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Manipulation of nanofiber-based β-galactosidase nanoenvironment for enhancement of galacto-oligosaccharide production

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ABSTRACT

The nanoenvironment of nanobiocatalysts, such as local hydrophobicity, pH and charge density, plays a significant role in optimizing the enzymatic selectivity and specificity. In this study, *Kluyveromyces lactis* β-galactosidase (Gal) was assembled onto polystyrene nanofibers (PSNFs) to form PSNF-Gal nanobiocatalysts. We proposed that local hydrophobicity on the nanofiber surface could expel water molecules so that the transgalactosylation would be preferable over hydrolysis during the bioconversion of lactose, thus improve the galacto-oligosaccharides (GOS) yield. PSNFs were fabricated by electro-spinning and the operational parameters were optimized to obtain the nanofibers with uniform size and ordered alignment. The resulting nanofibers were functionalized for enzyme immobilization through a chemical oxidation method. The functionalized PSNF improved the enzyme adsorption capacity up to 3100 mg/g nanofiber as well as enhanced the enzyme stability with 80% of its original activity. Importantly, the functionalized PSNF-Gal significantly improved the GOS yield and the production rate was up to 110 g/l/h in comparison with 37 g/l/h by free β-galactosidase. Our research findings demonstrate that the localized nanoenvironment of the PSNF-Gal nanobiocatalysts favour transgalactosylation over hydrolysis in lactose bioconversion.

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1. Introduction

Advances in biotechnology have led to rapid development of enzyme-catalyzed bioprocesses which are green and sustainable in comparison with chemical processes. Enzymes, a type of biocatalysts able to provide specific and selective activity, have been found in broad applications including biocatalysis, biosensors and medicines. Nevertheless, biocatalysts are no longer economically viable when they are used in large scale processes due to their low stability and reusability under reactor conditions (Illanes et al., 2012). Therefore, enzyme immobilization on support materials is gaining immense interest from both industry and academia. The immobilization facilitates recovery and recyclability of biocatalysts that enables a cost-effective operation process (Tran and Balkus, 2011). A further benefit of immobilization is to enhance enzymatic stability, under both storage or operational conditions, by sheltering and/or stabilizing enzymes against harsh chemical and environmental conditions including extreme pH and temperature (Misson et al., 2015a; Sheldon and van Pelt, 2013).

Various support materials have been employed for enzyme immobilization including natural polymers *e.g.* cellulose, starch, agarose and chitosan (Bryjak et al., 2007; Matto and Husain, 2009), synthetic polymers *e.g.* ion exchange resin and polyvinyl alcohol (Bergamasco et al., 2013; Guidini et al., 2011), or inorganic materials *e.g.* silica (Kim et al., 2009), zeolite (Chang and Chu, 2007) and mesoporous silica (Nabavi Zadeh et al., 2015). In recent years, assembly of enzymes on nanostructured materials to form nanobiocatalysts has been recognized as a promising approach to enhance enzyme performance. To date, functional nanomaterials such as nanofibers, nanoparticles, nanotubes, and nanocomposites (Cho et al., 2012; Crespilho et al., 2009; El-Aassar, 2013; Feng and Ji, 2011) have been used as enzyme carriers. The approaches of immobilization can be accomplished via covalent and non-covalent binding, cross-linking, entrapment or encapsulation (Misson et al., 2015b). Despite constant efforts into optimizing immobilization strategies, reduction of the enzyme activity upon immobilization remains one of key challenges. The activity reduction may be associated with alteration of the unique and native structures of the enzyme (Talbert and Goddard, 2012). As a consequence, it may significantly affect

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the performance of the immobilized enzymes in a reactor system. The methodologies for recovering nanobiocatalysts are also poorly developed. It is, therefore, a focal point to explore recyclable enzyme nanocarriers that can preserve or enhance the enzyme catalytic function, and are suitable for industrial processes.

Cheese whey is a main by-product in casein and cheese manufacturing processes. It has been regarded as organic pollutants in wastewater due to its high organic loading, which has contributed to significant eutrophication and toxicity in eco-steams (Watanabe et al., 2014). Worldwide, more than 10^8 tons of cheese whey are produced annually, thus a cost-effective treatment technology prior to safe disposal into the environment is highly pursued (Grba et al., 2002). Lactose is the major content (60–80%) of dairy effluents. Bioconversion of the lactose-rich wastes into valuable products endows a promising solution. Lactose can be transgalactosylated into galacto-oligosaccharides (GOS), which offer a range of important health functions in living systems such as prebiotics, low caloric sugar alternatives, and low cariogenicity (Ganzle, 2012).

β-Galactosidase is the catalyst for bioconversion of lactose into GOS (Zheng et al., 2006). The enzyme cleaves the lactose molecules forming enzyme-galactosyl complexes before entering either hydrolysis or transgalactosylation pathways. The hydrolysis reaction takes place when water acts a galactosyl acceptor yielding glucose and galactose, while GOS is generated in the transgalactosylation reaction when acceptor molecules, which derived from sugar molecules in reaction medium, surpress the water activity (Jovanovic-Malinovska et al., 2012). To enhance GOS production, a localized nanoenvironment can be optimized to promote the transgalactosylation. Meanwhile, due to high cost of β-galactosidase, development of technologies for immobilizing β-galactosidase and enhancing its enzymatic activity and stability has gained momentum in research and development for industrial GOS production.

In this work, we propose to manipulate the local nanoenvironment of β-galactosidase by increasing the local hydrophobicity to reduce the water activity around the enzyme catalytic sites, thus transgalactosylation becomes more preferable than hydrolysis, which leads to a high GOS yield. Polystyrene nanofibers (PSNF) were selected as enzyme nanocarriers because the nanostructured fibers offer intrinsic advantages including high surface area for enzyme loading, and high porosity and interconnectivity to promote low hindrance for mass transfer (Misson et al., 2015b). The nanofiber surface presents a hydrophobic surface and the solid nanofiber-based nanobiocatalysts can also be recycled. The nanofibers were synthesized using an electrospinning device, and the fabrication process was optimized to generate uniform sized fibers. The PSNF surface was then functionalized through the surface oxidation method to facilitate β-galactosidase binding. The immobilization efficiency was assessed using bovine serum albumin (BSA) and β-galactosidase. Finally, the bioengineering performance of the PSNF-Gal was evaluated in bioconversion of lactose into GOS by benchmarking with the free enzyme.

2. Materials and methods

2.1. Chemicals

Polystyrene (molecular weight: 350,000), *N,N*-dimethylformamide (DMF) and nitric acid (HNO_3) were of analytical grade without further purification. Bovine serum albumin (BSA), *Kluyveromyces lactis* β-galactosidase, O-nitrophenyl-β-D-glactopyranoside (ONPG), Coomassie Brilliant Blue G-250 and calcium carbonate ($CaCO_3$) were obtained from Sigma-Aldrich. Phosphate buffer saline (PBS), pH 7.2 (10×) were procured from Life Technologies.

