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A Novel, Efficient and Sustainable Strategy for the Synthesis of α-Glycoconjugates by Combination of a α-Galactosynthase and a Green Solvent

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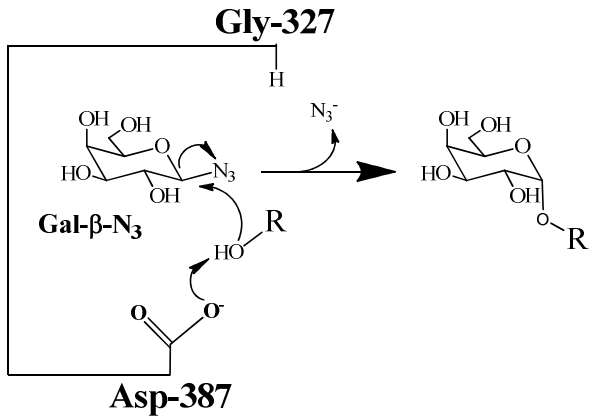
Glycosynthases are becoming important enzymatic tools for the synthesis of oligosaccharides. Herein, we explore for the first time the synthesis of α-glycoconjugates using a α-glycosynthase in green solvents. Using this biocatalysts, β-Gal-N3 as donor, pNP-Glc and pNP-Man as acceptors, and green co-solvents we obtained hight yields and excellent selectivities in the synthesis of α-glycoconjugates. In addition, reaction scaling up is feasible and co-solvent can be recovered and reused, increasing the sustainability of the reaction process. The results demonstrate that the combination of a glycosynthase and a green solvent is a promising alternative for the synthesis of glycoconjugates. The non-hydrolytic capability of this enzyme on the product obtained is a key feature that can be expanded to other glycosynthases.

Introduction

Oligosaccharides are involved in many biological processes such as cell-cell recognition and communication, growth regulation and antibody interaction, bacterial and viral infection and other crucial intercellular recognition events.[1-4] There is a growing interest in the synthesis of these compounds in order to increase our knowledge of their biological functions. Organic chemical methods for obtaining them have been developed[5-10] but they involve several elaborate protection and deprotection procedures.

On the other hand, enzymatic synthesis of carbohydrates, related to processes catalyzed by glycosyltransferases[11] and glycosidases working in transglycosylation mode,[12, 13] are often convenient, but present disadvantages. In the case of glycosyltransferases, showing high stereo- and regioselectivity, the issues are the high costs and poor availability of both the substrates and catalysts, while in the case of glycosidases the main drawback is that the newly formed product is a substrate for secondary hydrolysis with reduced final yields and problematic application in industrial procedures.[12, 13]

To overcome these limitations, glycosynthases, a new class of mutant glycosidases with no hydrolytic activity that synthesize glycans in enhanced yields, were obtained from β-[14-16] and α-glycosidases.[17-19] Recently, a new α-galactosynthase has been obtained for the first time: the TmGalA D327G,[18] mutant derived from the α-galactosidase from the thermophilic bacterium *Thermotoga maritima*.[20] This efficient α-galactosynthase produced different galactosylated disaccharides from β-galactosyl-azide donor and different *p*-nitrophenyl-α-glycosides acceptors (Scheme 1).[18]



Scheme 1. Mechanism followed by TmGalA D327G α-glycosynthase: the cavity created by the mutation that removed the side chain of Asp327 allowed the access to the active site of the β-Gal-N3. Then, the galactose moiety is transferred to the acceptor activated by the base catalysis of the Asp387 residue; the disaccharide product, showing the newly formed α-bond, cannot be hydrolyzed by the mutant and thus accumulates in the reaction mixture.[18]

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The use of green solvents,[21] such as solvents derived from biomass (SDB) or ionic liquids (ILs), as co-solvents in the enzymatic reactions in general, and in glycosidase-catalyzed reactions in particular, lead to an increase of the reaction yields and a noticeable change on the regioselectivity.[22-32]

SDB show some new and attractive advantages, as being made from a renewable source, they are less toxic, exhibit low volatility, and possess tuneable physicochemical properties.[21, 26, 33-37] In addition ILs, which are salts in liquid state, composed entirely of ions have remarkable special properties,[38-45] and have received increasing attention as new co-solvents for chemo- and biocatalytic organic synthesis. The use of them as co-solvents in enzymatic reactions, has already been applied to different kinds of enzymes[28, 30, 46-50] and whole cells.[51]

Despite these advantages, green solvents have never been used as co-solvents of glycosynthase-catalyzed reactions. In this work we analyze for the first time the effect of green solvents (SDB and ILs) on the activity of the galactosynthase TmGalA D327G. We explore the possibility of combining the non-hydrolytic capabilities of this kind of enzymes with the yield and regioselectivity enhancements in the synthesis of disaccharides previously obtained with conventional glycosidases in these conditions[26-30]. Furthermore, we show here that reaction scaling up and green solvent recycling can be conveniently obtained without losing catalytic action.

