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Two-step enzymatic strategy for the synthesis of a smart phenolic polymer and further immobilization of a β-galactosidase able to catalyze transglycosydation reaction

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ABSTRACT

A rapid and efficient enzymatic procedure for the preparation of an immobilized β-galactosidase has been described. In a first step, soybean peroxidase was used to catalyze the polymerization of a strategically activated phenol (*N*–Succinimidyl 3–(4–hydroxyphenyl)propionate, known as Bolton-Hunter reagent). The phenolic support was directly employed for immobilizing β–galactosidase from *Bacillus circulans* (ATCC 31382, β–Gal–3), giving rise to a new biocatalyst subsequently applied in the synthesis of a β–galatodisaccharide (Gal–β(1–3)–GlcNAc and Gal–β(1–3)–GalNAc). The reaction proceeded with high conversion rates and total regioselectivity. Reusability assays were performed with the same reaction conditions finding that the immobilized enzyme retains about 55% of its activity after eight batches. Finally and based on our results, the two-step enzymatic procedure presented here is a good and green alternative to the preparation of carbohydrates with biological activities.

1. Introduction

Biocatalytic synthesis of valuable products is a process where one or more enzymes can be involved and illustrate the vastness of the different activities of enzymes and their versatility to participate in smart synthetic process routes for valuable products. Polymers and carbohydrate are one of these valuable products that pervade our daily lives. These materials find specific use of new materials for chemical and biomedical applications. However, conventional methods for the production of these compounds are faced by numerous challenges, resulting in current technology being based on chemical synthesis that uses environmentally unfriendly reactants and solvents, as well as inefficient protocols [1,2]. In that context, phenol resins are synthetic polymers of common use in many domestic and industrial products such as carbon membranes, fibreglass or protein-based adhesive [3]. They are obtained by chemical sequential reactions using toxic chemical catalysts leading to potential environmental problems [4]. However, yields obtained are low and therefore becoming a significant limitation in the development of improved polymers.

In last decades, attempts to overcome this burden were made by using enzymes that catalyze polymerization reactions. Enzymatic catalysis offers great versatility since a wide range of phenolic derivatives can be recognised by enzymes able to catalyze the production of different polymers, avoiding the use of highly toxic reactives, such as, formaldehyde well known to have influence in the development of some carcinogenesis process [5]. In fact, oxidoreductases such as peroxidases are known to catalyze the polymerization of phenol and aniline derivatives afford novel polyaromatic structures. This strategy can also be applied for chemoselective polymerizations of phenol derivatives having different functional groups. Most research in the field of peroxidase-catalyzed polymerization of phenols has been carried out with *p*-substituted phenols. In this case, the reactive *para* position of the phenol is blocked, so that the recombination of the phenol should take place mainly at the *orto* positions of the phenols [6]. This approach can also be applied for chemoselective polymerizations of phenol derivatives having different functional groups. In particular, soybean peroxidase (SBP) and horseradish peroxidase (HRP) have been used to synthesize phenolic polymers and copolymers from a wide range of *p*-substituted phenols, including 4-hydroxybenzenesulfonicacid, *p*-cresol, *p*-phenylphenol and 4-fluorophenol, 2-(4-hydroxyphenul)ethyl methacrylate, among others [6–9]. These phenolic aromatic polymers funtionalized with different reactive groups could be applicable in several fields such as bioremediation, paper industry or diagnostic therapies [10,11]. Despite these applications, functionalized aromatic polymer prepared by enzymatic reactions has never been used for enzyme immobilization.

For this reason we propose here the enzymatic synthesis of a functionalized phenolic polymer to immobilize β–galactosidase from

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Bacillus circulans (ATCC 31382, β–Gal–3). This enzyme recognises *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-galactosamine (GalNAc) as acceptors and is able to use them to synthesize β-(1 → 3)-galacto-oligosaccharides, such as Gal–β(1–3)–GlcNAc and Gal–β(1–3)–GalNAc, [2,12–19] which are involved in different biological functions such as metastasis [20–22].

The system described in this work brings to light the biosynthesis of interesting medical carbohydrates in a sustainable way, based on the efficient combination of two enzymes.

2. Experimental

2.1. Materials

Hydrogen peroxide (H_2O_2) 50% w/t, Bolton-Hunter reagent, *N*-Succinimidyl 3-(4-hydroxyphenyl)propionate (NDHPP), *p*-nitrophenol-β-D-galactopyranoside (*p*NP–β–Gal), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc) and analytical standards of monosaccharides for HPLC were purchased from Sigma Aldrich. Soybean hulls were kindly donated by Entre Ríos Crushing S.A., Argentina and Guaiacol (>98%) was from Mallinckrodt Chemical Works (St. Louis, MO). All other chemicals were from analytical grade.

2.2. Purification of soybean hull peroxidase

This peroxidase (SBP) was extracted from soybean hulls as a single isoenzyme. Soybean hulls were soaked overnight with distilled water at 4 °C. After solids separation by centrifugation at 10000 rpm for 15 min, the supernatant was concentrated by ultrafiltration with a hollow-fibre cartridge of MWCO 10 kDa (Fresenius Polysulfone®). Then, the supernatant was loaded to a prepacked Q FF column (GE Healthcare, Piscataway, USA) previously equilibrated with 25 mM sodium phosphate buffer, pH 6.0. Elution was performed with a 20 min-gradient of NaCl from 0 to 1 M in equilibration buffer, at a flow rate of 1.0 ml/min. The effluent was monitored by absorbance at 280, 215 and 403 nm and measurement of peroxidase activity. The peak corresponding to peroxidase was pooled, added ammonium sulphate up to 0.75 M and loaded to a prepacked Phenyl HP column (GE Healthcare, Piscataway, USA) equilibrated with 50 mM sodium phosphate buffer, pH 6.0, ammonium sulphate 0.8 M. The elution was performed with distilled water, at a flow rate of 1.0 ml/min, and the effluent was monitored by absorbance at 280, 215 and 403 nm and measurement of peroxidase activity. All chromatographic runs were carried out with an ÄKTA Purifier equipment (GE Healthcare, Piscataway, USA).