2.2. Synthesis of nanofibrous polystyrene by electrospinning

The precursor for electrospinning was prepared by dissolving polystyrene (10–30%, w/v) in DMF with gentle stirring for overnight to form a homogenous solution. The resulting solution was placed inside a 5 ml syringe bearing a needle tip with 1 mm inner diameter, which was connected to a high voltage power supply. The electrospun nanofibers were cast onto a metal-surface collector with a distance of about 10–25 cm from the needle tip. The flow rate was fixed at 2.5 ml/h while the electrospinning was performed within a voltage range of 20–30 kV. The fibers were detached from the collector surface and stored at room temperature for further use.

2.3. Enzyme immobilization on nanofibers

The surface of polystyrene nanofibers (PS) was modified through surface oxidation to introduce oxygen-containing reactive groups of carboxyl (COOH) and hydroxyl (OH) for enzyme binding (Ros et al., 2002). An approximately 1 cm × 2.5 cm piece of nanofibers was immersed in HNO_3 (69%) for 2 h at room temperature (An et al., 2015). The acid-modified support was rinsed with water and PBS (pH 7.2) three times to remove excess acid. BSA and β-galactosidase immobilization were carried out by submerging the nanofibers into BSA (0–10 mg/ml) or β-galactosidase (0–9 mg/ml) PBS solution and gently mixed overnight at 4 °C. Finally, the nanofibers were rinsed thoroughly with water to remove free BSA and β-galactosidase. The supernatant and washing solution were collected to measure the concentration of non-adsorbed proteins.

2.4. Enzymatic activity assays

The activity assays of free and PSNF- β-galactosidase (PSNF-Gal) were measured according to the procedure described by Ansari and Husain (2012). The reaction mixture containing 0.1 ml of enzyme solution, 1.7 ml of PBS buffer (pH 7.2) and 0.2 ml of 20 mM ONPG were incubated with continuous agitation at 37 °C for 10 min. The reaction was stopped by adding 2 ml of 1 M sodium carbonate before measuring the liberated product spectrophotometrically at 405 nm. The concentration of product was calculated from the O-nitrophenol standard curve. One unit (1 U) of β-galactosidase is defined as the amount of enzyme which liberates 1 μmol of O-nitrophenol per min under the standard assay condition.

2.5. Production of galacto-oligosaccharides

Lactose (400 g/l) solution was prepared by dissolving lactose into PBS (pH 7.2) at 60 °C. Free or immobilized enzymes were added after the solution cooled down to 37 °C. The mixtures were incubated at 37 °C in an orbital shaker at 200 rpm. Samples were drawn at designed intervals for analysis during the 5 h reaction period and then immediately heated in boiling water for 5 min to deactivate the enzyme before analysis (Lu et al., 2012; Rodriguez-Colinas et al., 2014). Samples were filtered using 0.45 μm nylon filters and diluted 40 times prior to high performance liquid chromatography (HPLC) analysis.

2.6. Chemical analysis

The enzyme concentration was analysed using the Bradford method (Bradford, 1976). A total of 100 μl sample was added into 5 ml of the Bradford reagent, mixed homogenously and allowed to react for 5 min. The sample was then measured using a UV spectrophotometer (Shimadzu, Japan) at 595 nm. Protein concentration was determined from a calibration curve using BSA as a standard. The adsorption yield (%) and adsorption capacity (mg/g) of the immobilized enzyme were calculated using Eqs. (1) and (2), where

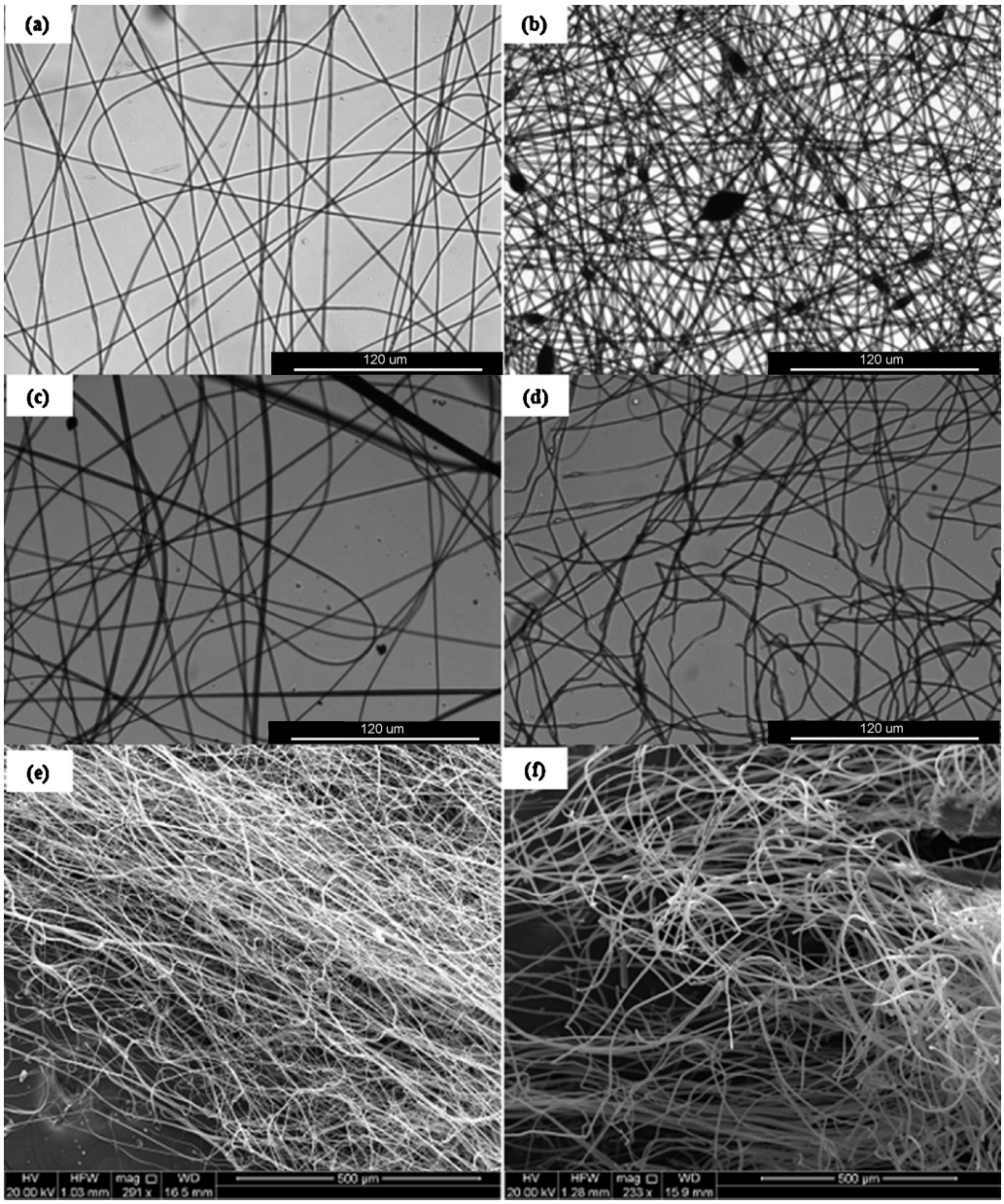

Fig. 1. (a–d) Microscopic images at 40× objective magnification of polystyrene nanofibers (PSNF): (a) nanofibers with uniform diameters (fabrication conditions: 20% w/v polymer concentration, 25 kV electric voltage, 15 cm distance), (b) nanofibers with beads (black dots) (fabrication conditions: 30%, w/v polymer concentration, 25 kV electric voltage, 10 cm distance), (c) nanofibers with heterogeneous diameter (fabrication conditions: 30%, w/v polymer concentration, 25 kV electric voltage, 20 cm distance), and (d) nanofibers with coil-like geometry (fabrication conditions: 30% w/v polymer concentration, 30 kV electric voltage, 25 cm distance). (e, f) SEM images of PSNF: (e) unmodified nanofibers and (f) HNO_3-modified nanofibers.

