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Stabilization of β–Gal-3 ATCC 31382 on agarose gels: synthesis of β-(1→3) galactosides under sustainable conditions

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β-Gal-3 ATCC 31382 (β-Gal-3) was immobilized by multipoint covalent attachment on an agarose support using different enzyme orientations on its surface. The derivatives that showed more activity and stability were the ones bound to a Lys rich region on a monofunctional glyoxyl-agarose (GA) support. Also, immobilization was performed using a Glu+Asp rich region on a heterofunctional amino-glyoxyl-agarose (AMGA) support. The immobilized enzyme was characterized in terms of optimal pH and thermal stability, and its catalytic efficiency was tested on the synthesis of β-(1→3) galactosyldisaccharides. Reactions were performed in the presence of green solvents ([Bmim][PF6] and 2HNND) with maximum conversion and mantained regioselectivity. Reusability assays under identical reaction conditions were also performed to find that GA immobilized enzyme retains about 90% of its activity after six batches with conversion yields above 75 % when [Bmin][PF6] was used as reaction media. Furthermore, green solvent recovery and recycling are achieved retaining catalytic activity and increased productivity.

Introduction

One of the key problems of enzymes as industrial biocatalysts is their moderate stability under industrially relevant conditions,[1] such as moderately high temperatures and presence of solvents.[2,3] Additionally, the use of free enzymes has other processing difficulties such as, reusability and product contamination, which enormously limit their industrial application.[4,5]

In recent decades, the improvement of operational and catalytic activity of enzymes for industrial purposes has been studied through several approaches which include process modifications, molecular biology strategies and protein immobilization. Therefore, enzyme immobilization rises as the most common strategy for this goal.[6-8] Enzyme immobilization may offer several advantages which include enhancement of stability, reusability of the enzyme and easy separation from the reaction media.[8-13] These advantages reduce operational costs and promote the use of immobilized enzymes for industrial uses.[6,12,14,15]

From the choice of polymeric supports for enzyme immobilization, glyoxyl-agarose is a widely used support because it provides a high degree of stabilization for many enzymes.[16,17] Immobilization in glyoxyl-agarose is normally carried out on the protein surface area with the highest concentration of lysine residues. Recent developments on glyoxyl-agarose supports include polymers that have been modified to promote other orientations of the enzyme based on the presence of anionic or cationic residues, or histidine tags.[18-20]

β-Gal-3 ATCC 31382 (EC 3.2.1.23) is an interesting glycosidase employed as biocatalyst in the synthesis of β-(1→3) galactosides such as, Galactic-*N*-biose (Gal-β-D-(1→3)GalNAc, GNB) and Lacto-*N*-biose (Gal-β-D-(1→3)GlcNAc, LNB).[21] Recently, we have reported the sustainable enzymatic synthesis of these disaccharides in presence of ionic liquids (ILs) or biosolvents, obtaining high conversion rates and excellent regioselectivity.[22-24] In a previously reported study, β-Gal-3 ATCC 31382 was covalently immobilized on the traditional CNBr-Sepharose support and catalyst efficiency was verified carrying out the synthesis of β-(1→3) galactosides with proceeded with a 62% yield.[25] However, there is still a need to improve the stability and reduction of production costs of this biocatalyst to meet industrial requirements.[26]

We present here the first example of a covalent immobilization of β-Gal-3 onto different heterofunctional glyoxyl-agaroses supports, showing how the best immobilized derivative can be usefully applied as a recoverable and reusable catalyst in green solvents (biosolvents and ionic liquids) to synthesize β-(1→3) galactosides with high yield and regioselectivity.

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Results and discussion

Optimization of immobilization under alkaline conditions

We first analyzed the immobilization of the β-Gal-3 on cross-linked agarose previously functionalized by four different methods (Figure 1). Agarose was selected as support because it comes from renewable sources, it can be degraded naturally, it is highly hydrophilic (and so is the enzyme) and as result of the process it generates a greener immobilized biocatalyst.[18,27]