2.3. Soybean peroxidase assay

The assay mixture contained 20 μM guaiacol and 8 mM hydrogen peroxide in 1 ml of 100 mM potassium phosphate buffer, pH 5.5, at 25 °C. After addition of a 50 μl SBP sample, absorbance at 470 nm was recorded. One unit of peroxidase activity represents the amount of enzyme that catalyses the oxidation of one μmole of guaiacol in 1 min, under the conditions of the assay.

2.4. Production of the β-Gal.3 and purification

Recombinant β–galactosidase from *Bacillus circulans* ATTC 31382 (β-Gal.3) was cloned in *Escherichia coli* BL21 (DE3) using pET28b(+) vector (Novagen). *E. coli* cultures were grown aerobically at 37 °C in LB broth with kanamycin (30 mg/l) and induced with IPTG (isopropyl–β–D–thiogalactopyranoside, 1 mM) at 37 °C for 5 h. Cells were broken by sonic disruption. Unbroken cells and insoluble debris were eliminated by centrifugation (14,000 rpm for 15 min at 4 °C). Supernatant was passed through a Ni^{2+}–agarose column (3 ml) according to manufacturer's protocol (BioRad). Fractions were monitored for absorbance at 280 nm. They were pooled, concentrated and desalted in an Amicon ultra centrifugal filter (Millipore) to eliminate remnant imidazole. The purification process was followed by SDS–PAGE [23]. Protein quantification was done by Bradford method, using bovine serum albumin (BSA) as standard [24].

2.5. Enzymatic reaction for polymer preparation

The enzymatic synthesis for oxidative polymerization was adapted from the related literature [25]. Phenolic polymerization was carried out in 30 mM sodium formic/formate pH 3.5 buffer and 30% v/v of THF as co-solvent using two procedures. Each reaction consisted of 4.5 mM of the NDHPP in the presence of 4.12 mg/ml SBP (2124 U/mg) an H_2O_2. In method I, 500 μl of 5% H_2O_2 was added dropwise at 50 μl each 2.5 min and in method II, 500 μl of 5% H_2O_2 was directly added at the beginning of reaction. Aliquots (20 μl) were withdrawn from reaction media at different times (0, 2.5, 5, 15, 20, 25, 45 and 60 min). Reaction was stirred for 60 min at 25 °C. Concentrations of phenols were determined by HPLC (Agilent 1100) with detection at 280 nm. Periodically, aliquots from the reaction mixtures were diluted 50:50 with acetonitrile and analyzed on a reversed phase C18 column (C-18 15 × 0,46 cm, Mediterranean column, Teknokroma). The eluent contained 0.1% (v/v) acetic acid in acetonitrile/water solution and was pumped with a linear gradient from 10 to 45% acetonitrile over 10 min and then isocratically at 45% acetonitrile for 20 min with a flow rate of 1 ml/min.

Reaction was stopped by physical separation of product by centrifugation of reaction media. Afterwards the product was filtered, recovered and gently washed with 30 mM sodium formic/formate pH 3.5 buffer and 20% v/v of THF. Finally, polymer was dried at 40 °C in vacuum until constant weight.

2.6. Characterization of succinimidyl-functionalized polymer

2.6.1. FT-IR-Spectroscopy

Infrared (IR) spectra were obtained in a FTIR spectrometer with a diamond ATR accessory for solid and liquid samples, requiring no sample preparation, and the major frequencies were reported in cm^{-1}. The synthesized polymer was examined as KBr pellets.

2.6.2. Size–exclusion chromatography

Size–exclusion chromatography (SEC/GPC) was employed to determine average molecular weight. Waters Styragel HR4E DMF (50-100000) column was connected to the HPLC (Waters 600 with Waters 2414 Reflective Index Detector) and 1 ml/min DMF was used as the mobile phase. Standards with peak molecular weights of 66000, 34800, 17600, 9130, 3470, 1250 and 474 were used as molecular weight calibrations and the molecular weight distribution of the polymer was determined based on these standards.

2.6.3. Thermal characterization

Thermogravimetric analysis (TGA) was performed using a RIGAKU Thermo plus EVO equipment. For each assay 1–2 mg of polymer were used. The heating rate was set at 10 °C/min and all the experiments were carried out under air atmosphere constant at flow 100 ml/min.

2.7. β–Gal-3 immobilization

The polymer (10 mg) was added to the β–Gal-3 solution (0.1 mg) in 1.5 ml of 50 mM MES (2-(*N*-morpholino)ethanesulfonic acid) buffer, pH 6.2. The reaction was carried out for 12 h at 25 °C with gentle shaking (200 rpm). After 12 h of incubation, polymer particles were collected on a sintered-glass filter, and the solution was removed by suction under vacuum. The polymer particles were washed thoroughly on the same filter with 10 ml of 50 mM MES buffer, pH 6.2 (the volume was divided into five aliquots, and one aliquot was used for each washing step). The immobilized β–Gal-3-polymer samples were stored at 4 °C until use.

2.8. Determination of the amount of immobilized β–Gal-3

The protein concentration was determined using Bradford's method, [24] following the manufacturer's protocol (Bio-Rad) and bovine serum albumin as standard. The coupling yield (%) of the β–Gal-3 was determined by the difference between the initial β–Gal-3 amount present in the β–Gal-3 coupling solution and the final β–Gal-3 amount present in the remaining coupling solution.