P_i is the initial protein content of the enzyme solution before immobilization, P_w and P_s are the amount of protein in washed solution and supernatant, respectively (Verma et al., 2012).

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

$$\text{Adsorption capacity(mg/g)} = \frac{[P_i - (P_w + P_s)]}{\text{nano fiber mass}} \quad (2)$$

The saccharides (lactose, glucose, galactose, GOS) were determined by HPLC (Agilent, Germany) using an Aminex HPX-87H column (300 × 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 was injected and the saccharides were detected with a refractive index detector. The column and the detector cell were maintained at 60 °C and 40 °C, respectively (Zheng et al., 2006). The conversion (%) was calculated using Eq. (3).

$$\text{Conversion}(\%) = \frac{[\text{initial lactose}] - [\text{final lactose}]}{[\text{initial lactose}]} \times 100 \quad (3)$$

Yield (Y) of glucose, galactose and GOS which represents the fraction with respect to total concentration of initial lactose, was calculated using Eqs. (4–6).

$$Y_{\text{glucose}}(\%) = \frac{[\text{glucose}]}{[\text{Initial Lactose}]} \times 100\% \quad (4)$$

$$Y_{\text{galactose}}(\%) = \frac{[\text{galactose}]}{[\text{Initial Lactose}]} \times 100\% \quad (5)$$

$$Y_{\text{gos}}(\%) = \%\text{Concersion} - \%Y_{\text{glucose}} - \%Y_{\text{galactose}} \quad (6)$$

2.7. Statistical analysis

Sample collection and analysis were conducted in triplicates. Results were analysed using the statistical tool in MS Excel 2010. Each value corresponds to the mean of independent experiments.

3. Results and discussion

3.1. Fabrication and functionalization of polystyrene nanofibers

Structures and the local nanoenvironment of nanocarriers can significantly influence enzyme activity and stability upon immobilization and its bioconversion performance in industrial applications. Large surface areas and minimal defects are two essential criteria for the nanofibers used for β-galactosidase immobilization. Nanofibers with a large surface area are vital to host a high load of enzymes. The presence of defects on the nanomaterial surface could possibly reduce its adsorption capacity. High quality nanofibers are required to be fabricated from an electrospinning process. During the electrospinning process, an electric field charges the polymer solution to create a repulsive force which overcomes the surface tension and leads to a polymer jet eruption from the spinneret tip, thus fiber mats are produced at the collector (Feng et al., 2013). A range of operating variables, which could affect the quality and localized physiochemical properties of PS nanofiber, was optimized to obtain high quality nanofibers. The variables comprise of polymer concentration (10–30%, w/v), electric voltage (20–30 kV) and the distance between needle tips and collector (10–25 cm).

A total of 36 trials were carried out based on different combinations of the synthesis conditions as summarized in Table S1. The electrospun homogeneity, fiber alignment and lack of macroscopic defects are normally employed to describe the quality of final products. Good quality nanofibers were obtained from the trials using 20% (w/v) polymer concentration, basing on homogeneous and droplet-free fiber mats spread on the collector surface by visual inspection. The uniform size and ordered alignment of fibrous mats are further confirmed from microscopic images (Fig. 1a). It was found that polymer concentration strongly influenced the nanofiber quality. Desirable PSNFs can be obtained using 20% (w/v) PS regardless of the applied voltage or distance. These findings were in agreement with the data reported by An et al. (2015). At the lowest polymer concentration (10%, w/v), liquid droplets were produced instead of fibers, probably due to a high surface tension which is associated with a low viscosity of the liquid (Deitzel et al., 2001). In this case, the process is called electrospraying which is used in many industries to obtain aerosols with narrow distributions. In contrast, condensed fibers with major defects were produced using the PS solution at a high concentration (30%, w/v). The defect formation is due to a high viscosity of the PS solution. The resultant PS fibers fused together into a network rather than individual fibers and generated rough and thick mats. This fiber morphology at a high polymer concentration was also demonstrated by Deitzel et al. (2001) due to its low surface tension. It showed formation of beads on their surface which was observed as black dots under microscopic image (Fig. 1b), heterogeneous fibers diameters (Fig. 1c) and disordered alignments (Fig. 1d), and all these represent a certain level of defects.

The fabricated electrospun mats with high quality were then modified by soaking in HNO_3 solution. Oxidation using HNO_3 has shown a favourable surface modification for protein adsorption (Kaur and Suri, 2007). In a previous study, different ratios of HNO_3/H_2SO_4 for modifying polystyrene nanofiber surface were reported (An et al., 2015) and an acid mixing ratio of 4:1 (HNO_3/H_2SO_4) was found to significantly improve the enzyme loading. However, the acidic solution containing sulphuric acid resulted in fiber aggregation. Therefore, surface modification with HNO_3 solution was selected for nanofiber surface treatment. Fig. 1e and f shows the SEM images of unmodified and HNO_3-treated nanofibers. The acidic oxidation of the fiber surface changes the morphology of the PS fibers. The electrsopun mats becomes shorter fibers as a consequence of the HNO_3 treatment. Such modifications have been proven to create oxygen-containing reactive groups, such as carboxyl (COOH) and hydroxyl (OH), which are able to establish stable binding with biomolecules (Ros et al., 2002). As demonstrated by An et al. (2015), the presence of COOH group on the PS nanofibers facilitated to assemble dehydrogenase on the nanofiber surface as a functional biocatalyst. The wettability and hydrophobicity have been improved for the modified nanofibers in comparison to the as-spun fibers. Our previous results (An et al., 2015) show that the as-spun fibers are hydrophobic and they float on the water surface. After acidic treatment, the nanofibers are well-dispersed in water.

3.2. Immobilization of BSA and β-galactosidase

Two proteins, BSA and β-galactosidase at a concentration range of 0–10 mg/ml, were used in order to evaluate the protein immobilization performance on the nanofibers before and after HNO_3 treatment (Fig. 2). BSA and β-galactosidase generally exhibited similar adsorption performance on the nanofibers at all tested concentrations. The maximum adsorption was achieved at protein concentrations around 2 mg/ml and 4 mg/ml for the enzyme and BSA, respectively. Fig. 2a and b shows that a smaller amount of the bound protein was observed in the case of using unmodified nanofibers. For the PS fibers without surface modification, the proteins may adsorb onto the fiber surface through weak binding such as hydrophobic interactions or van der Waals forces (Fernandez et al., 2013). Thus the hydrophobic nature of the nanofibers drives enzyme deposition. In addition, the enzyme itself contains approximately 28–33% of hydrophobic amino acids which are favourable for hydrophobic binding (Tudorache et al., 2012). Such protein adsorption through hydrophobic interactions has been reported for lipases by Palomo et al. (2002) and Chen et al. (2008).