Results and discussion

Addition of a histidine-tag to the extreme C-terminus of TmGalA and TmGal D327G

In previous works, TmGalA and TmGal D327G enzymes were purified by ion-exchange and hydrophobic-interactions columns.[20] To take advantage of the histidine tag carried by the pET24d(+) vector, in which the enzymes were cloned, we developed a Quick-Change protocol to remove the stop codon of the gene and merge the histidine tag at the *C*-terminus of the enzyme. The new enzymes obtained were named TmGalA-HisTag and TmGalA D327G-HisTag respectively.

In order to determine the effect of the histidine tag on the enzyme activity, cells containing TmGalA and TmGalA-HisTag plasmids were grown, induced with IPTG and disrupted. The hydrolytic activities of the two enzymes were assayed on the cell-free extracts using *p*NP-α-Gal 5 mM as substrate. Parental TmGalA and TmGalA-HisTag showed a specific activity of 45.4 and 44.6 U.mg of protein^{-1}, respectively, in good agreement with the previously reported activity of 48.2 U.mg of protein^{-1}.[20]

After purification through a Ni^{2+}agarose-column, hydrolytic activity of TmGalA-HisTag was assayed using different substrates: *p*NP-α-Fuc, *p*NP-β-Fuc, *p*NP-α-Gal, *p*NP-β-Gal, *p*NP-α-Glc, *p*NP-β-Glc, *p*NP-α-Man, *p*NP-β-D-Man, *p*NP-α-GlcNAc and *p*NP-β-GlcNAc. Only *p*NP-α-Gal was recognized by the enzyme, showing an activity of 216.3 U.mg^{-1}, which corresponds with the results previously reported.[20]

We concluded that the histidine tag did not affected the enzyme activity, demonstrating that the one-step purification procedure through a Ni^{2+}agarose-column was more efficient than the two-step procedure previously used (ion-exchange and hydrophobic-interactions columns).[20]

Galactosynthase activity screening of TmGalA D327G-HisTag

Transglycosylation activity of TmGalA-HisTag was tested using *p*NP-α-Gal as donor and different substrates as acceptors: Glc, Man, Gal, Fuc, Fru, GalNAc, GlcNAc, *p*NP-α-Fuc, *p*NP-β-Fuc, *p*NP-α-Gal, *p*NP-β-Gal, *p*NP-β-Glc, *p*NP-α-Man, *p*NP-β-Man, *p*NP-β-GalNAc, and *p*NP-β-GlcNAc. No synthesis of disaccharides was found, confirming previous results using this enzyme.[18]

The synthetic activity of the galactosynthase TmGalA D327G-HisTag was investigated at the conditions previously reported.[18] β-Gal-N_3 (14 mM) was used as donor and different sugars were used as acceptors (14 mM) at 1:1 molar ratio: Glc, Man, Gal, Fuc, Fru, GalNAc, GlcNAc, *p*NP-α-Fuc, *p*NP-β-Fuc, *p*NP-α-Gal, *p*NP-β-Gal, *p*NP-α-Glc, *p*NP-β-Glc, *p*NP-α-Man, *p*NP-β-Man, *p*NP-β-GalNAc, and *p*NP-β-GlcNAc, in 50 mM sodium acetate buffer pH 5, 65 ºC for 18 h. The analyses were run on an HPLC with LC detector (Figure 1). At these conditions, products were obtained exclusively in the reaction with *p*NP-α-Glc and *p*NP-α-Man as acceptors.

Reactions with these acceptors were scaled up to 80 ml in order to purify and characterize the products. Purifications were carried out through a silica column chromatography (Figure 2) and fractions containing purified products were analyzed by NMR.

NMR and HPLC results showed that the galactosynthase led to the production of Gal-α(1-6)-Glc-α-*p*NP and Gal-α(1-6)-Man-α-pNP, respectively, using Gal-β-N_3 as donor and *p*NP-α-Glc or *p*NP-α-Man as acceptors (Scheme 2).

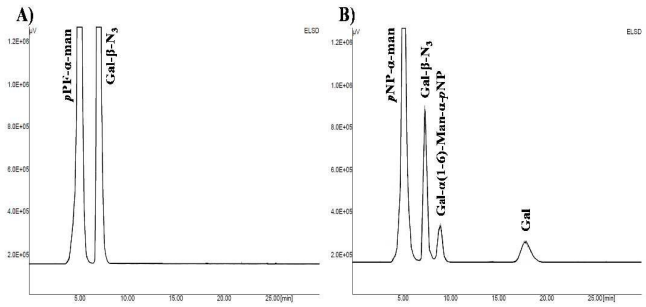


Figure 1. HPLC chromatogram of the reaction catalyzed by the α-galactosynthase using Gal-β-N_3 as donor and *p*NP-α-Man as acceptor. **A)** T 0h. **B)** T 18h.

