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Immobilized β-Galactosidase on Functionalized Nanoparticles and Nanofibers: A Comparative Study

Mailin Misson [1, 2], Bo Jin [1], and Hu Zhang [1+]

[1] School of Chemical Engineering, University of Adelaide, Adelaide, SA 5000, Australia
[2] Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400, KK, Sabah, Malaysia

Abstract. In this study, two β-galactosidase nanocarriers made from nanofibers and nanoparticles were fabricated for bioconversion of lactose to galacto-oligosaccharides (GOS). Polystyrene nanofibers (PSNF) were synthesized using electrospinning and functionalized through chemical oxidation. Amino-carrying dendrimer-like silica nanoparticles with hierarchical pores (HPSNs) were fabricated through a one-pot sol-gel approach. PSNF-β-galactosidase (PSNF-Gal) and HPSNs-β-galactosidase (HPSNs-Gal) nanobiocatalyst assembly promises excellent biocatalyst activity, stability and functionality. The findings demonstrated PSNF and HPSNs possess great potential as enzyme nanocarriers. It is evident by 80 % adsorption yield in both cases. PSNF-Gal was found 8-fold greater adsorption capacity than HPSNs-Gal. In comparison to free β-galactosidase, PSNF-Gal also exhibited exceptional catalytic ability by favouring transgalactosylation over hydrolysis. As a result, the GOS formation increased from 19 % to 28 % and reduced undesirable products in lactose bioconversion. HPSNs, on the other hand, yielded 25 % GOS with a significant amount of hydrolysis by-products at similar operating conditions.

Keywords: Nanoparticles, nanofibers, nanobicocatalyst, enzyme immobilization.

1. Introduction

The nanobiocatalyst has become prominent research interests in assembling enzyme onto nanomaterial carriers. It is an emerging innovation that combines nanotechnology and biotechnology advances, aiming to improve enzyme stability, capability, and engineering performances [1]. Nanomaterial supports provide a large surface area and a modifiable surface to tailor the biotechnological requirements. To date, functional nanomaterials, such as nanofiber scaffolds, nanotubes, nanoparticles, nanocomposites and nanosheets, have been used as enzyme carriers. Despite the constant efforts in optimizing the immobilization protocol, enzyme poor stability such as leakage and deactivation in a reactor system remain as the major challenges in nanobiocatalyst development. Therefore, it leads to the need of exploring new technology for nanobiocatalysts.

Among the nanostructured materials examined for the nanobiocatalyst assembly, nanostructured fiber promises high enzyme loading and homogenous dispersion in a liquid phase. Their low hindrance in mass transfer due to high porosity and interconnectivity suits for bioprocesses using bioreactor systems [2]. On the other hand, porous materials like nanoparticles offer a number of outstanding characteristics, including excellent mechanical strength and uniform nanopores. In addition, nanoparticles possess tuneable periodic nanostructures which favourable for hosting large and small biomolecules [3]. The success of nanobiocatalyst technology is determined by catalytic stability, recyclability, high throughput, and productivity for a large-scale process.

In this work, we fabricated two enzyme carriers, polystyrene nanofibers (PSNF) and dendrimer-like silica nanoparticles with hierarchical pores (HPSNs). The PSNF was fabricated using electrospinning and

[+]Corresponding author. Tel.: + 61 88313 3810
E-mail address: hu.zhang@adelaide.edu.au.

functionalized through chemical oxidation. The dendrimer-like silica nanoparticles with hierarchical pores (HPSNs) was synthesized using sol-gel method and functionalized with amino groups. The functional groups are important as anchoring sites for enzyme binding. The potential of the nanocarriers in immobilizing enzyme were evaluated and compared. Next, their bioengineering performance in lactose bioconversion was assessed and benchmarked with free enzyme as comparative study. Lactose is abundantly present in dairy wastewater which has been regarded as high organic loading wastewater, causing significant eutrophication and toxicity in water streams [4]. Their bioconversion into valuable products such as galacto-oligosaccharide (GOS) endows a promising solution. GOS possess demonstrable health benefits such as prebiotic function, low caloric sugar alternatives, and low cariogenicity [5]. Therefore, this study is not only beneficial for sustainable green waste management, but also for the production of value-added products. The nanofiber and nanoparticle-based nanobiocatalysts also may be potentially viable for a range of enzyme-catalysed processes.