It is notable that the surface modification leads to a substantial increase in the adsorption yield. Approximately 80% of BSA and β-galactosidase were adsorbed on the modified carrier, which was approximately four times that of the unmodified counterpart (20%). It has been recognized that surface oxidation of carbon nanofibers can create more active sites with a large number of carboxylic acid groups, epoxy and hydroxyl groups, which act as anchoring sites for binding protein molecules (Stavyiannoudaki et al., 2009). A redox enzyme has been successfully employed on electrospun nonwoven poly(acrylonitrile-co-acrylic acid) nanofiber meshes, carrying carboxylic functional groups (Wang et al., 2009). In the present work, the binding forces for interfacial reactions might come from hydrogen bonding, electrostatic force and some hydrophobic interactions (Secundo, 2013). As such, functional groups introduced through the HNO_3 treatment favour and stabilize enzyme adsorption (Stavyiannoudaki et al., 2009).

High adsorption capacity is one of important criteria for enzyme nanocarriers. Adsorption capacity is defined as the amount of adsorbed enzyme (mg) per gram of enzyme support. The experimental data presented in Fig. 2c and d shows the effect of acidic treatment of the PS nanofiber surface on the adsorption capacity of BSA and β-galactosidase. It is noted that the nanofibers after treatment by HNO_3 have the similar adsorption capacity for both BSA and β-galactosidase. The maximum adsorption capacity was found to be around 4000 mg/g and 3100 mg/g for BSA and β-galactosidase, respectively. The protein adsorption capacity on the treated surface is about 2-fold for BSA and 3-fold for β-galactosidase in comparison

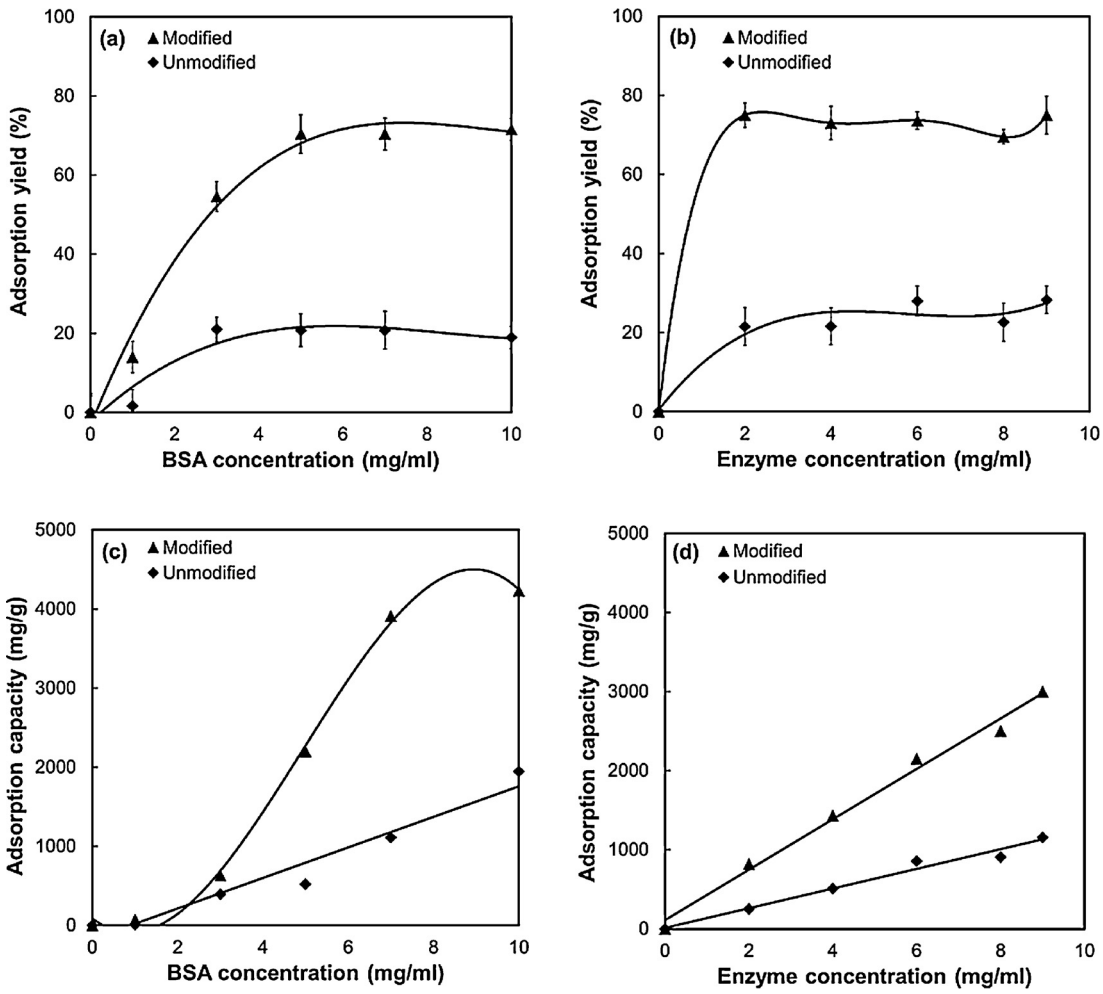

Fig. 2. Adsorption yield and adsorption capacity for (a,c) BSA and (b,d) PSNF-Gal on acid-modified and unmodified nanofiber surface.

with the untreated surface. The enhancement of adsorption could be ascribed to the protein binding through hydrogen bonding and electrostatic force.

The success in the development of the nanobiocatalysts towards bioprocess applications relies on their excellent performance, especially on enhancing or preserving their catalytic activity and stability upon immobilization. Fig. 3 presents the specific enzymatic activity of free and immobilized β-galactosidase at different concentrations. The activity of the enzyme immobilized on the nanofiber surface after acidic treatment was found to be comparable with the original activity of the free enzyme. Meanwhile, the decrease in enzyme activity for the unmodified carrier might be associated with the less availability of enzyme as evidenced by a lower adsorption yield as illustrated in Fig. 2b. In our previous study (Misson et al., 2015a), the PSNF-Gal has shown a promising recyclability. The nanobiocatalyst was demonstrated to retain 30% of its initial activity after nine operation cycles.

3.3. Production of galacto-oligosaccharides

The performance of PSNF-Gal was further evaluated for bioconversion of lactose into GOS. The reaction product stream from biocatalysis by PSNF-Gal includes glucose, galactose, GOS and the remaining lactose. The saccharides were analysed using HPLC with an Aminex HPX-87H column. GOS are eluted first in the chromatogram, followed by lactose, glucose and galactose (Zheng et al., 2006). Lactose, glucose and galactose are quantified using chemical standards. The amount of GOS is calculated based on the mass difference between the initial mass of lactose prior to the reaction and summation of the mass of glucose, galactose, and lactose after the reaction.