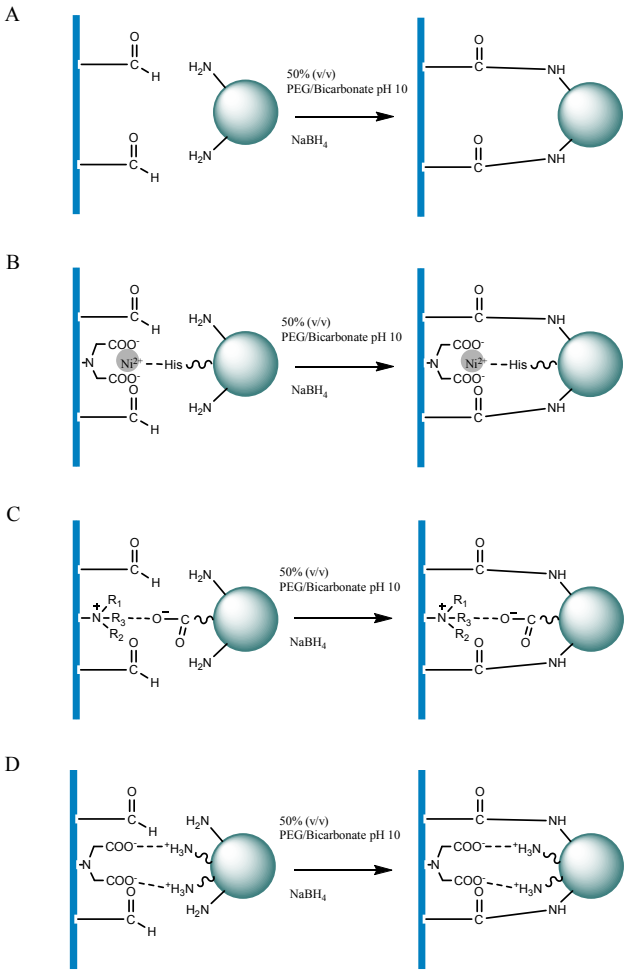


Figure 1. Immobilization strategies used. **A)** GA support; **B)** NiGA support chelated with nickel; **C)** AMGA support activated with aminoethyl residues; **D)** COGA support activated with carboxyethyl residues.

The covalent immobilization process on glyoxyl agarose (GA) and heterofunctional supports implies the reaction of ε-NH_2 groups of the lysine residues located on the enzyme surface with aldehyde groups of the carrier under alkaline conditions; whereas on amino glyoxal agarose (AMGA) the covalent binding was mediated also by reaction of the enzyme carboxyl groups with support aminoethyl groups. Immobilization on chelate glyoxyl agarose (NiGA) was based on the reaction of the enzyme HisTag with the resin nickel residues. The enzyme-carrier bonds generated are reversible imine bonds that can be reduced with $NaBH_4$ to secondary amines (Figure 1).[18,18]

GA support was prepared by activation of the matrix with glycidol and following oxidation with periodate.[16] Heterofunctional agarose supports were prepared via a two-step activation process: i) activation of agarose with epiclorohydrin yielding supports with epoxy and glyceryl residues; ii) and subsequently modification of epoxy groups with triethylamine, iminodiacetic or $NiCl_2$ and oxidation with sodium periodate for obtaining AMGA, COGA or NiGA respectively. Maximum binding capacity of 105±5 μmol activated residues/0,5 gr of support were obtained in all cases, allowing to intramolecular multi-point covalent binding.[19] The immobilization on these supports was evaluated using the β-Gal-3 enzyme as model.

On the other hand, it has been reported for other enzymes that alkaline conditions and reduction steps may lead to enzyme irreversible inactivation.[17,28] We evaluated the stability of the soluble enzymes under alkaline conditions. We observed that β-Gal-3 only kept 70.0 % of its initial activity after 30 min of incubation and 30.0 % of its initial activity after 2 h of incubation under such conditions. In order to avoid this, we screened different types of additives (glycerol, threalose and PEG 600) to improve enzyme stability under alkaline conditions (Figure 2). Several additives did have a strong effect on the stability of soluble β–Gal-3, but the enzymes increased their stability when 30 % of PEG600 solutions under alkaline conditions (pH 10.0) were used, obtaining a 100 % of activity after 2 h. These results agree with different studies that describe polyols as stabilizers for many enzymes.[29,30]

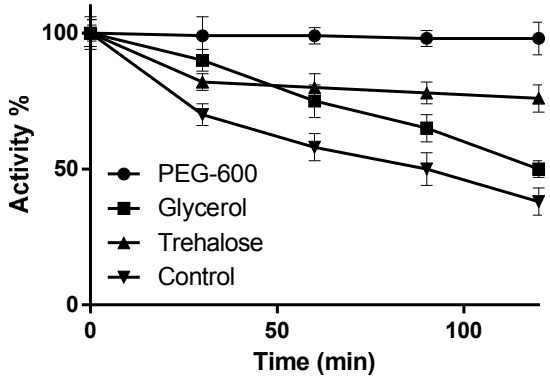


Figure 2. Inactivation profile of β-Gal 3 incubated along 120 minutes at 4ºC in the presence or absence of different additives.

The best conditions were found after a thorough screening of different additives to immobilize β-Gal-3 on agarose supports under alkaline conditions, and based on these results, PEG-600 was selected for further studies. In order to study the influence of PEG600 on the stability of the β-Gal 3 at pH 10.0, the activity was analyzed in the presence of different

percentages of PEG600 (5, 10, 20, 30 and 50 %). Assays were performed at room temperature and results obtained are shown in Figure 3. The best stabilization conditions were obtained with 50% (w/w) of PEG 600, where the enzyme retains 86% activity after 30 min and 56 % after 2h. The same effect was previously observed in a recent work using a commercial β-galactosidase from *B. circulans*, where addition of 20% glycerol to the immobilization media avoids protein inactivation.[31]