2. Materials and Methods

2.1. Preparation and functionalization of nanomaterials

Polystyrene (20 w/v) was dissolved in *N, N*-dimethylformamide (DMF) with gentle stirring for overnight to form a homogenous solution. The resultant solution was placed inside a 1-mm inner diameter needle tip of 5-mL syringe which was connected to a 25 kV voltage power supply. The flow rate was fixed at 2.5 ml/h while the electrospun polystyrene nanofibers (PSNF) were cast onto a metal-surface collector with a distance of about 10 cm from the needle tip. Approximately 1 cm x 2.5 cm piece of polystyrene nanofibers (PSNF) were immersed in nitric acid (HNO_3) (69%) for 2 h at room temperature [7]. The treated support was rinsed with water and PBS (pH 7.2) for three times to remove excess acids.

The preparation and functionalization of HPSNs were conducted using the methods developed by Du *et al.* [6]. The emulsion system comprised of 0.5 g of Cetyl trimethylammonium bromide (CTAB), 70 mL of H_2O, 0.8 mL of aqueous ammonia, 15 mL of ethyl ether and 5 mL of ethanol. The solution mixtures were vigorously stirred at 1000 rpm for 0.5 h at room temperature and stirred continuously for 4 h after addition of tetraethoxysilane (TEOS) (2.5 mL) and 3-aminopropyltriethoxysilane (APES) (0.1 mL). The reaction was stopped by adding a total of 1 mL of hydrochloric acid (HCl) (37%) and centrifuged for 12 min at 4200 rpm. The resultant white precipitate of HPSNs was washed with ethanol and water. The CTAB removal was conducted with ethanolic HCl (15 ml of concentrated HCl in 120 ml ethanol) at 70^{o}C for 24h.

2.2. Immobilization of β-galactosidase

Approximately 10 mg pre-treated PSNF were submerged into β-galactosidase (0-8 mg/ml) containing the PBS solution overnight at 4 ^{o}C. It was thoroughly rinsed with water to remove free β-galactosidase. The supernatant and washing solution were collected to measure the concentration of non-adsorbed proteins. HPSNs (10 mg) were dispersed in 1 ml potassium phosphate buffer (pH 7.2) and sonicated for 30 min. The mixtures were submerged into β-galactosidase (0-8 mg/ml) and gently mixed overnight at 4 ^{o}C. The particles were collected through centrifugation and washed with water to remove free BSA or enzyme.

2.3. Bioconversion of lactose into galacto-oligosaccharide

Lactose (400 g/l) solution was prepared by dissolving lactose into PBS solution (pH 7.2) at 60 ^{o}C. Free or immobilized enzymes were added after the solution cooled down to 37 ^{o}C. The mixtures were incubated at 37 ^{o}C in an orbital shaker at 200 rpm. Samples were drawn after 2 h reaction and then immediately heated in boiling water for 5 min to deactivate enzyme activity [9].

2.4. Chemical analysis

The protein content was assayed by Bradford reagent method, by mixing 100 µl sample with 5 ml of Bradford reagent which was composed of 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol, 100 ml of 85% phosphoric acid and then diluting to a final volume of 1 litre [8]. The sample was measured using a UV spectrophotometer (Shimadzu, Japan) at 595 nm. The protein concentration was determined from a calibration curve using BSA as a standard.