Fig. 4a and b shows the production profiles of lactose bioconversion using free β-galactosidase and PSNF-Gal. Free enzyme demonstrated a rapid lactose conversion during the first hour reaction (Fig. 4a). The major product was glucose (177 g/l), followed by galactose (80 g/l) and GOS at around 37 g/l. After extending the reaction time from 1 to 5 h, glucose leveled off while lactose decreased at a relatively small order. It is interesting to note that biocoversion using PSNF-Gal has a different reaction profile (Fig. 4b). The concentrations of glucose and galactose are remarkably lower than those produced by the free β-galactosidase. There is a sharp reduction in lactose concentration and a rapid increase in GOS production during the first hour, resulting in the highest conversion rate of 165 g/l/h, and the production rate of 110 g/l/h, respectively. GOS concentration reduces slightly beyond 2 h, while lactose concentation gradually increases. Lactose and isomers can be possibly re-synthesized through revesible reaction between the galactosyl-enzyme complex and the glucose molecules (Kim et al., 2004). Interestingly, the GOS concentration is much higher than that of glucose or galactose using PSNF-Gal up to a reaction time of 5 h. The amount of GOS produced was about 3-fold and 8-fold higher than glucose and galactose, respectively. The PSNF-Gal has the highest GOS production rate of 110 g/l/h in contrast with 37 g/l/h for the free counterpart.

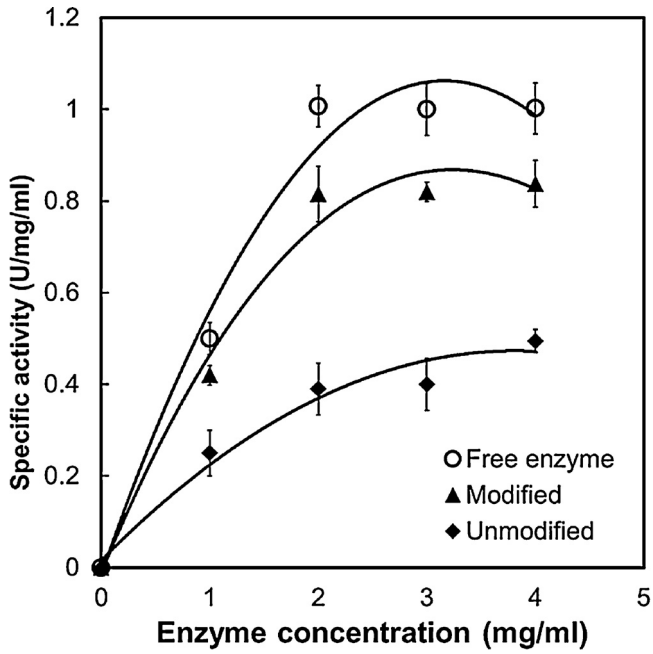

Fig. 3. Specific enzymatic activity for free β-galactosidase and PSNF-Gal on acid-modified and unmodified nanofiber surface.

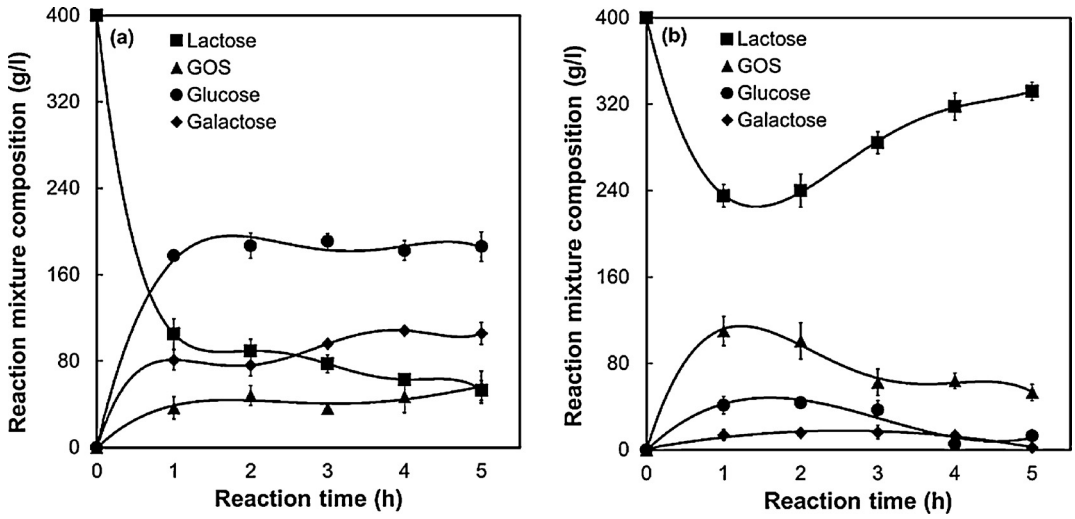

Fig. 4. Component concentration changes in the product stream at different reaction time for (a) free β-galactosidase and (b) PSNF-Gal. (Operation conditions: incubated at 37 °C using 400 g/l of initial lactose concentration).

Table 1
Comparison of various types of immobilized β-galactosidases for lactose conversion and GOS production yield.

Enzyme source	Support material	Initial lactose (g/L)	Conversion (%)	GOS (g/L)	Reference
Kluyveromyces lactis	Polystyrene nanofibers	400	41	110	This work
Bacillus circulans	Magnetic polysiloxane-polyvinyl alcohol	400	73	198	Rodriguez-Colinas et al. (2012)
Aspergillus oryzae	polyvinyl alcohol	400	75	120	Jovanovic-Malinovska et al. (2012)
Aspergillusoryzae	Magnetic polysiloxane-polyvinyl alcohol	400	50	104	Neri et al. (2009)
Aspergillus candidus	Alginate	400	–	87	Zheng et al. (2006)
Bacilus circulans	Membrane surface	200	–	40	Palai and Bhattacharya (2013)
Kluyveromyces lactis	Chitosan macroparticles	400	58	26	Klein et al. (2013)

The lactose conversion and the GOS yield are presented in Fig. 5 for both free β-galactosidase and the PSNF-Gal. Free β-galactosidase offers the advantage for lactose conversion (Fig. 5a). The amount of converted lactose by free β-galactosidase (86%) is higher than that by PSNF-Gal (41%). The PSNF-Gal, however, appears to have better catalytic capability for synthesizing GOS (Fig. 5b). A GOS yield of 28% is obtained in the first hour for the PNSF-Gal preparation in comparison with less than 9% for the free enzyme at the same reaction time. The GOS yield can also be calculated based on the consumed lactose, and this yield is estimated to be 67%. The difference in the GOS yield between the immobilized enzyme and its free counterpart is distinctive, increasing from