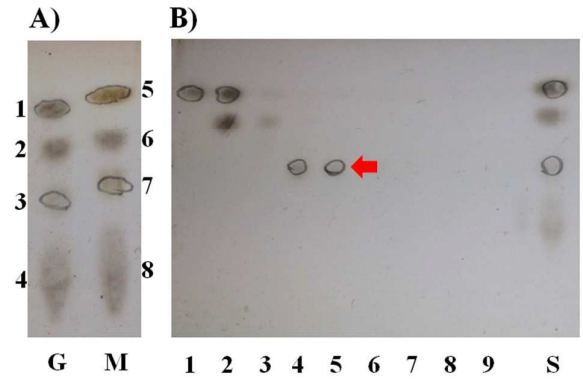
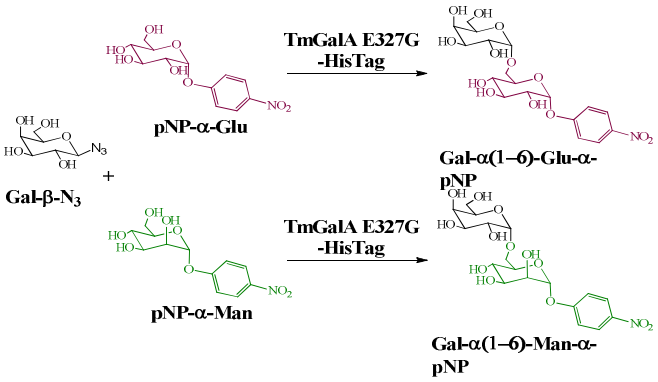


Figure 2. Panel A) TLC of the galactosynthase reaction by the TmGalA D327G mutant producing Gal-α(1-6)-Glc-α-*p*NP and Gal-α(1-6)-Man-α-*p*NP using Gal-β-N_3 as donor and *p*NP-α-Glc (G) or *p*NP-α-Man (M) as acceptors. **1)** *p*NP-α-Glc, **2)** Gal-β-N_3, **3)** Gal-α(1-6)-Glc-α-*p*NP, **4)** Gal, **5)** *p*NP-α-Man, **6)** Gal-β-N_3, **7)** Gal-α(1-6)-Man-α-*p*NP and **8)** Gal. **Panel B)** TLC of the purification through a chromatography silica column of the synthesis of Gal-α(1-6)-Man-α-*p*NP (red arrow). Mobile phase: ethyl acetate:methanol:water (70:20:10). Circles indicate active compounds under UV light.



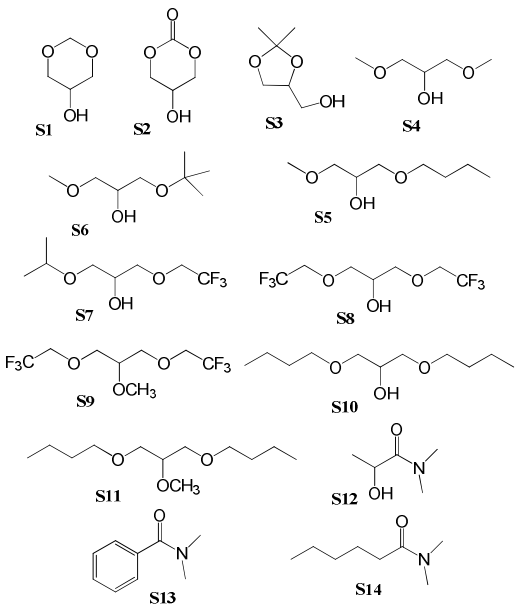
Scheme 2. Galactosynthase-catalyzed synthesis of Gal-α(1-6)-Glc-α-*p*NP and Gal-α(1-6)-Man-α-*p*NP using Gal-β-N_3 as donor and *p*NP-α-Glc or *p*NP-α-Man as acceptors.

When *p*NP-α-Glc was used as acceptor, 55% of the initial Gal-β-N_3, was converted, obtaining Gal-α(1-6)-Glc-α-*p*NP as a major product (35% yield) and 20% of hydrolytic product (Gal), while using *p*NP-α-Man as acceptor, the conversion obtained was 57% with yields of 25% and 32% of Gal-α(1-6)-Man-α-*p*NP and Gal, respectively.

Galactosynthase activity in the presence of biosolvents

We have previously described the effect of the use of SDB on the transglycosylation reactions catalyzed by the enzyme β-Gal-3 from *Bacillus circulans* using *p*NP-β-Gal as donor and GlcNAc or GalNAc as acceptors, obtaining an enhancement of the yields to almost 100% in the synthesis of Gal-β(1-3)-GlcNAc and Gal-β(1-3)-GalNAc respectively.[30] In addition, with Biolacta β-galactosidase, using *p*NP-*β*-Gal as donor and GlcNAc as acceptor, we improved the regioselectivity of the enzyme from a mixture of β(1→4) and β(1→6) towards the synthesis of only β(1→6) oligosaccharides.[26]

In this work, three different groups of solvents SDB (Scheme 3)were tested on the α-galactosynthase: glycerol based solvents with cyclic structures (**S1**-**S3**), glycerol based solvents with open chain (**S4**-**S11**) and 3-*N*,*N*- dimethylamide based solvents (**S12**-**S14**).



Scheme 3. Structure of the different SDB employed in transglycosilation reaction with TmGalA D327G-Histag.

Galactosynthase-catalyzed reactions were carried out in 50 mM sodium acetate buffer pH 5.0, 65ºC, and by following the general procedure described in the experimental section and monitored by HPLC. The concentration of green solvents in the sodium acetate buffered mixture was fixed to 2 M, which is the optimal concentration previously used by our research group for these solvents applied to different galactosidases.[29, 30, 52, 53] Results obtained are summarized in Figure 3 (*p*NP-α-Glc as acceptor) and Figure 4 (*p*NP-α-Man as acceptor).