2.9. Enzyme assays

Hydrolytic activity was determined by spectrophotometrical quantification of *p*NP liberated by the hydrolysis of *p*NP-β-galactopyranoside. Reactions were carried out in a 5 mM in sodium phosphate buffer 50 mM, pH 7.0 using a discontinuous method. 10 mg of immobilized enzyme or 2 μg of free enzyme were dissolved in 1 ml of 50 mM of each buffer and 5 mM of pNP-β-Gal were placed in a parallel reactor, the mixture was stirred (200 rpm) at 37 °C for 10 min. The reaction was stopped by adding 1 ml of 1 M Na_2CO_3. Samples were quantified at 410 nm comparing to a standard.

Each experimental assay was determinate at least three times with a standard deviation under 5%. One enzyme unit (U) was defined as the amount (mg) of protein that hydrolyses 1.0 μmol of substrate per minute. The β-Gal-3 showed an activity of 12.2 U/mg.

2.10. pH and thermal stability of free and immobilized enzyme

The pH stability of free and immobilized β–Gal-3 was studied by incubating the enzyme at 37 °C in buffer of varying pH in the range 4–9 for 20 min and then determining the catalytic activity.

10 mg of immobilized enzyme or 2 μg of free enzyme were dissolved in 1 ml of 50 mM of each buffer and 5 mM of *p*NP-β-Gal were placed in a parallel reactor, the mixture was stirred (200 rpm) at 37 °C for 10 min. The reaction was stopped by adding 1 ml of 1 M sodium carbonate. Samples were quantified at 410 nm comparing to a standard curve performed under the same experimental conditions.

Thermal stability was studied comparing remnant hydrolytic activity showed at different times for free or immobilized enzyme incubated in 50 mM of sodium phosphate buffer at 37 °C or 50 °C.

2.11. Transglycosylation reaction

Transglycosylation reactions were carried out using free and immobilized enzyme. *p*-NP-β-Gal (85 mM) and GlcNAc or GalNAc (425 mM) were dissolved in 500 μl of buffer sodium phosphate 50 mM pH 7.0 and pre-warmed at reaction temperature (37 °C). Reaction started by addition of biocatalyst to the mixture: 10 U of free enzyme and immobilized enzyme and the mixture was stirred (200 rpm) at 37 °C for 12 h. Aliquots (50 μl) were withdrawn from reaction media at different times to follow time course of product formation. Reaction was stopped by physical separation of biocatalyst by centrifugation of reaction media. Analytical determination of products was performed by HPLC using a NH2P50–4E amino column (Asahipak, Japan) whit the cooperative use of three detectors: ELSD (Evaporative Light Scattering Detector), UV–Vis at 317 nm and CD (Circular Dichroism). Finally, products were purified as following described: crude reaction mixture was loaded on carbon–celite column (50% m/m), eluted with a linear gradient from 0% to 15% (v/v) of ethanol in water. Solvents were extracted by vacuum and disaccharide powder was stored in a freezer. 30 mg of purified disaccharides were dissolved in D_2O to be characterized by ^1H NMR and ^{13}C NMR spectroscopy (D_2O, 500 MHz–500 MHz). Spectra were consistent with previous references [26, 27] (Fig. S1 in Supplementary information).

Gal-β(1–3)–GlcNAc: [26,27] ^1H NMR (500 MHz, D_2O): 1.96 (s, 3H, Ac), 5.11 (d, $J1\alpha,2 = 3.45$ Hz, H–1α). ^{13}C NMR (500 MHz, D_2O): 22.39 (Me of Ac, α), 22.64 (Me of Ac, β), 53.28 (C–2α), 56.02 (C–2β), 60.98 (C–6), 61.39 (C–6′), 68.94 (C–4′), 69.10 (C–4), 71.12 (C–2′), 71.62 (C–5α), 72.95 (C–3′), 75.63 (C–5′), 75.85 (C–5β), 80.57 (C–3α), 83.01 (C–3β), 91.42 (C–1α), 95.11 (C–1β), 103.83 (C–1′β), 103.96 (C–1′ α), 174.93 (C═O of Ac, α), 175.19 (C═O of Ac, β).

Gal-β(1A3)GalNAc: [27] ^1H NMR (500 MHz, D_2O): 1.92 (s, Ac), 4.58 (d, $J1\beta$, 2 = 8.47 Hz, H-1β), 5.11 (d, $J1\alpha$ 2 = 3.71 Hz, H-1α). ^{13}C NMR (500 MHz, D_2O): 21.96 (Me of Ac, α), 22.19 (Me of Ac, β), 48.92 (C-2α), 52.39 (C-2β), 60.89 (C6α), 60.93 (C-6β), 61.11 (C-6′), 68.01 (C-4′), 68.51 (C-4α), 68.68 (C-4β), 70.13 (C-5α), 70.57 (C-2′), 72.48 (C-3′), 74.78 (C-5β), 74.90 (C-5′), 76.99 (C-3α), 80.01 (C-3β), 91.13 (C-1, α), 95.12 (C1β), 104.64 (C-1′ β), 104.81 (C-1′ α), 174.61 (C═O of Ac, α), 174.91 (C═O of Ac, β).

2.12. Re-use cycles in transglycosylation reactions

To evaluate the reusability of immobilized biocatalyst in the synthesis of LNB, after the first reaction was stopped, the immobilized biocatalyst was recovered, washed and re-assayed with fresh substrate mixture under the same conditions as in the first experiment, and then the process was repeated eight times. The residual activity was calculated by taking the enzyme activity of the first cycle as 100%.

