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Improved synthesis of disaccharides with *Escherichia coli* β-galactosidase using bio-solvents derived from glycerol

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ABSTRACT

A noticeably increase in activity, keeping total regioselectivity was found in the synthetic behaviour of *Escherichia coli* β-galactosidase in glycerol-based solvents using a 1:7 molar ratio of donor (*p*NP-β-Gal): acceptor (GlcNAc). Yields of up to 97% of β(1→6) with different solvents were found. These reactions take place without noticeable hydrolytic activity and with total regioselectivity, representing a considerable improvement over the use of aqueous buffer or conventional organic solvents. There is a clear dependence of the catalytic results on the solvent structure, which is analysed in terms of polarity and hydrophobicity.

1. Introduction

Biocatalysis and solvents play an important role in green processes, since they provide a valuable alternative to classic organic chemistry.[1–4] First, enzymes offer suitable tools for industrial reactions, which can be carried out under milder conditions, without using of heavy metals and with a great control over chemo-, regio- and stereoselectivity using appropriate enzymes. Second, there are numerous potential advantages in employing enzymes in organic solvents, such as increased solubility of nonpolar substrates, shifting of thermodynamic equilibria to favour synthesis over hydrolysis, decrease of water-dependent side reactions, enhanced thermal stability of enzymes and elimination of microbial contamination, among others.[5] On the other hand, the presence of organic solvents in enzymatic reactions can alter the activity and specificity of the enzyme. There are numerous examples in the literature wherein solvents physical properties, such as dielectric constant, dipole moment and hydrophobicity are related to various effects on enzyme activity, specificity and enantioselectivity.[6–9] Among the different families of the so-called bio-solvents, organic solvents derived from renewable sources are receiving an increasing attention.[10] In particular, glycerol and glycerol derivatives present some interesting features, such as their ready availability from biodiesel production, easy derivatisation and tuneable properties.[11]

Oligosaccharides are involved in a wide range of biological processes including bacterial and viral infection, cancer metastasis, the blood-clotting cascade and many other crucial intercellular recognition events.[12–15] As the understanding of these biological functions increases, the need for practical synthetic procedures of oligosaccharides in large quantities has become a major subject. Organic chemical methods for obtaining them have been developed,[16–18] but they involve several elaborate protection and deprotection procedures. Glycosyl hydrolases (glycosidases) can be used to synthesize oligosaccharides in a kinetically controlled reaction, where a glycosyl donor is used to transfer its glycosyl residue to a sugar acceptor present in the reaction medium.

In this work, we present a study of the influence of the use of green solvents derived from glycerol on the activity and selectivity of β-galactosidase of *Escherichia coli* in the enzymatic synthesis of disaccharides.

2. Results and discussions

Novel carbohydrate compounds can effectively be synthesized using enzymatic methods. Glycosidases can be used to synthesize oligosaccharides in a kinetically controlled reaction, where a glycosyl donor is used to transfer its glycosyl residue to a sugar

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acceptor present in the reaction medium.[19] In this sense, β-galactosidase from *E. coli* has been proven to be a valuable biocatalyst for galactosyl transfer from suitable donors onto a *N*-acetylglucosamine as acceptor.[20] The regioselectivity of enzymatic transgalactosidation depends on the source of the glycosidase used. The synthesis of Gal-β-(1→6)-GlcNac or/and Gal-β-(1→4)-GlcNac employing β-galactosidase from *E. coli*, *A. oryzae* and *Bacillus circulas* have been previously reported.[20–22]

In a recent work we have shown that the use of co-solvents derived from glycerol in the reaction medium is able to direct the regioselectivity of the transglycosydation reaction, allowing to obtain only the β(1→6) regioisomer, in the case of the *B. circulans* β-galactosidase.[23]

In the present work, we have extended the use of this kind of glycerol-based solvents to the study of oligosaccharides synthesis catalysed by β-galactosidases from *E. coli*. Two commercial solvents (glycerol and 2,2,2-trifluoroethanol, TFE) have also been included for comparative purposes (Fig. 1).