Selection of agarose activation method

As it has already been mentioned, β-Gal-3 was immobilized in an agarose matrix previously functionalized by four different methods (Figure 1) analyzing their effect on immobilization yields. The enzyme load during the immobilization step was set to 0.5 mg of enzyme for 1 gr of support in 5 mL of 50% (v/v) PEG600-buffer bicarbonate pH 10.0, except for COGA support, where was 0,6 mg enzyme per 1 g of support. These conditions were selected according to previously described studies performed with another enzymes such as a commercial β-galactosidase from *B. circulans*, where, in order to prevent diffusional restrictions, low enzyme loading was selected as optimal.[31]

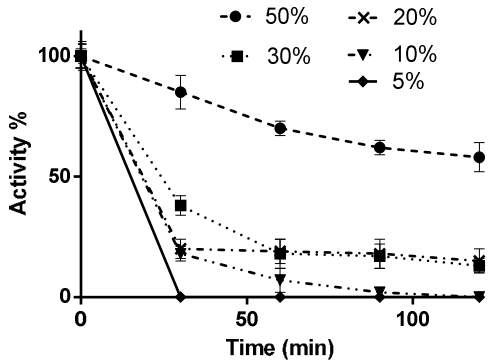


Figure 3. Stability of the free β-Gal-3 at different percentages of PEG-600. The β-Gal 3 was incubated along 120 minutes at 25ºC in the presence of different percentages of PEG-600.

As we can see in Table 1, 100 % binding of protein to glyoxyl agarose (GA), amino-glyoxyl agarose (AMGA) and chelate-glyoxyl agarose (NiGA) was observed. Only in the case of carboxy glyoxyl agarose (COGA) the enzyme loaded was 60 %. On the other hand, highest activity was found when GA was used (85%), compared to AMGA (53%), CGA (2.7%) and NiGA (0.96 %).

A key element in explaining these results is the first event for the incorporation of the enzyme on the support; *i*) the enzyme is directly immobilized on monofunctional GA by covalent immobilization through the region having the highest density of Lys residues (31 residues on β-Gal-3 surface) allowing multipoint binding; *ii*) the enzyme is ionically adsorbed on amino glyoxyl agarose (AMGA) through the region having the highest density of Glu+Asp residues and then an additional incubation under alkaline conditions promotes the multipoint covalent attachment between amino groups placed in that region and glyoxyl groups on the support surface. β-Gal-3 is rich in Asp (28 residues on β-Gal-3) and Glu (53 residues on β-Gal-3) on their surface (Figure 4) and with an pI of 5.13, which means that the enzyme is negative charged at immobilization pH, allowing immobilization to positive charged residues of AMGA; *iii*) the enzyme is ionically adsorbed on carboxy glyoxyl agarose (COGA) through the region having the highest density of Lys residues and then alkaline conditions promotes the covalent attachment between amino groups placed in that region and glyoxyl groups on the support surface.

Table 1. Immobilization of β-Gal 3 in different heterofunctional agarose supports.

Support	Protein Bounded (%)	Retained Activity (%)
GA	100 ± 5	85 ± 4
AMGA	100 ± 5	53 ± 2
COGA	60 ± 3	2.7 ± 14
NiGA	100 ± 5	0.96 ± 0.05

The low protein load obtained with COGA is explained by the fact that low amount of contacted enzyme was adsorbed to this anionic support as a consequence of its negative net charge at immobilization pH; *iv*) the immobilization of enzymes on chelate-glyoxyl-agarose (NiGA) was based in a first the adsorption of HisTag of enzyme with nickel residues of resin and a then alkaline conditions promotes the covalent attachment between Lys placed near the HisTag and the glyoxyl groups of the support. A much lower activity was obtained when β-Gal-3 was immobilized on NiGA support (Table 1). This drastic loss of activity suggests that the type of support on which the enzyme is immobilized could modify the surface region that was involved in the first adsorption and the further multipoint covalent attachment. In the case of NiGA derivative, adsorption on regions where the HisTag is situated may promote the most relevant changes on enzyme activity. Based on these results, all the following experiments were conducted with the immobilized derivatives GA and AMGA.

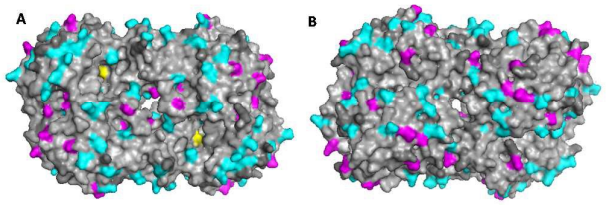


Figure 4. The representation of the surface of β-Gal-3, yellow active site, blue Glu and magenta Asp **A)** the front; **B)** the rear

Optimization of Enzyme Loading.