3. Results and discussion

A functionalized phenolic polymer having an *N*-succinimidyl group was designed as a support for enzyme immobilization and its enzymatic synthesis was executed in one step using SBP as a biocatalyst. The presence of the *N*-succinimidyl group allows easily and directly preparation

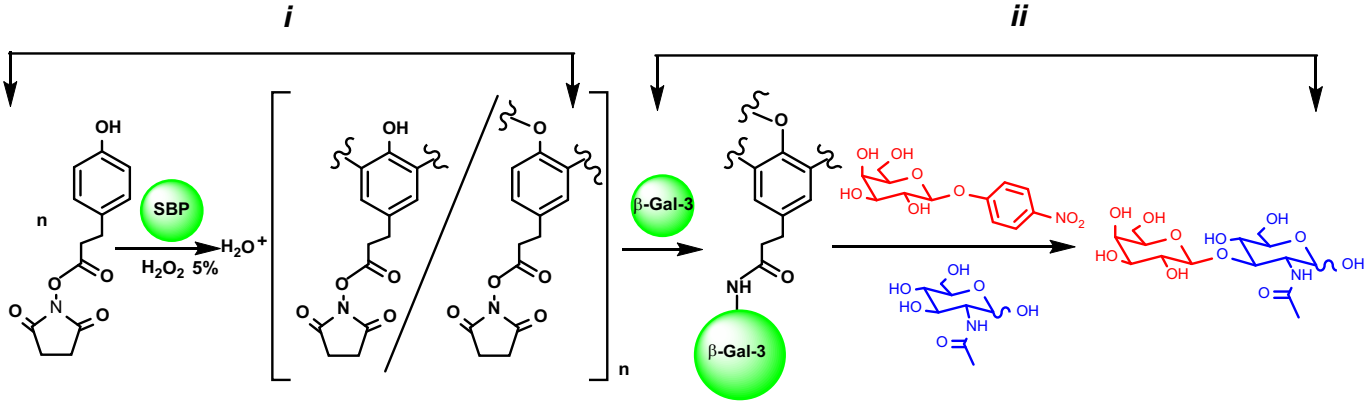

Fig. 1. Two-step reaction for the biosynthesis of: *i*) phenolic polymer mediated by the action of soybean hull peroxidase (SBP); *ii*) further immobilization of β–galactosidase from *B. circulans* (β -Gal-3), able to catalyze transglycosilation reaction.

of a functionalized polymer highly reactive, which can be covalently linked to the amino residues of β–galactosidase located in the surface [28–30]. For the enzyme immobilization we selected the β–galactosidase from *Bacillus circulans* (β -Gal-3 ATCC 31382, EC 3.2.1.23), this is an interesting glycosidase employed as biocatalyst in the synthesis of β-(1 → 3) galactosides such as lacto-*N*-biose (Gal-β-D-(1 → 3)GlcNAc) (Fig. 1) [31].

3.1. SBP–catalyzed polymerization

As has been described in experimental section phenolic polymerization was carried out using SBP and H_2O_2 in THF/buffer solution. Initially effect of H_2O_2 addition method on catalytic efficiency of SBP was investigated through analysis of substrate consumption during polymerization. The comparison of substrate measured at final time reveals that polymerization catalyzed by SBP is more efficient when the 5% of H_2O_2 is added dropwise to the reaction media (called method I), reporting a total consumption of substrate of 86%, compared with addition in one single step (method II), where the value of substrate consumption is 65%.

Inactivation effects of H_2O_2 over peroxidase activity are high documented in the bibliography [32–34]. In fact, as expected, dosing the addition of H_2O_2 brings better reaction yields, probably due to that the addition of a single dose likely produces an inhibitory effect on the peroxidase enzyme.

3.2. Polymer characterization

The obtained phenolic polymer was characterized using different techniques such FT-IR spectroscopy, thermogravimetric (TGA), differential scanning calorimetry (DSC) and gel permeation chromatography (GPC).

A comparison between infrared spectrum of Bolton–Hunter reagent (starting material) and the synthesized phenolic polymer was performed to verify the presence of functional groups. From the FT–IR spectra (Fig. S2 in Supplementary information) a disappearance of the broad peak at 3393 cm^{-1} of the polymer, assigned to phenolic O—H bond, suggest that the polymerization occurs by oxyphenylenes links. On the other hand, a strong signal at 1206 cm^{-1} was assigned to the asymmetrical stretching and vibrations of C–O–C and/or C–O–H links in the aromatic rings, and peaks showed at 1504, 1425 and 811 cm^{-1} confirm the presence of different vibration modes of C—H and C—C links to the aromatic rings.

Bands at 1780 and 1730 cm^{-1}, corresponding to the double bound of C═O, typical of the structure of cyclic imides are present in both spectrum, confirming that the enzymatic polymerization reaction did not affect the ester substituent structure, conserving its reactive capacity and be able to further use in the process of immobilization of the enzyme β–Gal–3 onto the polymer.

Thermogravimetric analysis (TGA) shows that polymer has excellent thermal stability up to 285 °C, where the polymers begin to lose weight drastically (Fig. 2).

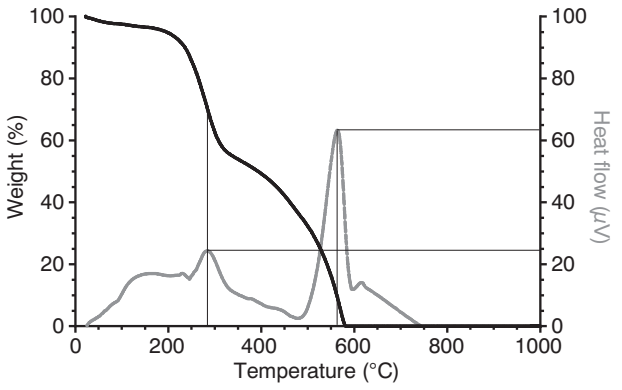

Fig. 2. Thermogravimetric analysis of the phenolic polymer.