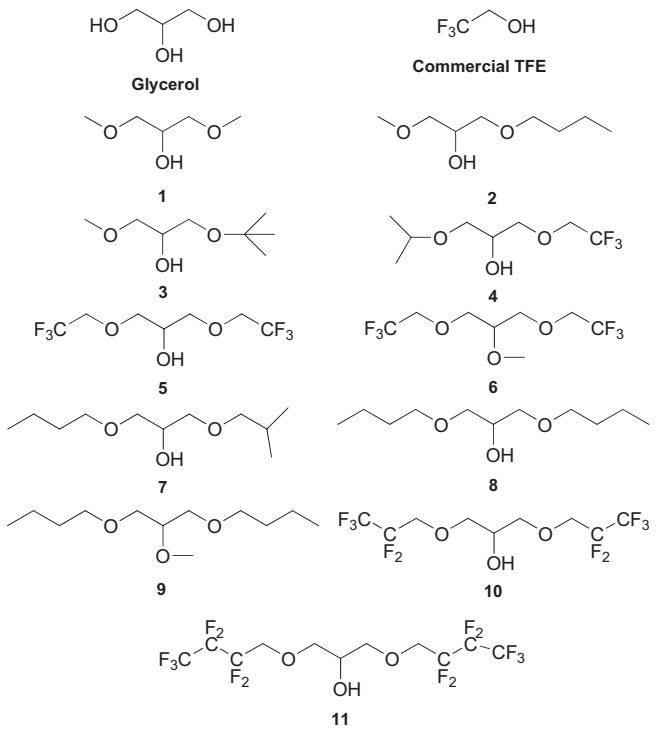
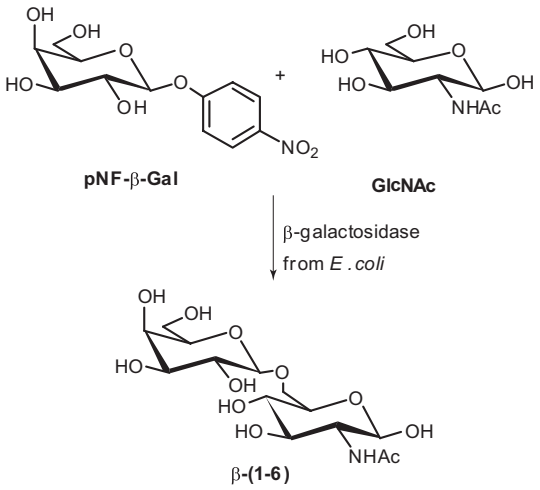


Fig. 1. Structure of different bio-solvents from glycerol employed in transglycosylation reaction with β-galactosidase from *E. coli*.

Transglycosylation reaction catalysed by a commercial preparation of the β-galactosidase from *E. coli* was carried out following the general procedure described in the Experimental section and monitored by HPLC (Scheme 1). The concentration of bio-solvent was fixed to 2 M in the mixture with the Tris/HCl buffer. The results obtained for the transglycosylation reaction with the β-galactosidase from *E. coli* in the presence of different solvents derived from glycerol are summarized in Table 1.

The use of only buffer as the reaction medium leads to a good conversion of the substrate (92%), but there is a considerable amount of hydrolytic product (galactose), so that the total yield in disaccharide is a moderate 68%. The use of either glycerol or TFE results in the total loss of transglycosylation activity, and only ca. 35% hydrolysis product is observed. The total activity shows therefore a marked decrease.

Most of glycerol derivatives (**1**, **2**, **4**, **7**, **8** and **9**) give rise to a total loss of enzymatic activity when used as co-solvents in this reaction.



Scheme 1. General scheme of transglycosylation reaction catalysed by β-galactosidase from *E. coli*.

Table 1
Transglycosylation yields obtained with β-galactosidase from *E. coli* using Tris/HCl pH 7.3 and 2 M of glycerol-based solvent in the same buffer

Solvent	log *P*	ε^{a}	E_T^{Nb}	*p*NP-Gal	Gal	Galβ(1→6) GlcNAc
Only buffer	0.00	78.50	1.00	8	24	68
Glycerol	−1.30	42.50	0.81	63	37	—
TFE	0.71	27.10	0.90	65	35	—
1	−0.60	13.00	0.61	100	—	—
2	0.14	7.00	0.48	96	—	4
3	0.27	6.60	0.44	73	—	27
4	1.14	12.00	0.59	100	—	—
5	1.42	14.60	0.70	7	—	93
6	1.71	13.70	0.55	15	—	85
7	1.93	5.20	0.46	100	—	—
8	2.07	5.60	0.45	100	—	—
9	2.48	4.70	0.15	100	—	—
10	2.56	10.40	0.70	81	—	19
11	3.77	8.60	0.07	27	58	15

[a] Dielectric constant of the pure solvent.
[b] E_T^N of the pure solvent.