The first variable studied which could affect the immobilization efficiency was the relative amounts of polymer and enzyme used in the coupling process. Thus, different enzymatic amounts were used, keeping a fixed quantity of supporting polymer (GA). As can be seen in Figure 5, using a 0.5 mg/g ratio of protein to polymer, 85% binding of protein to GA

support was observed. Nevertheless, when the amount of enzyme added to the immobilization mixture was increased, up to 60% of the protein added did not get linked to the support. This finding could be explained taking into account some diffusional restrictions previously described when using glyoxyl agarose as enzyme support, where particle size and enzyme protein loading were identified as critical factors to improve immobilization efficiency.[32]

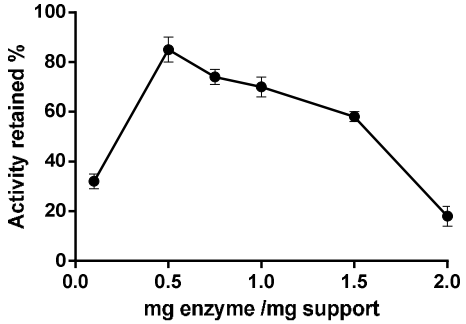


Figure 5. Optimal loading capacity assay of β-Gal-3 in GA support.

pH effects

The study of pH effects on the activity of free and immobilized enzyme on GA was performed in triplicated in the pH range from 4 to 9 (Figure 6). The enzyme immobilized on AMGA exhibited a shift in the optimal pH of about 1.0 unit toward basic pH values. In both cases, the activity decreases rapidly for free and immobilized enzymes between pH 8.0 and 9.0, being higher for the free enzyme that at pH 9.0 retains only a 12 % of activity. Changes in pH profile can be a consequence of internal changes of the pKa of the residues involved in the catalytic activity, as a result of immobilization.[14] Similar results were obtained in our group in previous immobilization of a commercial β-galactosidase Biolacta on poly(AGE-co-DVB)-27 or Eupergit C.[33,34]

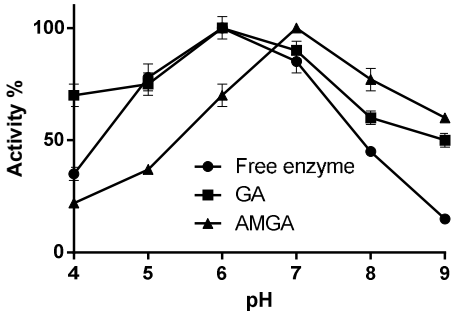


Figure 6. Effect of pH on the activity of free and immobilized β-Gal-3

Thermal stability of free and immobilized enzyme

The immobilized enzyme preparations can be stored at 4 °C for at least one month without appreciable deactivation (less than 5%). Thermal stability experiments were performed in triplicated as described in the experimental section. Figure 7A and 7B show the thermal stability behavior of the free and immobilized enzymes. As it can be seen, native enzyme shows a clear deactivation with half-life times of 1 h at 50ºC. This loss of activity for the free enzyme upon heating at 50ºC has been previously described for the β-galactosidase Biolacta.[33,34] After 24h at 50ºC, the residual activity for amine support was about 65% and 46% in GA, where soluble enzyme was inactivated. This enzyme stabilization could be explained by the benefits obtained by the multipoint covalent attachment suffers by the enzyme in these supports, mediated by, among others, the Lys residues located in the enzyme's surface without any conformational changes when GA was used.[17,18,35]

Transglycosylation in buffer and biosolvents

The β-Gal-3 has proven to be a valuable biocatalyst for galactosyl transfer from a suitable donor (*p*NP-gal) to an N-acetyl glucosamine (GlcNAc) as acceptor. The synthesis of Gal-β-(1→3)-GlcNAc employing β-Gal-3 have been previously reported (Scheme 1).[21] This reaction can afford Gal-β-(1→3)-GlcNAc as a major product and Gal-β-(1-6)-GlcNAc as a minor product in some cases.[25] Recent work from our group demonstrates that β-Gal-3 can catalyze the synthesis of Galβ-(1→3)-GlcNAc in the presence of different green solvents.[22,23] The results clearly showed an important increase in enzymatic activity with full retention of β-(1→3) regioselectivity. Yields of

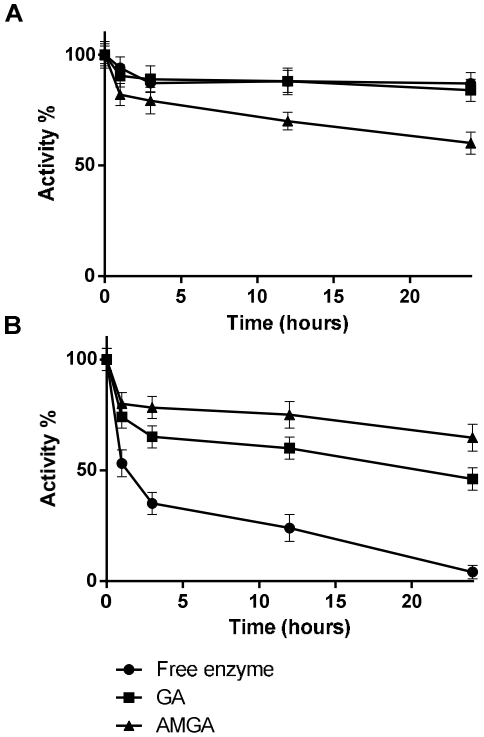
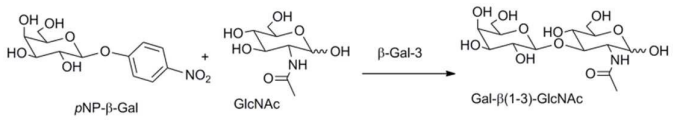


Figure 7. Thermal stability of free and immobilized β-Gal-3 at 37°C (**A**) and 50 °C (**B**).