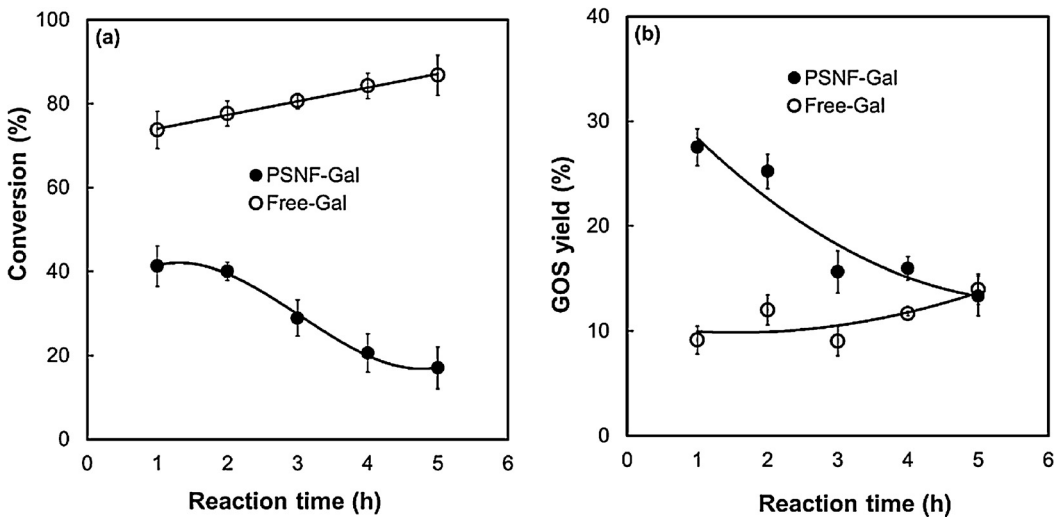

Fig. 5. (a) Lactose conversion and (b) GOS yield using free β-galactosidase (free-Gal) and PSNF-Gal. (Operation conditions: incubated at 37 °C within 5 h reaction time using 400 g/l of initial lactose concentration).

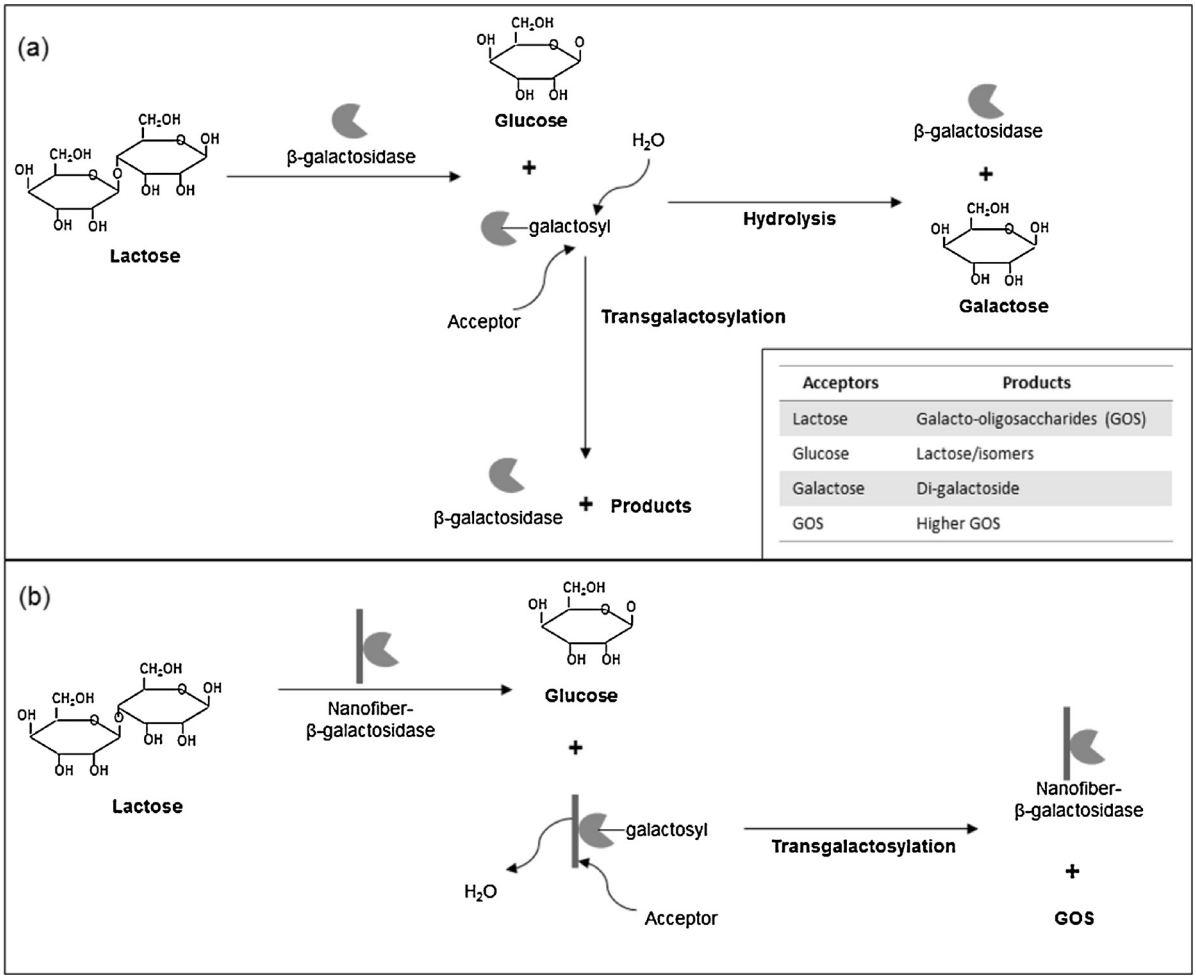

Fig. 6. (a) The reaction pathway of lactose hydrolysis and transgalactosyslation using free β-galactosidase (Shen et al., 2012). (b) Proposed reaction pathway of lactose bioconversion using the PSNF-Gal due to localized nanoenvironment.

12% to 67%. The functionalized polystyrene nanofibers-based β-galactosidase has shown comparable bioconversion performance to some of reported immobilized enzyme on various supports for GOS production (Table 1) (Jovanovic-Malinovska et al., 2012; Klein et al., 2013; Neri et al., 2009; Palai and Bhattacharya, 2013; Rodriguez-Colinas et al., 2012; Zheng et al., 2006). This present study used a similar source of enzyme (*K. lactis*) as employed by Klein et al. (2013) but a different type of enzyme immobilization matrix was selected: polystyrene nanofibers in this work while chitosan macroparticles in Klein et al. (2013). The matrix difference can

be ascribed to morphology, size, functional groups on the matrix, hydrophobicity, surface charge and others. The local hydrophilic nanoenvironment of chitosan macroparticles could be the key factor for the low GOS yield as water molecules are preferable in the enzyme's active sites. In addition, a packed bed reactor was used by Klein et al. and a low yield of GOS from a continuous operation can be expected. Lactose conversion catalyzed by β-galactosidase has two different reaction pathways: hydrolysis to glucose and galactose and transgalactosylation to GOS (Gaur et al., 2006; Shen et al., 2012), which are sketched in Fig. 6a. According to the proposed pathways, enzyme-galactose complex is formed before entering either hydrolysis or transgalactosylation. Hydrolysis takes place when water acts a galactosyl acceptor, while transgalactosylation occurs when saccharides molecules succeed competing with the water activity (Jovanovic-Malinovska et al., 2012; Rodriguez-Colinas et al., 2012). In the free enzyme reaction system, both water and nucleophilic molecules have the equivalent opportunity to enter the enzyme's active site and react with the galactosyl-enzyme complex. Therefore, in the first hour, concentrations for both products from hydrolysis and transgalactosylation increase. After the first hour, the product glucose concentration keeps a constant value, while GOS fluctuates because GOS may be synthesized or hydrolyzed in the system. The increase or decrease of the GOS concentration depends on which reaction becomes preferable in the system.