The enzyme activity were assayed in a reaction mixture containing 0.1 ml of enzyme solution, 1.7 ml of phosphate buffer saline (PBS) (pH 7.2) and 0.2 ml of 20 mM O-nitrophenyl-β-d-glactopyranoside (ONPG) at 37 ^{o}C for 10 mim. The reaction was stopped by adding 2 ml of 1M sodium carbonate. The liberated product was measured spectrophotometrically at 405 nm and the concentration was calculated from the O-nitrophenol standard curve.

Saccharides (lactose, glucose, galactose, GOS) were determined by high performance liquid chromatography (HPLC) (Agilent, Germany) using an Aminex HPX-87H column (300 x 7.8 mm). The flow rate of a pre-degassed 8 mmol/L-H_2SO_4 mobile phase was set at 0.5 ml min^{-1}. A total of 5 µl samples were injected and the saccharides were detected with a refractive index detector. The column and the detector cell were maintained at 60 ^{o}C and 40 ^{o}C, respectively.

2.5. Statistical analysis

Experimental works were carried out in triplicates and data were presented as a mean value with an average standard deviation of <5%.

3. Results and Discussion

3.1. Immobilization of β-galactosidase

Integration of a biological entity on a nanomaterial carrier is becoming of biotechnological interest. Owing to their exquisite properties as enzyme carriers, two potential materials, nanofibers and nanoparticles, were fabricated and evaluated in this study. The polystyrene nanofibers (PSNF) were fabricated using electrospinning system. Their surface was oxidised using HNO_3 which has been proven to create oxygen-containing reactive groups, such as carboxyl (COOH) and hydroxyl (OH), for protein adsorption [10]. Meanwhile, the dendrimer-like silica nanoparticles with hierarchical pores (HPSNs) were synthesized through a one-pot sol gel approach using CTAB as a template. The nanocarrier was functionalized with amino group to facilitate enzyme binding. Their performance in immobilizing enzyme is presented in Figure 1. Adsorption yield was determined by dividing the amount of adsorbed protein on the nanocarriers with the total protein for immobilization. The highest adsorption yield was determined to be 80 % at 2 mg/ml enzyme concentration for both nanocarriers. Increasing the enzyme concentration caused a slight decrease to PSNF-Gal while a sharp reduction trend shown by HPSNs-Gal. It indicates the PSNF provides a larger surface area to accommodate higher concentrations of enzyme.

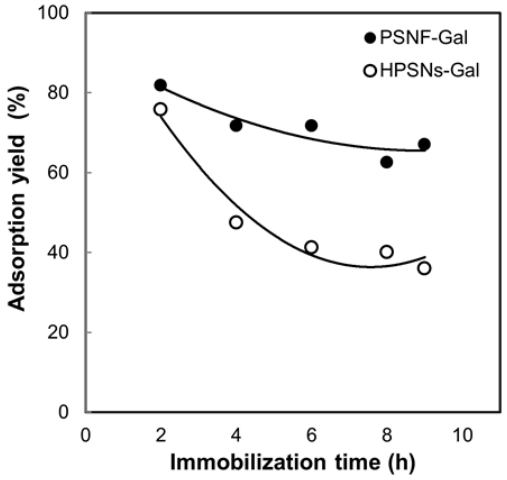

Fig. 1: The effect of enzyme concentration on the adsorption yield of HPSNs-Gal and PSNF-Gal.

Development of enzyme carriers with a high adsorption capacity is highly pursued. Adsorption capacity is defined as the amount of adsorbed enzyme (mg) per gram of enzyme support. Figure 2 compares the adsorption capacity between HPSNs-Gal and PSNF-Gal at different enzyme concentrations. It can be seen that the maximum adsorption capacity was 2500 mg/g and 320 mg/g for the PSNF-Gal and HPSNs-Gal, respectively. It indicates the PSNF carrier demonstrated nearly 8-fold higher capacity than the HPSNs. The

porosity of PSNF might have beneficially enhanced the penetration of biomolecules into all available surfaces. In addition, the surface modification through a specially determined chemical oxidation also probably has favoured the enzyme adsorption [7]. Nevertheless, it is also noticeable that HPSNs were promising to carry lower concentration of enzyme as indicated by the 80 % yield at 2 mg/ml (Figure 1). In previous study, the HPSNs have been proven as a remarkable carrier for small molecules like anticancer drugs and nucleic acids [6]. Their small particle size with open pores and uniform mesopores act as nanocontainers which may only suit for loading of small amount but potent biomolecules.