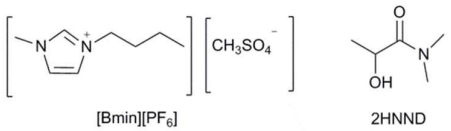
up to 99% of Gal-β-(1→3)-GlcNac were obtained with full substrate conversion. These reactions take place without noticeable hydrolytic activity and retaining regioselectivity, representing a considerable improvement over the use of aqueous buffers or conventional organic solvents.



Scheme 1. General scheme of transglycosylation reaction catalyzed by β-Gal-3

The influence of the immobilization of the β–Gal-3 (GA and AMGA derivatives) on transglycosylation reactions was studied. The reactions were carried out following the procedure described in the experimental section. The amount of Galβ-(1→3)-GlcNAc was monitored as a function of time, and samples were analyzed by HPLC. As we can see in Figure 8, with free enzyme, production of Galβ-(1→3)-GlcNAc reached a maximum of 51% in 3 h, in buffer. As for the immobilized enzyme in buffer, a yield of 34% was observed when GA support was used, and 25% in the case of AMGA.

Previous work from our laboratory has shown that the use of certain green solvents (biosolvents or IL) as cosolvents in the reaction media increases enzyme activity and regioselectivity is not affected. Yields of up to 99% of Gal-β-(1→3)-GlcNac were obtained with full substrate conversion.[22,23] Based on these results, solvents **1** and **2** (Scheme 2) were selected for further studies with the β-Gal-3 enzyme immobilized on GA and AMGA.



Scheme 2. Structure of green solvents employ in this work.

Transglycosylation reactions catalyzed by immobilized β-Gal-3 (GA and AMGA) were carried out following the general procedure described in the experimental section and monitored by HPLC. The concentration of green solvent was set to 2 M for 2HNND and 1.46 M of [Bmin][PF6], each of them was added to the reaction media (sodium phosphate buffer, 50 mM at pH 6.0). The results obtained for the transglycosylation reactions with the immobilized β-Gal-3 derivatives (AMGA and GA) in the presence of different green solvents ([Bmin][PF6] and 2HNND) are summarized in Figure 8.

Initially, the regioselectivity and conversion shown by GA and AMGA derivatives was investigated when the reaction was carried out in a 50 mM sodium phosphate buffer at pH 6.0 (Figure 8). For the GA derivative reaction affords Gal-β-(1→3)-GlcNAc as major product (34% conversion) using GlcNAc as acceptor. For the AMGA derivative, reaction carried out in the same buffer yields a 25% conversion when GlcNAc was used as acceptor

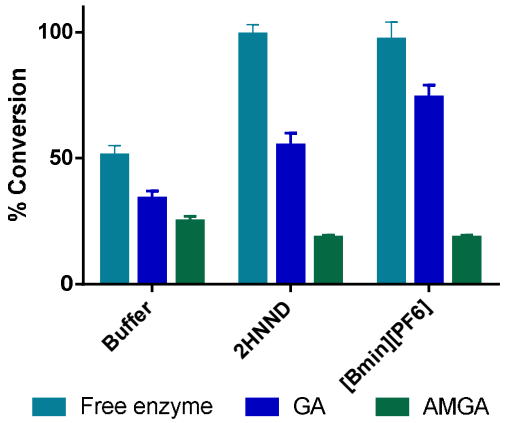


Figure 8. Transglycosylation yields obtained with free and immobilized β-Gal-3

In the presence of green solvent 2HNND, the GA derivative reaction yields 55% of Gal-β-(1→3)-GlcNAc and for AMGA derivative, reaction carried out in the dame green solvent gives a 18,5% yield of Gal-β-(1→3)-GlcNAc (Figure 8).

In [Bmin][PF6], GA derivative gave a 75% yield for Gal-β-(1→3)-GlcNAc and AMGA gave a 18,5% yield for Gal-β-(1→3)-GlcNAc.