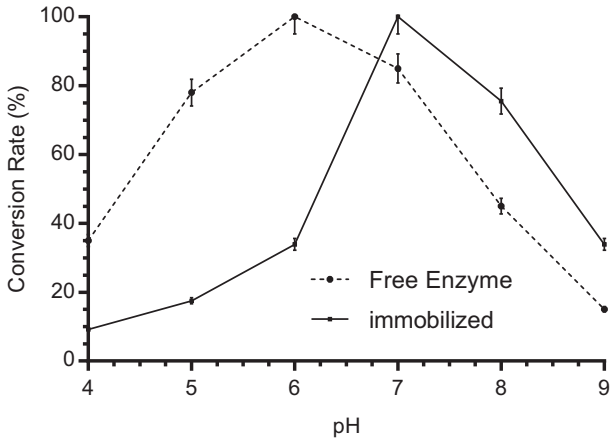

Fig. 3. Effect of pH on the activity of the enzyme β–Gal–3 free and immobilized monitoring the hydrolysis of the substrate *p*NP–β–Gal 5 mM at 37 °C.

Differential scanning calorimetry (DSC) was used to determine a glass transition temperature (Tg) of 272 °C (Fig. S3 in Supplementary information).

Finally, the gel permeation chromatography (GPC) allowed determining an average molar mass of the polymer of 12.6 kDa with a polydispersity index of 1.08. (Mn = 11.5 kDa, Mw = 12.5 kDa, Mz = 13.6 kDa).

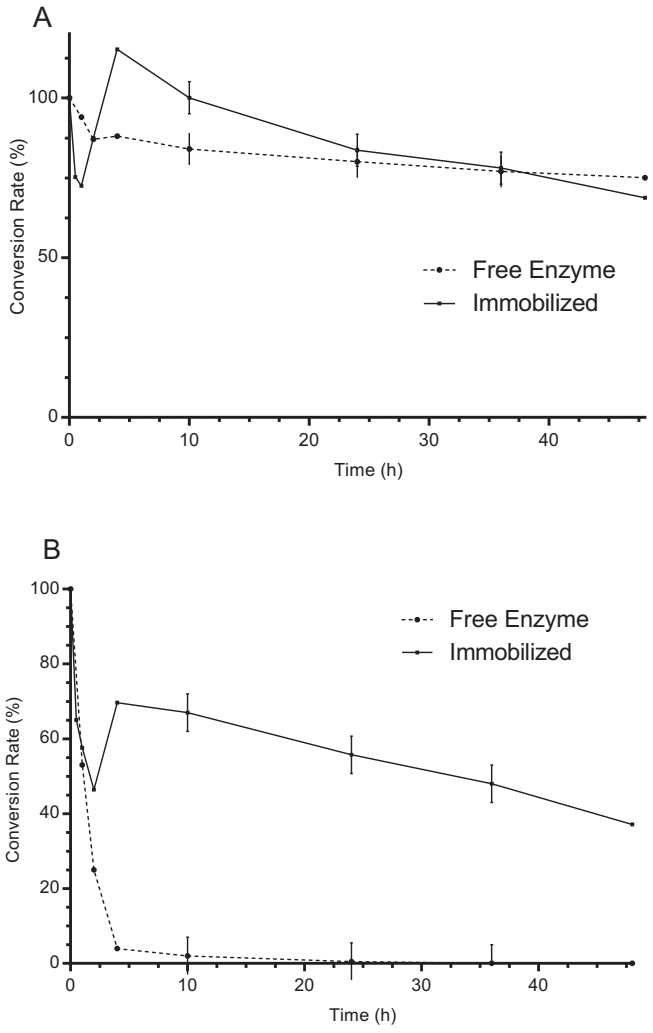

Fig. 4. Thermal stability of free and immobilized β–Gal–3 at 37 °C (A) and 50 °C (B).

3.3. Immobilization of β–Gal–3

The ability to use the synthesized polymer as support for enzyme immobilization was evaluated. Following the procedure described in material and methods, β-Gal–3 was successfully immobilized, with 64% of the enzyme added covalently linked to the support and the new immobilized biocatalyst retains about 35% of the hydrolytic activity of the free enzyme. Immobilization process usually produces alterations in enzyme activity, mainly caused by possible changes in the enzyme structure due to immobilization per se. However, majority of these alterations are considered as positive in total terms, because are related to high stabilization in extreme conditions such as high temperatures or pH [35].

3.4. Effect of pH and thermal stability on enzyme activity

The study of pH effect on the activity of the β-Gal–3 free and immobilized was performed in a pH range covers from 4 to 9, expressing the enzyme activity obtained for each pH value as relative activity compared with the maximum value (Fig. 3). β-Gal–3 immobilized enzyme exhibited a shift of about 1 unit toward basic pH values. Similar results had been shown in our group with the immobilization of other β-galactosidase isoforms from *B. circulans* in different supports such as porous polymers or Eupergit–C, and a possible reason may be under the slightly change of pK_a of different internal residues showed as result of the immobilization process [30,36,37].