In presence of SDB and *p*NP-α-Glc acceptor (Figure 3), almost all the solvents (**S1**-**S7**, **S12** and **S13**) led to a total loss of enzyme activity. **S9**, **S10**, **S11** and **S14** did not inhibit the enzyme, but the glycosynthetic yields of Gal-α(1-6)-Glc-α-*p*NP and the donor conversion were lower than those the obtained in the reaction with only conventional buffer (Figure 3).

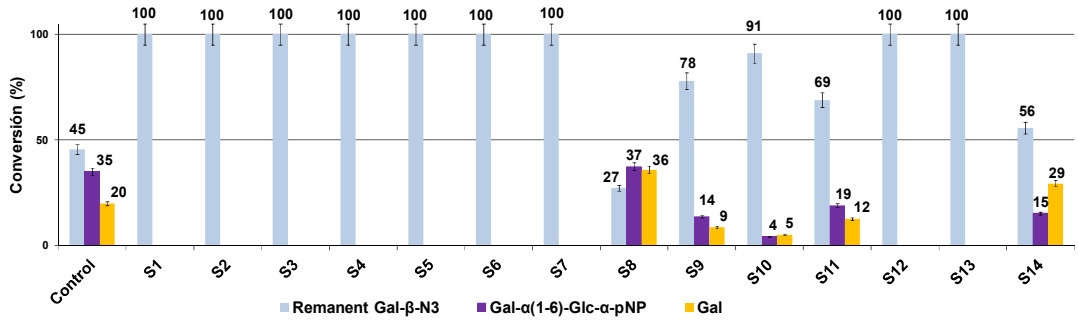


Figure 3. Reaction yields obtained with α-galactosynthase using buffer-2 M SDB with β-Gal-N_3 as donor and *p*NP-α-Glu as acceptor

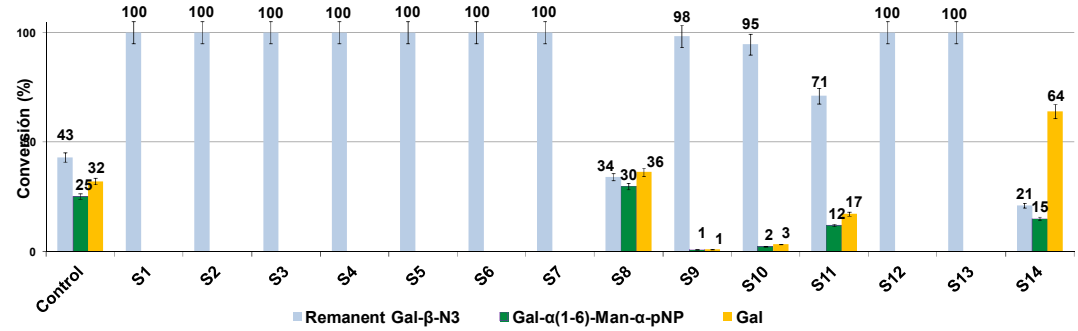


Figure 4. Reaction yields obtained with α-galactosynthase using buffer-2 M SDB with β-Gal-N_3 as donor and *p*NP-α-Man as acceptor

The best solvent was **S8**, leading to a better conversion (73%), but higher hydrolysis (36% compared with the 20% obtained in the reaction with only buffer). The yield of disaccharide obtained with **S8** as co-solvent was almost the same than that obtained in the reaction with only buffer (37% and 35% respectively).

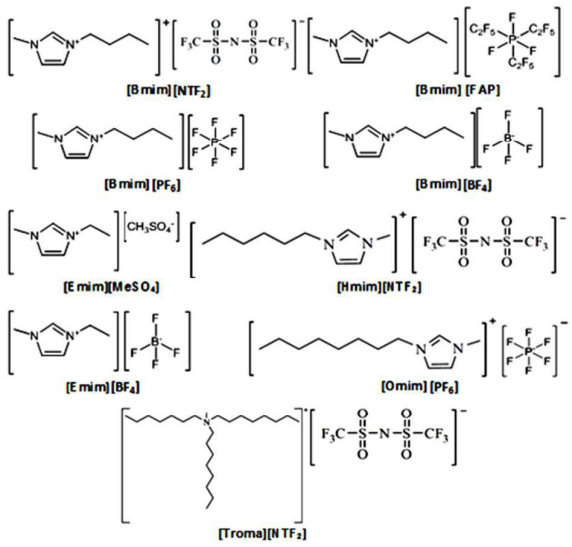
With *p*NP-α-Man (Figure 4) acceptor, the results were very similar. Solvents **S1**-**S7**, **S9**, **S10**, **S12** and **S13** inactivated the enzyme. **S11** and **S14** resulted in a donor conversion of 29% and 15 %, respectively. Again, the best results in the synthesis of the Gal-α(1-6)-Man-α-*p*NP disaccharide were obtained with **S8**, but with only a 5% yields enhancement compared to the reaction with conventional buffer (30% and 25 % respectively).