To this date, the only work reported in similar system is the one performed by Naundorf *et al.* in 1998.[25] They analyzed the transglycosylation efficiency of β-Gal-3 as an immobilized derivative using CNBr-Sepharose as support, obtaining a 62% molar ratio in β(1→3) derivatives when the reaction was carried out in a 100 mM sodium phosphate buffer at pH 6.0 with 20% of DMF as cosolvent, employing *p*NP-β-Gal as donor and benzyl-α-D-N-acetylgalactosamine as acceptor. Compared to our results, the presence of [Bmin][PF6] as cosolvent in the reaction media gives a higher Gal-β-(1→3)-GlcNAc yield (75%), comparable with maximum yield levels obtained with free enzyme (97% with [Bmin][PF6]). Also, [Bmin][PF6] and 2HNND could be recovered from the reaction media, following the procedure described in experimental section, and reused in following reactions, decreasing the total cost associated with the process and therefore increasing the productivity in terms of substrate and solvent consumption.

Thus, Gal-β-(1→3)-GlcNAc were easily isolated and purified by column chromatography (carbon–celite) and structural characterization was performed by ^{1}H-NMR and ^{13}C-NMR. Spectra were identical to the ones previously reported.[36,37]

Re-use of immobilized β-gal-3 from *B. circulans* on GA and AMGA in the enzymatic synthesis of Gal-β-(1→3)-GlcNAc with green solvents

The potential re-use of supported enzymes in the synthesis of Gal-β-(1→3)-GlcNAc 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 6 times, using biosolvents 2HNND, [Bmin][PF6] and 50 mM sodium phosphate buffer at pH 6.0 (Figure 9). The GA derivative in the presence of [Bmin][PF6] and 2HNND showed an excellent reusability and high stability. 90 % of its initial activity over 6 reaction cycles was retained when [Bmin][PF6] or 2HNND were used as reaction media. When the GA derivative was placed in the presence of only buffer, it showed 70 % of its initial activity over 6 reaction cycles. On the contrary, when AMGA derivative was used in buffer or green solvent ([Bmin][PF6] or 2HNND), a continuous reduction in activity was observed until the immobilized enzyme lost the initial activity over 6 reaction cycles, probably due to AMGA inactivation.

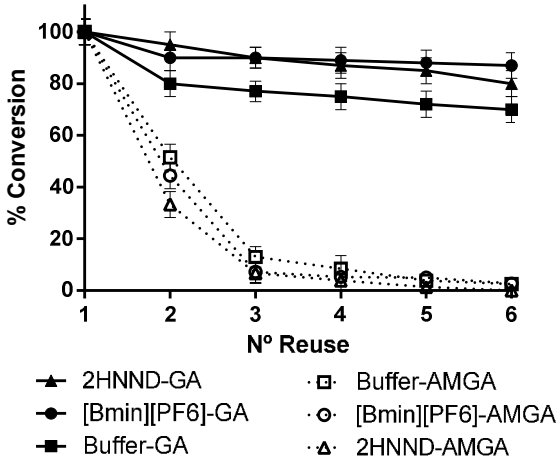


Figure 9. Reusability of β-Gal-3 immobilized on GA and AMGA in presence of buffer and green cosolvents

The results indicated the excellent reusability and stability for the GA, which retained ~90% of its initial activity after 6th catalytic cycles when [Bmin][PF6] is used as reaction media. These results suggest that, over 6 reaction cycles under the above reaction conditions, no significant leaching of enzyme from GA or denaturation of immobilized β-galactosidase occurred. Nevertheless, the loss of activity observed for AMGA derivative may be ascribed to the blocking of some β-galactosidase active sites or to the gradual loss of bound β-galactosidase during catalysis.[38]

It was also observed that the selectivity for Gal-β-(1→3)-GlcNAc formation remains almost unchanged after re-use. The main advantage in the enzyme reuse in green solvent compared to buffer is the high yield obtained for the product Gal-β-(1→3)-GlcNAc over 6 reaction cycles. In the presence of 2HNND biosolvent the GA derivative present 80% of its initial activity after 6th catalytic cycles and 90% when [Bmin][PF6] is used as cosolvent.

On the other hand, an advantage of using [Bmin][PF6] is that under these experimental conditions there is a biphasic mixture between these solvents and the aqueous buffer, allowing solvent separation from the reaction media by centrifugation.[23] Moreover, carbohydrates remain exclusively media in the aqueous phase. Centrifugation becomes then a very useful tool for the isolation of these solvents from the reaction media.[22,23]

2HNND is water soluble and cannot be eliminated by lyophilisation, percolation, rotary evaporation or organic extraction. The protocol for green solvent elimination is based on direct hexane precipitation at -196 °C without prior extraction. The pellets obtained (composed by the mixture of saccharides) were purified by filtration. 2HNND was separated by rotary evaporation of the hexane, recovering the total amount of both solvents.[22] After the purification process, 2HNND was reused in further reactions.

The new immobilized enzyme was recycled 6 times (six catalytic cycles), meaning that the same amount of GA was fully operational during a 18 h period (3 h, initial cycle, plus (6 × 3 h), 6 reuses) at 37 °C, still retaining a 70-87% of residual activity. In order to calculate the productivity of the immobilized derivatives, the results of the catalytic performance of GA compared to both native and AMGA enzymes (Table 2).