Thermal stability assay was performed as described in the experimental section (Fig. 4). As can be seen, immobilized and free enzyme are very stable at 37 °C, retaining up to 88% of activity after 48 h of incubation. At 50 °C, free enzyme lost drastically activity after 5 h of incubation, becoming inactive. Immobilized enzyme show higher thermostability, where enzyme activity remains close to 47% after 48 h of incubation. It was previously described that immobilization of these enzymes on agarose gels increase enzyme stability preserving enzyme activity under drastic conditions such as high temperatures [11]. Similar results had been showed in the immobilization of other β-galactosidase isoforms from *B. circulans* in different supports such as porous polymers or Eupergit–C [36,37].

3.5. Synthesis of Gal–β(1–3)–GlcNAc and Gal–β(1–3)–GalNAc catalyzed by β-Gal-3 immobilized

The transglycosylation reaction of *p*-nitrophenyl-β-D-galactopyranoside (*p*-NP-β-Gal) as donor and *N*-acetyl glucosamine (GlcNAc) or *N*-acetyl galactosamine (GalNAc) as acceptors, catalyzed by β-Gal-3 immobilized in the phenolic polymer will be used throughout this work.

This reaction can afford Gal-β-(1-3)-GlcNAc as a major product (99% conversion) after 5 h, when GlcNAc was used as acceptor, while with GalNac the main product formed was Gal-β-(1-3)-GalNAc with similar yields (97%) (Fig. 5). Substrate consumption was followed through HPLC, where total consumption of the substrate *p*NP–Gal was achieved after 5 h, coinciding with the maximum yield of desired product.

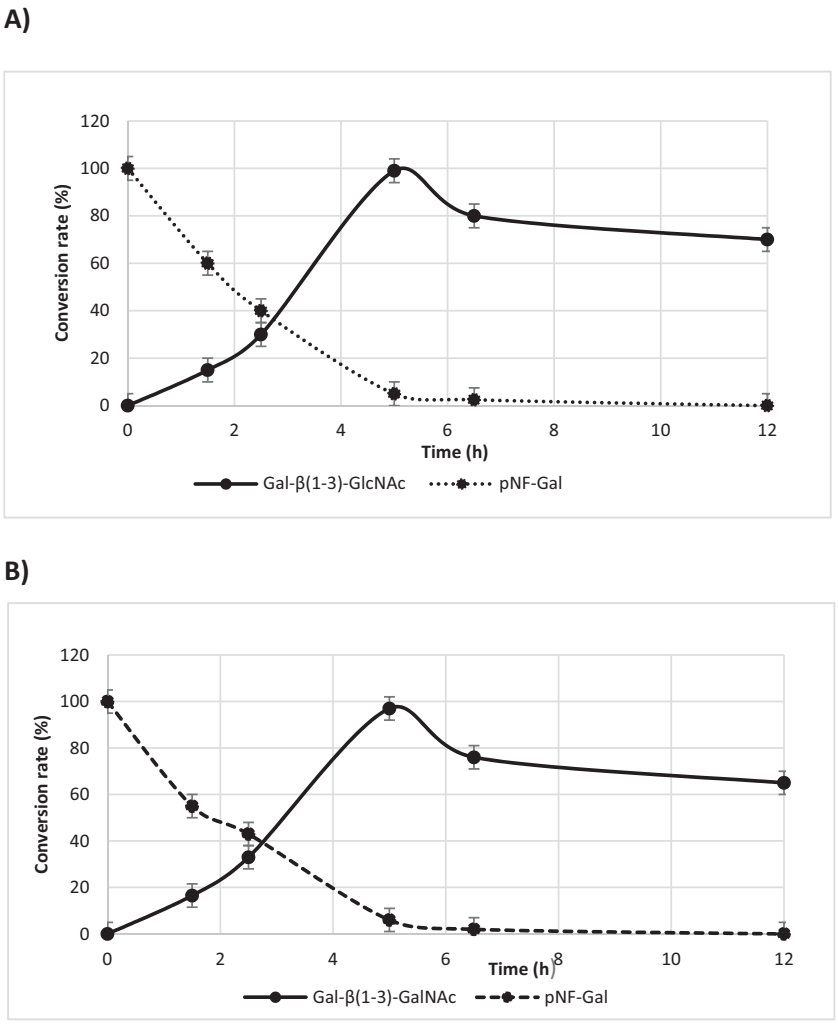

Fig. 5. Kinetics of transglycosylation reaction catalyzed by β–Gal–3 immobilized using *p*NF–Gal as donor and GlcNAc (**A**) or GalNAc (**B**) as acceptors.

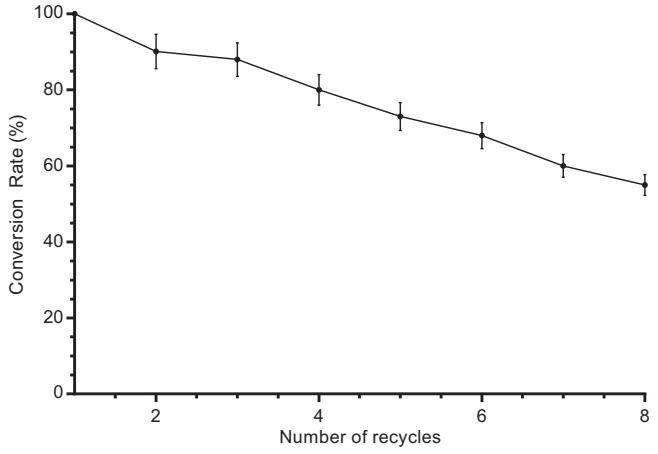

Fig. 6. Repetitive synthesis of catalyzed by immobilized β-Gal-3.

Beyond 6.5 h, the product begins to be hydrolyzed as consequence of enzyme inverse reaction, establishing optimal reaction time as 5 h for total substrate consumption, maximum product obtaining and total regioselectivity.