These results showed that SDB were not appropriate as co-solvents in the reaction with the α-galactosynthase. As can be seen in Scheme 3, the structural diversity of the SDB results in a wide range of physico-chemical parameters (log P, dipole moment or dielectric constant).[26, 30] Some of the SDB used, namely those bearing fluorinated chains, display simultaneously both high hydrophobicity and hydrogen-bond donor ability, a rather unusual combination in conventional organic solvents. In fact, this negative result cannot be easily related to the physico-chemical parameters, but these features could be of importance for solvent-substrate and/or solvent-enzyme interactions. According to this, the effect of SDB on the enzyme's structure could be attributed to a conformational change of the enzyme that would destabilize the protein. For that reason most of them inhibited the enzyme and the best achievement was the reproduction of the yields obtained in the reaction with only buffer. This prompted us to test the activity of the enzyme in the presence of ILs.

Galactosynthase activity in presence of ILs

In a recent work we have shown that the use of ILs as co-solvents in the reaction medium allows to increase the regioselectvity of transgalactosylation reactions in order to obtain only the β(1-4) regioisomer when *Thermus thermophilus* β-galactosidase was employed.[28] Also, we have reported that ILs as co-solvents in the reactions of the enzyme β-Gal-3 from *Bacillus circulans* using *p*NP-β-Gal as donor and GlcNAc or GalNAc as acceptors, enhanced the disaccharide yields to almost 100% in the synthesis of Gal-β(1-3)-GlcNAc and Gal-β(1-3)-GalNAc respectively.[30] Here, we have extended the use of these ILs to the study of oligosaccharide

synthesis catalyzed by the α-galactosynthase. Several ILs have been included in this study: [Bmim][BF4], [Bmim][FAP], [Bmim][NTf2], [Bmim][PF6], [CPMA][MeSO4], [*Emim*][BF4], [Omim][PF6], [Troma][NTf2], [Hmim][NTf2], [Emim][MeSO4] (Scheme 4).



Scheme 4. Structure of the different ILs tested on the α-galactosynthase

Reactions with TmGalA D327G-Histag were run as described in the experimental section, using β-Gal-N_3 as donor and *p*NP-α-Glc or *p*NP-α-Man as acceptors, adding 30% IL to the reaction media, which is the optimal concentration used in previous works. [28, 30, 54] Reactions were monitored by HPLC and results are summarized in Figure 5 and 6 (*p*NP-α-Glc and *p*NP-α-Man acceptors, respectively).

In reactions using [Bmim] [BF_4], [Bmim] [PF_6], [Bmim] [$MeSO_4$], [Emim] [BF_4], [Hmim] [Ntf_2] y [Omim] [PF_6] as co-solvents, lower disaccharide yields were obtained compared with control reaction (Figure 5). Specifically, in the cases of [Bmim] [PF_6] and [Emim] [BF_4] the conversion of the donor β-Gal-N_3 was higher than that obtained in the reaction with buffer (93% and 89% respectively, compared with 55 %), but the enhancement is directed to the hydrolysis. The yield of Gal-α(1-6)-Glc-α-*p*NP disaccharide using [Bmim] [Ntf_2] and [Emim] [$MeSO_4$] was almost the same of that obtained in the control reaction. [Bmim] [FAP] enhanced the yield to 43% and the best result was obtained with [Troma] [Ntf_2], with which the conversion of the substrate β-Gal-N_3 was almost total and the disaccharide yield was 50% keeping total regioselectivity to the α–(1→6) isomer

Results obtained when *p*NF-α-Man was used as acceptor for the synthesis of Gal-α(1-6)-Man-α-*p*NP in presence of 30% of IL are summarized in Figure 6. [Bmim] [BF_4], [Bmim] [$MeSO_4$] and [Omim] [PF_6] inhibited the enzyme, showing a 92%, 66% and 82% of unreacted Gal-β-N_3 respectively. [Bmim] [FAP], [Bmim] [Ntf_2], [Bmim] [PF_6] and [Hmim] [Ntf_2] led to a yield of disaccharide similar to that observed in control reaction and to an increased hydrolysis. [Emim] [BF_4] enhanced the hydrolysis over the synthesis, while [Emim] [$MeSO_4$] showed a better disaccharide yield (45% compared with 25% of the control reaction) and a lower hydrolysis (16% against 32%). The best results were obtained again with [Troma] [Ntf_2], showing an almost total conversion of the donor Gal-β-N_3, a lower hydrolysis (17%), and a boost on the yield of Gal-α(1-6)-Man-α-*p*NP up to 80%, which was the only disaccharide obtained.

In a recent work we have shown that the use of ILs as cosolvents in the reaction medium allows to increase the activity retaining total regioselectivity of the transgalactosylation reactions when *Bacillus circulans* ATCC 31382 β-galactosidase was employed.[30] According to these results the solvent could play a key role in the interaction pattern between IL and enzyme and this fact could explain the results obtained. These results reinforce the concept of driving enzymatic syntheses to the desired product simply by adjusting the reaction medium with small amounts of ILs.

These remarkable results prompted us to select IL [Troma] [Ntf_2] for further analyses in which the reaction time was extended to up to 36 h. Results showed that once the reaction reached the maximal synthetic yield (50% of Gal-α(1-6)-Glc-α-*p*NP and 80% of Gal-α(1-6)-Man-α-*p*NP), products accumulated in the medium with no hydrolysis (Figure 7).