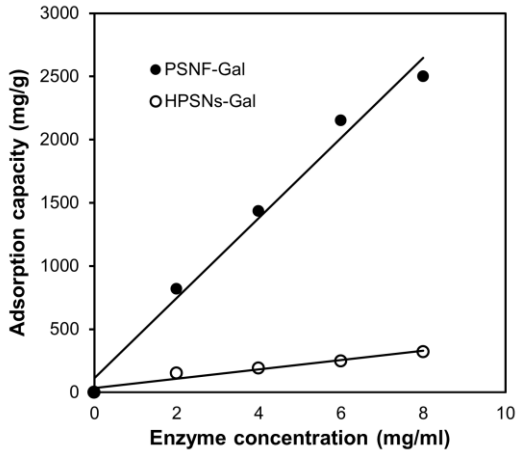

Fig. 2: The comparison of adsorption capacity between HPSNs-Gal and PSNF-Gal at different enzyme concentrations.

3.2. Production of galacto-oligosaccharide

After evaluating the immobilization capability, the performance of both PSNF and HPSNs nanobiocatalysts in lactose bioconversion into GOS was investigated. The findings were benchmarked with free β-galactosidase as a comparative study (Fig. 3). Free β-galactosidase was found to yield the highest bioconversion determined at 74 %. It was followed by the HPSNs-Gal and PSNF-Gal with respective yield of 59 % and 41 %. While free β-galactosidase could freely diffuse inside reaction medium to have greater interactions with substrates, both nanobiocatalysts might encounter mass transfer resistance thus resulted in lower bioconversion as shown in Fig. 3. Wu *et al.* [11] also has reported such unfavourable diffusion resistance created by enzyme carriers that were made from nanofibrous polyvinyl alcohol.

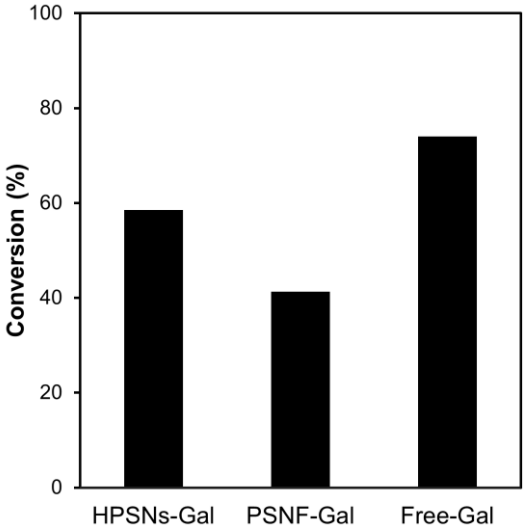

Fig. 3: The lactose bioconversion by HPSNs-Gal, PSNF-Gal and free-Gal at 400 g/l initial lactose concentration. (Enzyme concentration: 2 mg/ml in pH 7 phosphate buffer solution, incubated at 37 ^{o}C).

The profiles of product distribution in lactose bioconversion using β-galactosidase are illustrated in Fig. 4. The products comprise a mixture of glucose, galactose, galacto-oligosaccharide (GOS) and the remaining

lactose. Hydrolysis and transgalactosylation are the two possible pathways in β-galactosidase reactions [12]. Glucose and galactose are yielded in hydrolysis when water acts as a galactosyl acceptor, while transgalactosylation occurs for synthesis of GOS when nucleophilic molecules succeed to compete with the water activity [13]. Transgalactosylation is preferable because the GOS product is a beneficial functional food ingredient. It is noticeable that the highest hydrolysis products, glucose (44 %) and galactose (10%), was produced by the free β-galactosidase. It indicates the free enzyme favoured the hydrolysis pathway. This also further explains why the GOS yield by the free β-galactosidase was lower than the immobilized enzyme.