3.6. Re-use cycles in transglycosylation reactions

The potential re-use of supported enzymes in the synthesis Gal–β(1–3)–GlucNAc was investigated. After the first assay, immobilized enzyme was recovered, washed and re-assayed with fresh substrate mixture under the same experimental conditions, and this procedure was repeated 8 times. The results obtained in the reuse of immobilized enzyme in transglycosylation process are shown in Fig. 6. As can be seen, the new immobilized enzyme was recycled seven times (eight catalytic cycles), so this mean that the same amount of immobilized enzyme was working 40 h (5 h, initial cycle, plus 7 × 5 h, 5 reuses) at 37 °C, still retaining a 50% of residual activity. However, the selectivity with respect to Gal–β(1–3)–GlucNAc formation remained after re-use. Similar stability data have been obtained using this enzyme immobilized on agarose gel [14] or the immobilization of other β-galactosidase isoforms from *B. circulans* in different supports such as porous polymers or Eupergit–C [36,38].

4. Conclusions

In conclusions, an efficient and sustainable enzymatic procedure to obtain an immobilized biocatalyst was successfully developed. In a first step SBP was able to catalyze the enzymatic polymerization of a functionalized phenol which was used in a second step, as an activated support for the covalent immobilization of a β–galactosidase. This new biocatalyst could be usefully applied as a recoverable and reusable biocatalyst for the synthesis of Gal–β(1–3)–GlucNAc and Gal–β(1–3)–GlucNAc with high yield and regioselectivity.

This approach represents a useful two-step system involving SBP and β–Gal-3 and thus widens the range of applications of these valuable biocatalysts in preparative biotransformations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.05.177.

References

[1] S. Shoda, H. Uyama, J. Kadokawa, S. Kimura, S. Kobayashi, Enzymes as green catalysts for precision macromolecular synthesis, Chem. Rev. 116 (4) (2016) 2307–2413.
[2] A. Farran, C. Cai, M. Sandoval, Y.M. Xu, J. Liu, M.J. Hernáiz, R.J. Linhardt, Green solvents in carbohydrate chemistry: from raw materials to fine chemicals, Chem. Rev. 115 (14) (2015) 6811–6853.
[3] A. Knop, L.A. Pilato, Phenolic Resins: Chemistry, Applications and Performance, Springer Science & Business Media, 2013.
[4] R.A. Gross, A. Kumar, B. Kalra, Polymer synthesis by in vitro enzyme catalysis, Chem. Rev. 101 (7) (2001) 2097–2124.
[5] J.A. Swenberg, B.C. Moeller, K. Lu, J.E. Rager, R.C. Fry, T.B. Starr, Formaldehyde carcinogenicity research: 30 years and counting for mode of action, epidemiology, and cancer risk assessment, Toxicol. Pathol. 41 (2) (2013) 181–189.
[6] M. Reihmann, H. Ritter, Synthesis of phenol polymers using peroxidases, Adv. Polym. Sci. 194 (2006) 1–49.
[7] A. Kumbul, E. Gokturk, E. Turac, E. Sahmetlioglu, Enzymatic oxidative polymerization of para-imine functionalized phenol catalyzed by horseradish peroxidase, Polym. Adv. Technol. 26 (2015) 1123–1129.
[8] B. Eker, D. Zagorevski, G. Zhu, R.J. Linhardt, J.S. Dordick, Enzymatic polymerization of phenols in room-temperature ionic liquids, J. Mol. Catal. B Enzym. 59 (2009) 177–184.
[9] S. Kobayashi, A. Makino, Chem. Rev. 109 (2009) 5288–5353.
[10] G. Hommes, C.A. Gasser, C.B. Howald, R. Goers, D. Schlosser, P. Shahgaldian, P.F. Corvini, Production of a robust nanobiocatalyst for municipal wastewater treatment, Bioresour. Technol. 115 (2012) 8–15.
[11] M. Hamid, Potential applications of peroxidases, Food Chem. 115 (4) (2009) 1177–1186.
[12] C. Bayon, A. Cortes, A. Aires-Trapote, C. Civera, M.J. Hernáiz, Highly efficient and regioselective enzymatic synthesis of β-(1 → 3) galactosides in biosolvents, RSC Adv. 3 (30) (2013) 12155–12163.
[13] C. Bayon, A. Cortes, J. Berenguer, M.J. Hernáiz, Highly efficient enzymatic synthesis of Gal β-(1 → 3)-GalNAc and Gal β-(1 → 3)-GlcNAc in ionic liquids, Tetrahedron 69 (24) (2013) 4973–4978.
[14] S. Gomez, C. Bayon, S. Navarrete, J.M. Guisan, M.J. Hernáiz, Stabilization of β-Gal-3 ATCC 31382 on agarose gels: synthesis of β-(1 → 3) galactosides under sustainable conditions, RSC Adv. 6 (83) (2016) 79554–79562.
[15] M. Perez-Sanchez, M. Sandoval, M.J. Hernáiz, P.D. de Maria, Biocatalysis in biomass-derived solvents: the quest for fully sustainable chemical processes, Curr. Org. Chem. 17 (11) (2013) 1188–1199.
[16] M. Perez-Sanchez, M. Sandoval, A. Cortes-Cabrera, H. Garcia-Marin, J.V. Sinisterra, J.I. Garcia, M.J. Hernáiz, Solvents derived from glycerol modify classical regioselectivity in the enzymatic synthesis of disaccharides with Biolacta beta-galactosidase, Green Chem. 13 (10) (2011) 2810–2817.
[17] M. Sandoval, A. Cortes, C. Civera, J. Trevino, E. Ferreras, M. Vaultier, J. Berenguer, P. Lozano, M.J. Hernáiz, Efficient and selective enzymatic synthesis of *N*-acetyllactosamine in ionic liquid: a rational explanation, RSC Adv. 2 (15) (2012) 6306–6314.
[18] R.S. Loka, C.M. Sadek, N.A. Romaniuk, C.W. Cairo, Conjugation of synthetic *N*-acetyllactosamine to azide-containing proteins using the Staudinger ligation, Bioconjug. Chem. 21 (10) (2010) 1842–1849.
[19] P.K. Mandal, W.B. Turnbull, Studies on the synthesis of Lewis-y oligosaccharides, Carbohydr. Res. 346 (14) (2011) 2113–2120.
[20] K. Klisch, E. Jeanrond, P.C. Pang, A. Pich, G. Schuler, V. Dantzer, M.P. Kowalewski, A. Dell, A tetraantennary glycan with bisecting *N*-acetylglucosamine and the Sd (a) antigen is the predominant N-glycan on bovine pregnancy-associated glycoproteins, Glycobiology 18 (1) (2008) 42–52.
[21] V. Engelstaedter, B. Fluegel, S. Kunze, D. Mayr, K. Friese, U. Jeschke, F. Bergauer, Expression of the carbohydrate tumour marker Sialyl Lewis A, Sialyl Lewis X, Lewis Y and Thomsen-Friedenreich antigen in normal squamous epithelium of the uterine cervix, cervical dysplasia and cervical cancer, Histol. Histopathol. 27 (4) (2012) 507–514.
[22] H. Ishida, A. Togayachi, T. Sakai, T. Iwai, T. Hiruma, T. Sato, R. Okubo, N. Inaba, T. Kudo, M. Gotoh, J. Shoda, N. Tanaka, H. Narimatsu, A novel β-1,3-*N*-acetylglucosaminyltransferase (β3Gn-T8), which synthesizes poly-N-acetyllactosamine, is dramatically upregulated in colon cancer, FEBS Lett. 579 (1) (2005) 71–78.
[23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (5259) (1970) 680–685.
[24] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[25] B. Eker, D. Zagorevski, G. Zhu, R.J. Linhardt, J.S. Dordick, Enzymatic polymerization of phenols in room-temperature ionic liquids, J. Mol. Catal. B Enzym. 59 (1) (2009) 177–184.
[26] A. Vetere, M. Miletich, M. Bosco, S. Paoletti, Regiospecific glycosidase-assisted synthesis of lacto-*N*-biose I and (Galβ1-3GlcNAc) and 3′-sialyl-lacto-*N*-biose I (NeuAcα2-3Galβ1-3GlcNAc), Eur. J. Biochem. 267 (4) (2000) 942–949.
[27] H. Yu, V. Thon, K. Lau, L. Cai, Y. Chen, S. Mu, Y. Li, P.G. Wang, X. Chen, Highly efficient chemoenzymatic synthesis of β1-3-linked galactosides, Chem. Commun. 46 (40) (2010) 7507–7509.
[28] C. Mateo, O. Abian, M. Bernedo, E. Cuenca, M. Fuentes, G. Fernandez-Lorente, J.M. Palomo, V. Grazu, B.C. Pessela, C. Giacomini, Some special features of glyoxyl supports to immobilize proteins, Enzym. Microb. Technol. 37 (4) (2005) 456–462.