Preparative Galactosynthesis and Solvent Recycling

With this optimized separation procedure in hand, we proceeded to the systematic semi-preparative experiments in 80 ml of reaction media in presence of [Troma] [Ntf2]. This scaling up of the reaction was carried out using β-Gal-N_3 as donor and pNP-α-Glc and *p*NP-α-Man as acceptors. The yields of pure disaccharides obtained after purification through a silica column, compared with initial β-Gal-N_3, were 50% for Gal-α-(1-6)-Glc-α-pNP (259,5 mg) and 80% for Gal-α-(1-6)-Man-α-pNP (415 mg). Remarkably confirming that the scale-up produced no loss in synthetic yields making this method available for further enhancement.

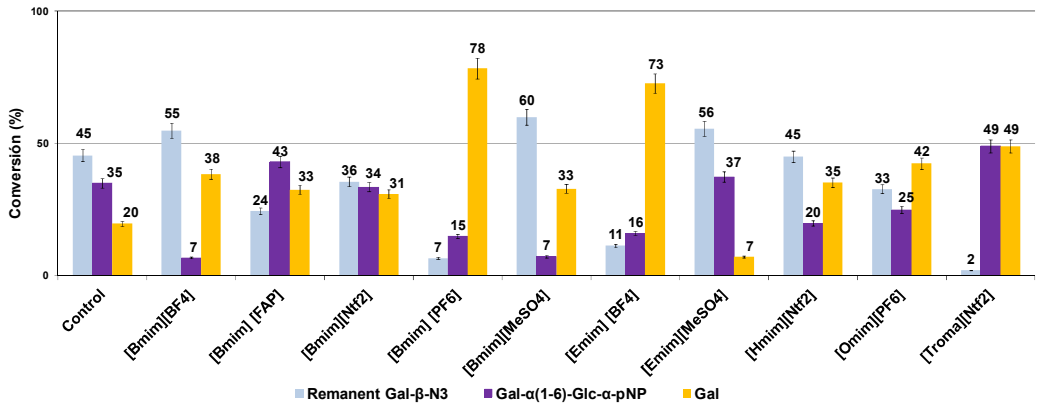


Figure 5. Transglycosylation yields obtained with TmGalA D327G-Histag using buffer-30% Ils with β-Gal-N_3 as donor and pNP-α-Glc as acceptor

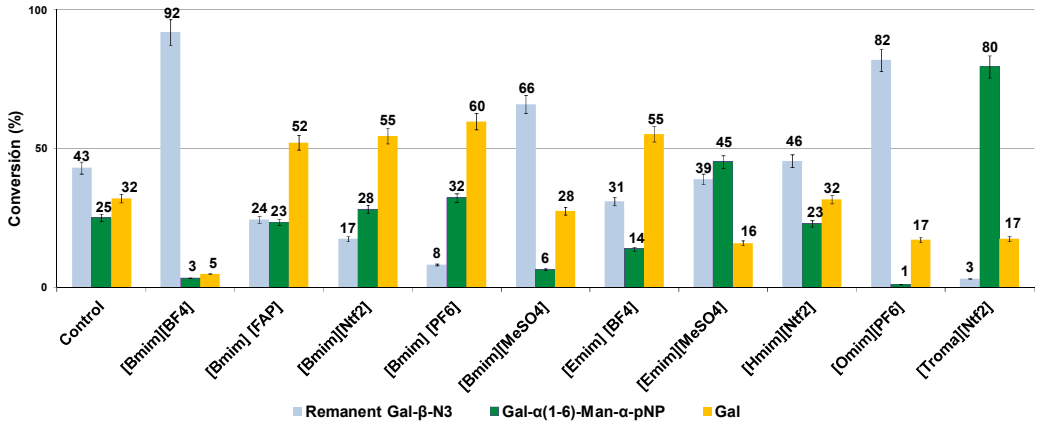


Figure 6. Transglycosylation yields obtained with TmGalA D327G-Histag using buffer-30% Ils with β-Gal-N_3 as donor and pNP-α-Man as acceptor.

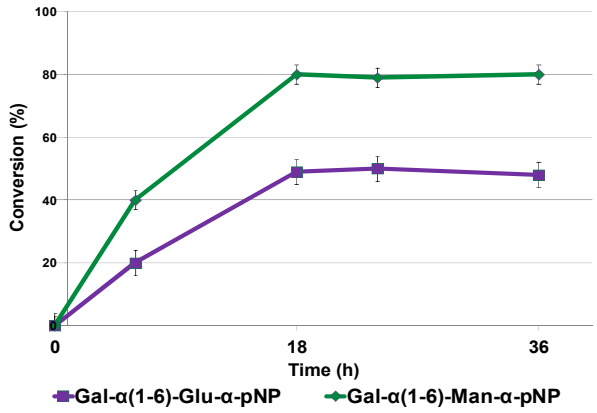


Figure 7. Evolution of the Gal-α(1-6)-Glc-α-*p*NP and Gal-α(1-6)-Man-α-*p*NP synthesized in presence of the IL [Troma] [Ntf_2] extending the reaction time up to 36 h.