Meanwhile, the HPSNs-Gal exhibited an equal production of GOS and glucose determined about 25 %. Interestingly, the lactose biocoversion using PSNF-Gal showed very prominent reaction profiles (Fig 4). Firstly, the concentration of glucose and galactose were remarkably lower than 10 %. Secondly, the GOS yield was significantly higher than glucose (3-fold) and galactose (8-fold), signifying a preferance towards transgalactosylation over hydrolysis. The PSNF surface possesses hydrophobic properties [14] that could potentially repel water from the PSNF-Gal surface. As a result, it might reduce the water-driven hydrolysis activity and ultimately lead to the catalytic pathway of transgalactosylation. Thirdly, the highest GOS was obtained when using PSNF-Gal (28%) comparing with the HPSNs-Gal (25 %) and the free enzyme (19 %). In fact, the production of GOS was higher than employing β-galactosidase on other reported carriers such as polymeric membrane surfaces (13 %) and polysiloxane-polyvinyl alcohol (26 %) [15, 16].

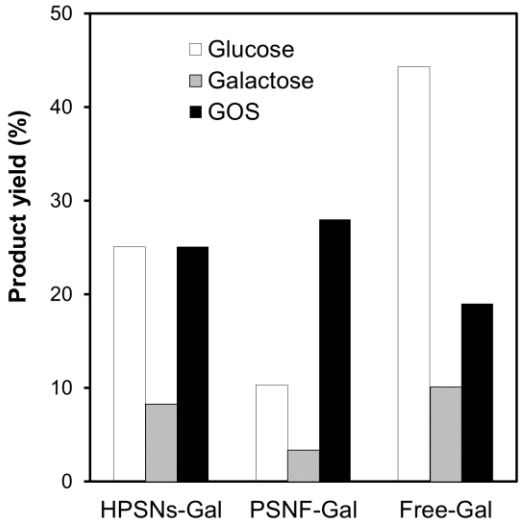

Fig. 4: The profile of product distributions by HPSNs-Gal, PSNF-Gal and free-Gal at 400 g/l initial lactose concentration. (Enzyme concentration: 2 mg/ml in pH 7 phosphate buffer solution, incubated at 37 ^{o}C).

3.3. Assessment of nanocarriers for bioprocessing application

Selection of nanomaterials to assemble enzyme forming nanobiocatalyst largely depends on many factors including adsorption capability, enzyme stability and productivity. Moreover, nanobiocatalyst with significant recyclability is a biotechnological interest. Besides of the recyclability of the enzyme-carrier assembly, developing recyclable support materials has gained increasing attention. HPSNs can be easily separated through sedimentation or centrifugation due to their high density. Nevertheless, their small size might cause the downstream process for biocatalyst recovery highly complex [17]. The particles tend to aggregate and may create a significant pressure drop in packed bed reactor system. It has become a major concern for industries besides the uneconomical cost for bulk materials synthesis. Meanwhile, a continuous fabrication of PSNF can be easily carried out using electrospinning device. The abundance of polymer resources in nature making the synthesis is considerably cheap. Importantly, enzyme-carrying PSNF can be readily located in a scalable reactor and promote easy separation. Hence, it endows potential translation of these bench-scale technologies into commercial practices. With regard to its bioengineering performance and its potential for scaling-up and reutilization, we propose the nanofiber-based nanobiocatalyst may beneficial for large scale enzyme-catalysed applications.