Table 2. Results in terms of productivity obtained from the transglycosylation reaction mediated by β-Gal-3 free or immobilized. * Overall productivity (mmol LNB ·mg^{-1} enzyme)

	Solvent	Overall productivity*
Free enzyme	Buffer	(1.08 ± 0.05) x 10^{-1}
	2HNND	(2.10 ± 0.12) x 10^{-1}
	[Bmin][PF6]	(2.06 ± 0.11) x 10^{-1}
GA	Buffer	1.39 ± 0.07
	2HNND	2.55 ± 0.10
	[Bmin][PF6]	3.47 ± 0.18
AMGA	Buffer	(3.99 ± 0.20) x 10^{-1}
	2HNND	(2.24 ± 0.13) x 10^{-1}
	[Bmin][PF6]	(2.72 ± 0.08) x 10^{-1}

As it can be seen, the overall productivity (calculated as mmol LNB·mg^{-1} enzyme considering all cycles of enzyme reused in the immobilized biocatalyst) obtained with covalent derivative GA was higher than that obtained with AMGA or free enzyme. On the other hand for GA derivative the presence of a green solvent increases this productivity specially in presence of [Bmim][PF6] (2.5 times in [Bmim][PF6] and 1.8 times in 2HNND). Also, taking account of the improved thermal and operational stability properties offered by the covalent immobilized derivative, development of column or batch designs can be considered as potential alternatives for further investigations.

Materials and methods

1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF6]) was obtained from Merck. 2-hydroxy-*N*,*N*-dimethylpropanamide (2HNND) biosolvent was a gift from COGNIS IP Management GmbH (Germany), now part of BASF.

Bovine serum albumin (BSA), *p*-nitrophenol (*p*NP), *p*-nitrophenyl-β-D-galactopiranosyde (*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. All other chemicals were analytical grade.

Production of the enzyme and purification

Recombinant β-Gal ATTC 31382 was cloned in *Escherichia coli* BL21 (DE3) using pET28b+ vector (Novagen). *E. coli* cultures were grown aerobically at 37ºC in LB broth with kanamycin (30 mg/L) and induced with IPTG (isopropyl-β-D-thiogalactopyranoside, 1 mM) at 37ºC for 5 hours. Cells were broken by sonic disruption, and solution was clarified by centrifugation (14.000 g for 15 min at 4ºC). The solution obtained was passed through a Ni^{2+}agarose column (3 mL) according to manufacturer's protocol (BioRad). Fractions were monitored for absorbance at 280 nm, pooled, concentrated and desalted in an Amicon ultra centrifugal filter (Millipore) to eliminate remnant imidazole. The purification process was followed by SDS-PAGE.[39] Protein quantification was done by Bradford method, using bovine serum albumin (BSA) as standard.[40]

Hydrolytic reactions

Hydrolytic activity was determined by spectrophotometrical quantification of *p*NP liberated by the hydrolysis of *p*NP-β-galactopyranoside 5 mM in sodium phosphate buffer 50 mM, pH 7.0 in a 300 µl cell by measuring the increase in absorbance at 410 nm during 3 min. 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.

β–Gal-3 immobilization

Stabilization of the enzyme in alkaline media (pH 10.0)

The β–Gal-3 ATCC 31382 solution (0.5 mg) was prepared in 100 mM sodium bicarbonate buffer, pH 10.0, with 20 % (v/v) of stabilizing agent (glycerol, trehalose or polyethylene glycol-600 (PEG-600)) at 4ºC. Samples (10 µL) were taken at different intervals and assayed for hydrolytic activity, and the best results were obtained with PEG-600. Optimization of PEG % were done at room temperature as described above using 0%, 5%, 10%, 20%, 30%, 40 % and 50% of PEG-600.

Supports preparation

The supports were prepared as previously described.[16,19] For the glyoxal supports, an agarose matrix was activated with glycidol and further oxidized with peryodate. Heterofunctional supports were prepared from agarose activated with epoxy groups.

Immobilization of the enzyme on glyoxyl-agarose support

The enzyme was immobilized in glyoxyl agarose (GA), amino glyoxyl agarose (AMGA), carboxy glyoxyl agarose (COGA) and chelate glyoxyl agarose (NiGA) containing 5 mL of enzyme (1mg/mL) per 1 g of support at 25ºC. The β-galactosidase solution was prepared in 100 mM sodium bicarbonate buffer, pH 10.0, with 50 % (v/v) of PEG-600, which was used to avoid enzymes inactivation during immobilization. Finally, to obtain stable bonds between the enzyme and the support, the Schiff bases formed were reduced by adding $NaBH_4$ to reach a final concentration of 1 mg/mL. The suspension was maintained under stirring during 30 min at 25 °C, and the biocatalyst was thoroughly washed with distilled water. The percentage of bound enzyme was determined by the difference between the initial amount of protein in the solution of the native enzyme and in the filtrate. The protein quantification was determined according to the same methodology utilized for the soluble enzyme. The immobilized enzyme was stored at 4 ºC. In all cases, assays were conducted by triplicate and the error was <5%.