Another important point that must be evaluated after the synthesis of a disaccharide of applicative interest is the feasibility of isolation of the target molecule from the reaction mixture. In this respect, an advantage of [Troma] [Ntf2] compared with other ILs, is that the reaction is carried on under stirring conditions and a two phase systen is created between IL and aqueous buffer. After the reaction was completed, this IL was separated from reaction media by simple centrifugation. Moreover, carbohydrate compounds in the reaction media are not soluble in the IL phase and remains in the aqueous phase. Centrifugation becomes a very useful tool for the separation of the IL from the reaction media, allowing its reuse in further reactions.

Conclusions

In this work, we describe an efficient enzymatic approach for the synthesis of α–glycoconjugates bearing a *p*NP group at the anomeric position. The use of green solvents as co-solvents of glycosynthetic reactions carried out by a mutant enzyme was assessed for the first time. Glycosynthase technology has been

exploited so far to transform a glycosidase with no transglycosylation activity into an active oligosaccharide-synthesizing catalyst. However, in some cases, glycosynthases show lack of total conversion and rather low yields.[19] This problem has been addressed by tuning the pH of the reaction and by directed evolution of the glycosynthase obtaining increased efficiency and yields of the synthetic reaction.[55-57] Here, we demonstrated through the screening of several green solvents, that the α-galactosynthase TmGalA D327G achieves a total substrate conversion and an enhancement of the yield in the synthesis of the glycoconjugates Gal-α(1-6)-Glc-α-*p*NP and Gal-α(1-6)-Man-α-*p*NP. In addition, reaction scaling up is feasible and co-solvents can be recovered and reused, increasing the sustainability of the reaction process. Thus, reactions with green co-solvents represent a considerable improvement over the use of aqueous buffers, creating new options for the use of glycosynthase enzymes.

Experimental section

Materials and reagents

Solvents **S1**, **S2**, **S3**, **S4**, **S5**, **S6**, **S7**, **S8**, **S9**, **S10** and **S11** were synthesized using the same procedures previously described.[58] **S12**, **S13** and **S14** were a gift from COGNIS IP Management GmbH (Germany).

1-Butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]), 1-butyl-3-methylimidazolium tris (pentafluoroethyl) trifluoro-phosphate ([Bmim][FAP], 1-butyl-3-methylimidazolium bis-(trifluoromethylsulfonyl)-imide ([Bmim][NTf_2]), 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6], 1-Ethyl-3-methylimidazolium methylsulfate ([Emim][$MeSO_4$]) 1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)-imide ([Hmim] [NTf_2]), 99% purity were obtained from Merck., 1-Ethyl-3-methylimidazolium tetrafluoroborate ([Emim] [BF_4]), 1-methyl-3-octylimidazolium hexafluoro phosphate ([Omim][PF_6]) and methyltrioctylammoniumbis (trifluoromethylsulfonyl)-imide, ([Troma][NTf_2], were obtained from Solvent Innovation GmbH (Germany).

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

Addition of histidine-tag by site-directed mutagénesis

Recombinant TmGalA and TmGalA D327G were cloned in *Escherichia coli* BL21 using pET24b(+) vector (Novagen). TmGalA-HisTag and TmGalA D327G-HisTag were generated using the Quick-Change site-directed mutagenesis kit (Stratagene) and synthetic oligonucleotides (Sigma-Aldrich): TmGalA-HisTag-Fw: 5'-acgaagagggtgagagagaaaagcttgcggc-3'TmGalA-HisTag-Rv: 5'-gccgcaagcttttctctctcaccctcttcgt-3'.

The gene containing the desired mutation was identified by direct sequencing and completely re-sequenced

Production of the enzymes and purification

E. coli cultures were grown aerobically at 37ºC in LB broth with kanamycin (30 $mg.l^{-1}$) and induced with IPTG (isopropyl *β*-*D*-thiogalactopyranoside, 1 mM) at 37ºC for 16 hours. Cells were disrupted by sonication, unbroken cells and insoluble debris were eliminated by centrifugation (14.000 g for 10 min at 4ºC). The solution obtained was incubated at 70ºC for 30 minutes to denature most of *E. coli* proteins, centrifuged (14.000 g for 20 minutes) and passed through a Ni^{2+}-agarose column (3 ml) according to manufacturer's protocol (BioRad). Fractions were monitored by absorbance at 280 nm, pooled, and concentrated and desalted in an Amicon ultra centrifuge filter (Millipore). Protein quantification was done by Bradford method,[59] using BSA as standard.

Hydrolytic reactions

Hydrolytic activity of the enzymes TmGalA and TmGalA-HisTag were determined by spectrophotometrical quantification of *p*NP liberated by the hydrolysis of different *p*NP-substrates: *p*NP-α-Fuc, *p*NP-β-Fuc, *p*NP-α-Gal, *p*NP-β-Gal, *p*NP-α-Glc,*p*NP-β-Glc, *p*NP-α-Man, *p*NP- β-D-Man, *p*NP-β-GalNAc and pNP-β-GlcNAc. Reactions were carried out using a solution of *p*NP-substrate 5 mM in sodium acetate buffer 50 mM, pH 5 in 300 µl during 5 minutes at 65 ºC and 10 µg of enzyme. Reactions were stopped by addition of 300 µl of sodium carbonate 0.5 M and its absorbance measured at 410 nm. Each assay was determined at least three times with standard deviation under 5% of the average of samples. One enzyme unit (U), was defined as the amount (mg) of protein that hydrolyzes 1.0 µmol of substrate per minute.