4. Conclusion

Surface-oxidized polystyrene nanofibers (PSNF) and amino-carrying dendrimer-like silica nanoparticles with hierarchical pores (HPSNs) exhibited great potential as enzyme nanocarriers. The employment of β-galactosidase on PSNF and HPSNs carriers as nanobiocatalysts assembly was beneficial not only for sustainable green waste management, but also for production of valuable products. PSNF was found to be relatively superior to HPSNs in hosting high enzyme loads. PSNF-Gal also exhibited distinguished reaction profiles by favouring transgalactosylation over hydrolysis, thus enhancing the GOS yield and reducing undesirable by-products in lactose bioconversion. The HPSNs-Gal, however, had an equal preference between the two reactions. Our research findings from experimental data exemplified a great potential of a nanofiber-based enzyme carrier for bioprocessing applications.

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6. References

[1] M. Wang, Advances in carrier-bound and carrier-free immobilized nanobiocatalysts. *Chemical Engineering Science*. In Press (0):

[2] M. Misson, Nanobiocatalyst advancements and bioprocessing applications. *Journal of Royal Society Interface*. 2015, 12 (102):

[3] J. Liu, Magnetic nanocomposites with mesoporous structures: Synthesis and applications. *Small*. 2011, **7** (4): 425-443.

[4] T. Watanabe, Production of a biodegradable plastic-degrading enzyme from cheese whey by the phyllosphere yeast Pseudozyma antarctica GB-4(1)W. *Journal of Bioscience and Bioengineering*. 2014, 118 (2): 183-187.

[5] M. G. Ganzle, Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose. *International Dairy Journal 22 (2012)*. 2012, 22: 116-122.

[6] X. Du, Developing functionalized dendrimer-like silica nanoparticles with hierarchical pores as advanced delivery nanocarriers. *Advanced Materials*. 2013, 25 (41): 5981–5985.

[7] H. An, Fabricating polystyrene fiber-dehydrogenase assemble as a functional biocatalyst. *Enzyme and Microbial Technology*. 2015, 68 (0): 15-22.

[8] M. M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976, 72: 248-254.

[9] L. Lu, Synthesis of galactooligosaccharides by CBD fusion beta-galactosidase immobilized on cellulose. *Bioresour Technol*. 2012, 116: 327-33.

[10] T. G. Ros, Surface Oxidation of Carbon Nanofibres. *Chemistry – A European Journal*. 2002, 8 (5): 1151-1162.

[11] Wu, L. *et al.*, Immobilization of cellulase in nanofibrous PVA membranes by electrospinning. *Journal of Membrane Science*. 2005, 250: 167-173.

[12] A. Gosling, Recent advances refining galactooligosaccharide production from lactose. *Food Chemistry 121*. 2010, 121: 307-318.

[13] B. Rodriguez-Colinas, Galacto-oligosaccharide Synthesis from Lactose Solution or Skim Milk Using the β-Galactosidase from Bacillus circulans. *Journal of Agricultural and Food Chemistry*. 2012, 60 (25): 6391-6398.

[14] J. Liu, Hydrophobic Electrospun Polyimide Nanofibers for Self-cleaning Materials. *Macromolecular Materials and Engineering*. 2015, 300 (3): 358-368.

[15] D. F. M. Neri, Galacto-oligosaccharides production during lactose hydrolysis by free Aspergillus oryzae b-galactosidase and immobilized on magnetic polysiloxane-polyvinyl alcohol. *Food Chemistry 128*. 2009, 115: 92-

99.

[16] H. A. Güleç, Immobilization of β-galactosidase from Kluyveromyces lactis onto polymeric membrane surfaces: Effect of surface characteristics. *Colloids and Surfaces B: Biointerfaces*. 2013, 104 (0): 83-90.

[17] T. P. N. Ngo, Reversible clustering of magnetic nanobiocatalysts for high-performance biocatalysis and easy catalyst recycling. *Chemical Communications*. 2012, 48 (38): 4585.