In order to promote transgalactosylation catalyzed by β-galactosidase, the local nanoenvironment of the nanofibers is tuned with the hydrophobicity so that acceptors such as lactose or smaller units of GOS are preferable at the enzyme catalytic sites. This hydrophobic property of the nanofiber surface also reduces the water-driven hydrolysis activity. Therefore, the hydrophobic nature of PS nanofibers could be a key factor for GOS formation as presented in Figs. 4 and 5, particularly during the first hour of reaction. Another contributing factor in GOS synthesis may be associated with the reduced diffusion path for biomolecules. Galactose forms a galactosyl—enzyme complex at the enzyme's active sites, and this complex reacts with the acceptors which are produced from the enzymatic reactions and do not immediately diffuse out of the nanofiber surfaces, leading to a preference towards transgalatcosylation reaction. This factor could be used to explain the reduced gap in GOS formation between PSNF-Gal and its free counterpart after 3–5 h reaction as presented in Fig. 5. After the nanofibers are exposed to aqueous solution for a few hours, water molecules start to penetrate into the enzymatic catalytic sites as the galactosyl acceptor, and hydrolysis competes with transgalactosylation. When the dynamic balance between both reactions is reached, enzyme on the nanofibers shares the similar performance as free enzyme.

Since PSNF-Gal is able to produce a high GOS yield during the first hour, residence time in the system can be reduced to meet the requiremets of continuous operations in large scale processes. Optimization of the catalytic process in a bioreactor system for continuous operation will be carried out to maximize the GOS yield, which will be expected to be much higher than the initial reported value (110 g/l).

4. Conclusion

Polystyrene nanofibers (PSNF) with excellent quality as enzyme carriers were successfully fabricated using electrospinning process. The PSNF surface was modified through chemical oxidation to improve enzyme loading and maintain a high enzyme activity. The lactose conversion by PSNF-Gal catalysis results in a high GOS yield as well as a high production rate. PSNF-Gal may beneficially promote transgalactosylation due to the hydrophobic nature of the polysterene surface. Another contributing factor could be the reduced diffusion path of nuclephilic molecules to facilitate transgalactosylation because these molecules could easily bind to the galactosyl-enzyme complex in such a nanoenvironment. This simple but effective nanobiocatalyst-assisted process may be economically viable for large scale GOS production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2016.02.014.

References

An, H., Jin, B., Dai, S., 2015. Fabricating polystyrene fiber-dehydrogenase assemble as a functional biocatalyst. Enzyme Microb. Technol. 68, 15–22.
Ansari, S.A., Husain, Q., 2012. Lactose hydrolysis from milk/whey in batch and continuous processes by concanavalin A-Celite 545 immobilized *Aspergillus oryzae* β-galactosidase. Food Bioprod. Process. 90, 351–359.
Bergamasco, J., de Araujo, M.V., de Vasconcellos, A., Luizon Filho, R.A., Hatanaka, R.R., Giotto, M.V., Aranda, D.A.G., Nery, J.G., 2013. Enzymatic transesterification of soybean oil with ethanol using lipases immobilized on highly crystalline PVA microspheres. Biomass Bioenergy 59, 218–233.
Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Bryjak, J., Aniulyte, J., Liesiene, J., 2007. Evaluation of man-tailored cellulose-based carriers in glucoamylase immobilization. Carbohydr. Res. 342, 1105–1109.
Chang, Y.K., Chu, L., 2007. A simple method for cell disruption by immobilization of lysozyme on the extrudate-shaped NaY zeolite. Biochem. Eng. J. 35, 37–47.
Chen, H., Yuan, L., Song, W., Wu, Z., Li, D., 2008. Biocompatible polymer materials: role of protein–surface interactions. Prog. Polym. Sci. 33, 1059–1087.
Cho, E.J., Jung, S., Kim, H.J., Lee, Y.G., Nam, K.C., Lee, H.J., Bae, H.J., 2012. Co-immobilization of three cellulases on Au-doped magnetic silica nanoparticles for the degradation of cellulose. Chem. Commun. Cambridge 48, 886–888.
Crespilho, F.N., Iost, R.M., Travain, S.A., Oliveira, O.N., Zucolotto, V., 2009. Enzyme immobilization on Ag nanoparticles/polyaniline nanocomposites. Biosens. Bioelectron. 24, 3073–3077.
Deitzel, J.M., Kleinmeyer, J., Harris, D., Beck Tan, N.C., 2001. The effect of processing variables on the morphology of electrospun nanofibers and textiles. Polymer 42, 261–272.
El-Aassar, M.R., 2013. Functionalized electrospun nanofibers from poly (AN-co-MMA) for enzyme immobilization. J. Mol. Catal. B: Enzym. 85–86, 140–148.
Feng, W., Ji, P., 2011. Enzymes immobilized on carbon nanotubes. Biotechnol. Adv. 29, 889–895.
Feng, C., Khulbe, K.C., Matsuura, T., Tabe, S., Ismail, A.F., 2013. Preparation and characterization of electro-spun nanofiber membranes and their possible applications in water treatment. Sep. Purif. Technol. 102, 118–135.
Fernandez, M.F., Sanroman, M.A., Moldes, D., 2013. Recent developments and applications of immobilized laccase. Biotechnol. Adv. 31, 1808–1825.
Ganzle, M.G., 2012. Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose. Int. Dairy J. 22, 116–122.
Gaur, R., Pant, H., Jain, R., Khare, S.K., 2006. Galacto-oligosaccharide synthesis by immobilized *Aspergillus oryzae* β-galactosidase. Food Chem. 97, 426–430.
Grba, S., Stehlik-Tomas, V., Stanzer, D., Vahčić, N., Škrlin, A., 2002. Selection of yeast strain Kluyveromyces marxianus for alcohol and biomass production on whey. Chem. Biochem. Eng. Q. 16, 13–16.
Guidini, C.Z., Fischer, J., Resende M.M. d. Cardoso, V.L., Ribeiro, E.J., 2011. β-galactosidase of *Aspergillus oryzae* immobilized in an ion exchange resin combining the ionic-binding and crosslinking methods: kinetics and stability during the hydrolysis of lactose. J. Mol. Catal. B: Enzym. 71, 139–145.
Illanes, A., Cauerhff, A., Wilson, L., Castro, G.R., 2012. Recent trends in biocatalysis engineering. Bioresour. Technol. 115, 48–57.

