml of aqueous ammonia, 15 ml of ethyl ether and 5 ml of ethanol. The mixture was vigorously stirred at 1000 rpm for 0.5 h at room temperature. The stirring was continued for 4 h after addition of a mixture of TEOS (2.5 ml) and APES (0.1 ml). After 4 h, 1 ml of HCl (37%) was added in order to stop the base-catalyzed reaction. A white precipitate of amino-functionalized silica nanoparticles (HPSNs-NH_2) was obtained from 12 min centrifugation at 4200 rpm. The precipitate was washed with ethanol and water. Treatment with ethanoic HCl (15 ml of concentrated HCl in 120 ml ethanol) was conducted under stirring at 70 °C for 24 h to extract CTAB template. The extracted particles, obtained from centrifugation at 4200 rpm for 12 min, were washed with water for three times. HPSNs with the carboxyl group (HPSNs-COOH) was synthesized by reacting HPSNs-NH_2 (50 mg in 20 ml DMF) with succinic anhydride (0.45 g in 5 ml DMF) in the presence of TEA (0.45 ml) for 5 h. Non-functionalized HPSNs was obtained by calcining HPSNs-NH_2 at 550 °C for 4 h.

2.3. Characterization of nanoparticles

The characterization of HPSNs-NH_2 and HPSNs-COOH using scanning electron microscopy (SEM) was performed under a Philips XL30 field emission scanning electron microscope operated at 10 kV. Fourier transform infrared (FTIR) spectra of samples were recorded on a Thermo Scientific NICOLET 6700 FTIR spectrometer at room temperature. TriStar II surface area and porosity analyzer from Micromeritics at −196 °C was used for nitrogen adsorption–desorption measurements using the volumetric method. Brunauer–Emmett–Teller (BET) specific surface areas were calculated by using adsorption data at a relative pressure range of P/P_0 range of 0.05–0.25. The Barrett, Joyner, and Halenda (BJH) method was used to estimate pore size distributions from adsorption branch of isotherm. The amounts of N_2 adsorbed at the single point of $P/P_0 = 0.99$ was used to determine pore volumes. The particle sizes and zeta-potentials dispersed in PBS (pH 7.5) were measured using a Malvern Zetasizer Nano ZS (Malvern Inst. Ltd., U.K.) equipped with four-side clear cuvettes or the ZET 5104 cell at room temperature. S60/51920 TGA/DSC (thermogravimetric/differential scanning calorimeter) analyzer from Setaram Instrumentation was used for thermogravimetric analysis (TGA) of samples using an oxidant atmosphere (air, 30 ml/min) (heating ramp of 10 °C/min from room temperature to 800 °C).

2.4. Enzyme immobilization on nanoparticles

Silica nanoparticles (10 mg) were dispersed in 1 ml PBS buffer (pH 7.2) and sonicated for 30 min. The mixtures were mixed into BSA (0–10 mg/ml) or β-galactosidase (0–9 mg/ml) and gently stirred overnight at 4 °C [21]. The particles were recovered through centrifugation at 4200 rpm for 10 min and washed with water to remove free BSA or enzyme on the surface of nanoparticles. The supernatant and washing solution were collected to measure the concentration of non-adsorbed protein.

2.5. Production of galacto-oligosaccharides

Lactose (50–400 g/l) was prepared by dissolving into PBS (pH 7.2) at 60 °C. After the solution temperature cooled down to room temperature, free or immobilized enzyme were added and incubated at 37 °C in an orbital shaker at 200 rpm. Samples were drawn at pre-set intervals for analysis (1–5 h) and immediately heated in boiling water for 5 min to deactivate the enzyme activity [22]. The mixture was centrifuged to separate the supernatant and nanoparticles. The resultant supernatant was filtered using 0.45 μm nylon filters, diluted 40 times and analyzed by high performance liquid chromatography (HPLC).

2.6. Chemical analysis

The enzyme concentration was assayed using the method described by Bradford [23]. A total of 100 mg Coomassie brilliant blue G-250 were dissolved in 50 ml of 95% ethanol, 100 ml of 85% phosphoric acid and diluted with water to a final volume of 1 l with gentle mixing. A total of 100 μl sample was added into 5 ml of the Bradford solution, mixed homogenously, and allowed to react for 5 min and measured at a 595 nm spectrophotometer. Protein concentration was determined based on the calibration curve using BSA as a protein standard. The adsorption yield and adsorption capacity of the immobilized enzyme were calculated using Eqs. (1) and (2), respectively, where P_i is the initial concentration of protein that is subjected for immobilization, P_w and P_s are the amount of protein in washing solution and supernatant, respectively [24].

$$\text{Adsorption yield}\,(\%) = \frac{\left[P_i - (P_w + P_s)\right]}{P_i} \times 100 \quad (1)$$