[29] C. Mateo, J.M. Bolivar, C.A. Godoy, J. Rocha-Martin, B.C. Pessela, J.A. Curiel, R. Munoz, J.M. Guisan, G. Fernández-Lorente, Improvement of enzyme properties with a two-step immobilizaton process on novel heterofunctional supports, Biomacromolecules 11 (11) (2010) 3112–3117.
[30] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzym. Microb. Technol. 40 (6) (2007) 1451–1463.
[31] Y. Ito, T. Sasaki, Cloning and characterization of the gene encoding a novel β-galactosidase from *Bacillus circulans*, Biosci. Biotechnol. Biochem. 61 (8) (1997) 1270–1276.
[32] B. Valderrama, Deactivation of hemeperoxidases by hydrogen peroxide: focus on compound III, in: E. Torres, M. Ayala (Eds.), Biocatalysis Based on Heme Peroxidases, Springer, Berlin Heidelberg 2010, pp. 291–314.
[33] B. Valderrama, M. Ayala, R. Vazquez-Duhalt, Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes, Chem. Biol. 9 (5) (2002) 555–565.
[34] L. Mao, S. Luo, Q. Huang, J. Lu, Horseradish peroxidase inactivation: heme destruction and influence of polyethylene glycol, Sci. Rep. 3 (2013) 3126.
[35] R.C. Rodrigues, C. Ortiz, A. Berenguer-Murcia, R. Torres, R. Fernandez-Lafuente, Modifying enzyme activity and selectivity by immobilization, Chem. Soc. Rev. 42 (15) (2013) 6290–6307.
[36] A. Aires-Trapote, A. Tamayo, J. Rubio, A. Rumbero, M.J. Hernáiz, Sustainable synthesis of *N*-acetyllactosamine using an immobilized β-galactosidase on a tailor made porous polymer, RSC Adv. 5 (50) (2015) 40375–40383.
[37] M.J. Hernaiz, D.H. Crout, Immobilization/stabilization on Eupergit C of the β-galactosidase from *Bacillus circulans* and an α-galactosidase from Aspergillus oryzae, Enzym. Microb. Technol. 27 (1–2) (2000) 26–32.
[38] M.J. Hernaiz, D.H.G. Crout, A highly selective synthesis of *N*-acetyllactosamine catalyzed by immobilised β-galactosidase from *Bacillus circulans*, J. Mol. Catal. B Enzym. 10 (4) (2000) 403–408.