Jovanovic-Malinovska, R., Fernandes, P., Winkelhausen, E., Fonseca, L., 2012. Galacto-oligosaccharides synthesis from lactose and whey by beta-Galactosidase immobilized in PVA. Appl. Biochem. Biotechnol. 168, 1197–1211.

Kaur, J., Suri, C.R., 2007. Direct hapten coated ELISA for immunosensing of low molecular weight analytes. Protoc. Exch., http://dx.doi.org/10.1038/nprot.2007.508.

Kim, C., ES, J., DK, O., 2004. A new kinetic model of recombinant betagalactosidase from *Kluyveromyces lactis* for both hydrolysis and transgalactosylation reactions. Biochem. Biophys. Res. Commun. 316, 738–743.

Kim, H., Kwon, H.-S., Ahn, J., Lee, C.-H., Ahn, I.-S., 2009. Evaluation of a silica-coated magnetic nanoparticle for the immobilization of a His-tagged lipase. Biocatal. Biotransform. 27, 246–253.

Klein, M.P., Fallavena, L.P., Schöffer J. d.N. Ayub, M.A.Z., Rodrigues, R.C., Ninow, J.L., Hertz, P.F., 2013. High stability of immobilized β-D-galactosidase for lactose hydrolysis and galactooligosaccharides synthesis. Carbohydr. Polym. 95, 465–470.

Lu, L., Xu, S., Zhao, R., Zhang, D., Li, Z., Li, Y., Xiao, M., 2012. Synthesis of galactooligosaccharides by CBD fusion beta-galactosidase immobilized on cellulose. Bioresour. Technol. 116, 327–333.

Matto, M., Husain, Q., 2009. Calcium alginate–starch hybrid support for both surface immobilization and entrapment of bitter gourd (Momordica charantia) peroxidase. J. Mol. Catal. B: Enzym. 57, 164–170.

Misson, M., Jin, B., Chen, B., Zhang, H., 2015a. Enhancing enzyme stability and metabolic functional ability of β-galactosidase through functionalized polymer nanofiber immobilization. Bioprocess Biosyst. Eng. 38, 1915–1923.

Misson, M., Zhang, H., Jin, B., 2015b. Nanobiocatalyst advancements and bioprocessing applications. J. R. Soc. Interface 12, 1–8.

Nabavi Zadeh, P.S., Mallak, K.A., Carlsson, N., Åkerman, B., 2015. A fluorescence spectroscopy assay for real-time monitoring of enzyme immobilization into mesoporous silica particles. Anal. Biochem. 476, 51–58.

Neri, D.F.M., Balcao, V.M., Costa, R.S., Rocha, I.C.A.P., Ferreira, E.M.F.C., Torres, D.P.M., Rodrigues, L.R.M., Jr, L.B.C., Teixeira, J.A., 2009. Galacto-oligosaccharides production during lactose hydrolysis by free Aspergillus oryzae β-galactosidase and immobilized on magnetic polysiloxane-polyvinyl alcohol. Food Chem 115, 92–99, 128.

Palai, T., Bhattacharya, P.K., 2013. Kinetics of lactose conversion to galacto-oligosaccharides by β-galactosidase immobilized on PVDF membrane. J. Biosci. Bioeng. 115, 668–673.

Palomo, J.M., Muñoz, G., Fernández-Lorente, G., Mateo, C., Fernández-Lafuente, R., Guisán, J.M., 2002. Interfacial adsorption of lipases on very hydrophobic support (octadecyl–Sepabeads): immobilization, hyperactivation and stabilization of the open form of lipases. J. Mol. Catal. B: Enzym. 19–20, 279–286.

Rodriguez-Colinas, B., Poveda, A., Jimenez-Barbero, J., Ballesteros, A.O., Plou, F.J., 2012. Galacto-oligosaccharide synthesis from lactose solution or skim milk using the β-galactosidase from *Bacillus circulans*. J. Agric. Food Chem. 60, 6391–6398.

Rodriguez-Colinas, B., Fernandez-Arrojo, L., Ballesteros, A.O., Plou, F.J., 2014. Galactooligosaccharides formation during enzymatic hydrolysis of lactose: towards a prebiotic-enriched milk. Food Chem. 145, 388–394.

Ros, T.G., van Dillen, A.J., Geus, J.W., Koningsberger, D.C., 2002. Surface oxidation of carbon nanofibres. Chem. Eur. J. 8, 1151–1162.

Secundo, F., 2013. Conformational changes of enzymes upon immobilisation. Chem. Soc. Rev. 42, 6250–6261.

Sheldon, R.A., van Pelt, S., 2013. Enzyme immobilisation in biocatalysis: why, what and how. Chem. Soc. Rev. 42, 6223–6235.

Shen, Q., Yang, R., Hua, X., Ye, F., Wang, H., Zhao, W., Wang, K., 2012. Enzymatic synthesis and identification of oligosaccharides obtained by transgalactosylation of lactose in the presence of fructose using beta-galactosidase from *Kluyveromyces lactis*. Food Chem. 135, 1547–1554.

Stavyiannoudaki, V., Vamvakaki, V., Chaniotakis, N., 2009. Comparison of protein immobilisation methods onto oxidised and native carbon nanofibres for optimum biosensor development. Anal. Bioanal. Chem. 395, 429–435.

Talbert, J., Goddard, J., 2012. Enzymes on material surfaces. Colloids and surfaces. B. Biointerfaces 93, 8–19.

Tran, D.N., Balkus, K.J., 2011. Perspective of recent progress in immobilization of enzymes. ACS Catal. 1, 956–968.

Tudorache, M., Protesescu, L., Negoi, A., Parvulescu, V.I., 2012. Recyclable biocatalytic composites of lipase-linked magnetic macro-/nano-particles for glycerol carbonate synthesis. Appl. Catal. A: Gen. 437–438, 90–95.

Verma, M.L., Barrow, C.J., Kennedy, J.F., Puri, M., 2012. Immobilization of beta-d-galactosidase from *Kluyveromyces lactis* on functionalized silicon dioxide nanoparticles: characterization and lactose hydrolysis. Int. J. Biol. Macromol. 50, 432–437.

Wang, Z.G., Wan, L.S., Liu, Z.M., Huang, X.J., Xu, Z.K., 2009. Enzyme immobilization on electrospun polymer nanofibers: an overview. J. Mol. Catal. B: Enzym. 56, 189–195.

Watanabe, T., Shinozaki, Y., Suzuki, K., Koitabashi, M., Yoshida, S., Sameshima-Yamashita, Y., Kuze Kitamoto, H., 2014. Production of a biodegradable plastic-degrading enzyme from cheese whey by the phyllosphere yeast Pseudozyma antarctica GB-4(1)W. J. Biosci. Bioeng. 118, 183–187.

Zheng, P., Yu, H., Sun, Z., Ni, Y., Zhang, W., Fan, Y., Xu, Y., 2006. Production of galacto-oligosaccharides by immobilized recombinant beta-galactosidase from *Aspergillus candidus*. Biotechnol. J. 1, 1464–1470.