Transgalactosylation

*p*NP-α-Gal and Gal-β-N_3 (14 mM) were used as donors for the reactions with TmGalA-HisTag and TmGal D327G-HisTag, respectively. Different substrates were used as acceptors (14 mM): Glc, Man, Gal, Fuc, Fru, GalNAc, GlcNAc, *p*NP-α-Fuc, *p*NP-β-Fuc, *p*NP-α-Gal, *p*NP-β-Gal, *p*NP-β-Glc, *p*NP-α-Man, *p*NP-β-Man, *p*NP-β-GalNAc and *p*NP-β-GlcNAc. Substrates were dissolved in 100 µl of buffer sodium acetate 50 mM, pH 5. In reactions with SDB, they were added to a final concentration of 2M. In reaction with ILs, they were added to a 30% concentration and pre-warmed at reaction temperature (65 ºC). Reaction started by addition of 10 µg enzyme to the mixture and were stopped after 16 hours by addition of 400 µl of methanol and conserved immediately at -20ºC. Analytical determination of products were performed by HPLC using a NH2P50-4E amino column (Asahipak, Japan) using three detectors: ELSD (Evaporative Light Scattering), UV-Vis at 317nm and CD (Circular Dicroism).

Preparative Transgalactosylation and Solvent Recycling

The scaled-up reaction mixture was composed by β-Gal-N_3 14 mM as donor and *p*NP-α-Glc or *p*NP-α-Man 14mM as acceptors, dissolved in 80 ml buffer sodium acetate 50 mM pH

5 at 65 ºC with [Troma][Nft2] (30 %) and 10 mg of TmGal D327G-HisTag. The reaction was stopped after 16 hours and the crude was centrifuged at 14,000 r.p.m with the aim of separating the aqueous phase (containing carbohydrate compounds) from the IL. After that, aqueous phase was frozen and lyophilized to eliminate the water. Powder was loaded on a silica chromatography column, using ethyl acetate:methanol:water (70:20:10) as eluent. Disaccharide enriched fractions were collected, solvent was removed by rotatory evaporation. The purity of the solid powder was analyzed by HPLC. Structural determination was done by ^{1}H-NMR and ^{13}C-NMR (D_2O, 500 MHz). Spectra were consistent with previous references.[18]

Gal-α(1-6)-Glc-α-*p*NP**.** ^{1}H NMR (500 MHz, D_2O): δ 5.87 (d, 1H, JH-1,H-2 = 3.6 Hz, H-1A), 4.88 (d, 1H, JH-1,H-2 = 3.7 Hz, H-1B), 3.95 (t, 1H, H-3A), 3.93 (d, 1H, H-4B), 3.90 (t, 1H, H-5A), 3.87 (d,1H, H-6aA), 3.87 (d, 1H, H-5B), 3.82 (dd, 1H, H-2A), 3.75 (d, 1H, H-6aB), 3.74 (d, 1H, H-2B), 3.72 (d, 1H, H-6bB), 3.71 (d, 1H, H-6bA), 3.58 (dd, 1H, H-3B), 3.56 (t, 1H, H-4A); ^{13}C NMR (500 MHz, D_2O): δ 99.2 (C-1B), 97.7 (C-1A), 74.5 (C-3A), 73.2 (C-5B), 72.2 (C-2A), 72.2 (C-5A), 70.8 (C-4A), 70.8 (C-3B), 70.5 (C-4B), 69.6 (C-2B), 66.9 (C-6A), 62.4 (C-6B).

Gal-α(1-6)-Man-α-*p*NP. ^{1}H NMR (500 MHz, D_2O): δ 5.87 (bs, 1H, H-1A), 4.95 (d, 1H, $J_{H\text{-}1,H\text{-}2}$ = 3.8 Hz, H-1B), 4.30 (dd, 1H, H-2A), 4.14 (dd, 1H, H-3A), 4.00 (d, 1H, H-4B), 3.98 (t, 1H, H-5A), 3.97 (dd,1H, H-6aA), 3.90 (t, 1H, H-5B), 3.89 (t, 1H, H-4A), 3.82 (dd, 1H, H-6aB), 3.81 (dd, 1H, H-2B), 3.78 (dd, 1H, H-6bB), 3.74 (dd, 1H, H-6bA), 3.66 (dd, 1H, H-3B); ^{13}C NMR (500 MHz, D_2O): δ 99.3 (C-1B), 99.2 (C-1A), 73.8 (C-5B), 72.3 (C-5A), 72.0 (C-3A), 71.1 (C-2A), 71.0 (C-3B), 70.6 (C-4B), 69.8 (C-2B), 68.0 (C-4A), 67.0 (C-6A), 62.4 (C-6B).

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Table of Contents

A Novel, Efficient and Sustainable Strategy for the Synthesis of α-Glycoconjugates by Combination of a α-Galactosynthase and a Green Solvent

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