Influence of the amount of enzyme loaded in the immobilization process

Effect of protein loaded in the supports was evaluated using different amounts of enzyme loaded per mass of support: 0.1, 0.5, 0.75, 1 and 2 mg enzyme /g support. Total immobilized protein and remaining activity were measured. All assays were conducted in triplicate.

pH and thermal stability of free and immobilized enzyme

The pH stability on free and immobilized enzyme was studied by incubating the enzyme at 25 ºC in buffers of varying pH in the range of 4-9 for 20 minutes and then determining the catalytic activity quantifying absorbance at 410 nm and comparing to a standard curve performed under the same experimental conditions.

Thermal stability experiments were performed with free and immobilized enzymes which were incubated in the absence of substrate at 37 ºC. The immobilized enzymes were placed in 50 mM sodium phosphate buffer (pH 6.0) and the specific enzymatic activities were measured at different storage times. All assays were conducted in triplicate and standard deviation was calculated.

Transglycosylation reactions

Transglycosylation reactions were carried out using soluble and immobilized enzymes. A 85 mM solution pNP-β-Gal (donor), 425 mM of GlcNAc (acceptor) in 1 mL of 50 mM sodium phosphate buffer (pH 6.0), 2 M of 2HNND biosolvent or 1.46 M of [Bmim][PF6] were preequilibrated to 37 ºC. Afterwards, 10 U of free enzyme or immobilized enzyme respectively were added to the reaction mixture. The reaction was monitored by HPLC-LS using a NH2P50-4E amino column (Asahipak, Japan) and was stopped after 3 hours by heating to 100 ºC for 5 min for the free enzyme and by filtration for the immobilized enzyme. All assays were conducted in triplicate and standard deviation was calculated.

Solvent Recycling

Upon reaction completion, biosolvents were recovered. For 2HNND, reaction media was lyophilized. The crude obtained was mixed with 30 mL of hexane and freeze at -196 ºC using liquid nitrogen. The resulting pellet was recovered by filtration and dried. Biosolvent was separated from hexane by rotary evaporation, recovering the total amount.

[Bmim][PF6], as water immiscible solvent, was easy recovered by physical methods. Reaction media was centrifuged at 14,000 rpm, separating the aqueous phase (containing carbohydrate compounds) from the IL. After that, aqueous phase was lyophilized.

Product purification and characterization

The reaction mixture was then directly loaded onto a carbon–celite column. The column was first eluted with water (200 mL) and then with a linear gradient of 0% to 15% (v/ v) of ethanol. Solvents were eliminated and disaccharides were dissolved in D_2O to be characterized by H^1 and C^{13} NMR spectroscopy on a Bruker 500 MHz spectrometer.

Spectra were consistent with previous references.[22,23,41,42]

Gal-β(1→3)GlcNAc (Lacto-N-Biose, LNB):[41,42] 1H-NMR (500 MHz, D2O): 1.96 (s, 3H, Ac), 5.11 (d, J1α,2=3.45Hz, H-1α). 13C-NMR (500 MHz, D2O): 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).

Re-use cycles in transglycosilation reactions

To evaluate the reusability of immobilized biocatalyst in the synthesis of LNB, each reaction was washed with the assay buffer after each use and then suspended again in a fresh reaction mixture to measure enzyme activity in transglycosilation, as previously described. The residual activity was calculated by taking the enzyme activity of the first cycle as 100%. Due to the capability of the reuse of solvent [Bmim][PF6], no addition of this solvent was necessary.

Conclusions

A complete study of the production of a highly active and stable immobilized β-gal-3 ATCC 31382 was carried out to be tested on the synthesis of β-(1→3) galactosides. Functionalization of agarose with four different methods (GA, NiGA, COGA and AMGA), resulted in two immobilized biocatalysts (GA and AMGA) with the highest enzyme loading and retained activity. Alkaline pH has been used to promote the immobilization reaction, however, the low stability of enzymes in such systems still remains a critical issue, limiting its industrial application. Here, we have reported a polyethylene glycol (PEG) pretreated stabilized β-gal-3 in alkaline pH that allows its immobilization on an agarose support. Results show that these conditions allowed high enzyme loading and excellent catalytic activity of β-galactosidase, favoring its thermal stabilization at 37ºC and 50 °C. At the same time, the immobilized enzyme was found to be highly efficient and regioselective in the enzymatic synthesis of Gal-β-D-(1→3)GlcNAc in the presence of green solvents ([Bmim][PF6] and 2HNND). The immobilized enzyme and green solvents could be reused up to 6 cycles with excellent catalytic activity and regioselectivity therefore increasing its productivity. This immobilized biocatalyst shows promising properties that could be used in the future development of an efficient bioprocess at a medium to large scale.

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Table of Contents Graphic

Stabilization of β–Gal-3 ATCC 31382 on agarose gels: synthesis of β-(1→3) galactosides under sustainable conditions

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