However, solvents **5** and **6** lead to the best yields for the synthesis of Gal-β-(1→6)-GlcNac (93% and 85%, respectively), with no traces of hydrolysis product. This means an important increase of activity according to natural behaviour of this enzyme in buffer 10 mM Tris/HCl buffer at pH 7.3, but also an improvement in the selectivity of the reaction (no hydrolysis product), keeping total regioselectivity to the β-(1→6) isomer. Solvents **5** and **6** have two trifluoromethyl (CF_3) groups in their structure, which seems to have a beneficial effect to the transglycosylation reaction. The presence of only one of these groups (solvent **4**) has no effect at all. On the other hand, an increase in the length of fluorinated chains (solvents **10** and **11**) was not beneficial for the activity in transglycosylation, either. This result is difficult to rationalize in terms of solvent polarity only, given the similar values displayed by **5**, **6**, **10** and **11**. A factor that could have influence would be solubility in water. Solvents with long fluorinated chains tend to be immiscible with water, so the presence of two separated liquid phases (buffer and fluorinated solvent) cannot be discarded in the case of **10** and **11**, which would explain the different behaviour obtained.

In order to check the influence of the co-solvent proportion on the enzymatic activity, different concentrations of **5** (0.5, 1 and 2 M) and **6** (0.5, 1, 2 and 3 M) were also tested, and the results obtained in these experiments are shown in Table 2.

As can be seen, in the case of solvent **5** the best yield in disaccharide was obtained with a concentration 1 M in glycerol-based solvent (97% yield with total substrate conversion).

Table 2
Transglycosylation yields obtained with β-galactosidase from *E. coli* using Tris/HCl 10 mM pH 7.3 and glycerol-based solvents **5** and **6** in the same buffer at different concentrations

Solvent (concn)	*p*NP-Gal	Gal	Galβ(1→6)GlcNAc
5 (0.5 M)	76	—	24
5 (1.0 M)	—	3	97
5 (2.0 M)	7	—	93
6 (0.5 M)	75	—	25
6 (1.0 M)	40	3	57
6 (2.0 M)	16	—	84
6 (3.0 M)	100	—	—

However, with lower concentrations (0.5 M), the transglycosylation yield downs to ca. 25%. In the case of solvent **6**, the maximum yield in the transglycosylation reaction is obtained with a concentration of green solvent of 2 M (84%), and this yield systematically decreases with solvent concentration. On the other hand, no activity was observed when the concentrations of this green solvent were increased up to 3 M, indicating the necessity of working at the adequate co-solvent concentration to obtain optimal results.

To get some insights on the origin of the activity boost observed in some of the glycerol-derived solvents tested, a molecular dynamics study was carried out, comparing pure water with the **5**–water mixture, as solvating media of the β-galactosidase of *E. coli*. Stability of the enzyme–substrate complex was analysed in both media measuring the root mean square deviation (RMSD) of α carbons through the simulation time. Both solvated systems have similar values of RMSD and radius of gyration, indicating that there is no difference in the protein flexibility (see Supplementary data, Fig. S1).

To investigate any possible displacement or motion that could explain the enhancement of the reaction, the RMS fluctuations (RMSF) for C-α atoms were computed (Supplementary data, Fig. S2). The results show that some parts of the protein may have more flexibility in the water system (Fig. 2). However, the surrounding environment of the active site remains with similar flexibility in both systems. The superimposition of the average structures of water-only and water–solvent **5** complexes on the original structure shows little displacement on active site residues confirming the absence of difference between systems.

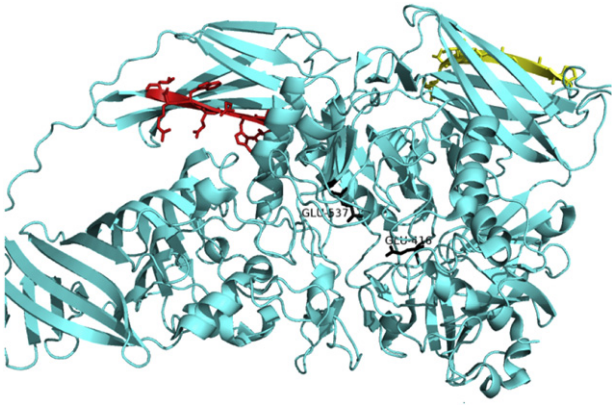


Fig. 2. *LacZ* monomer of *E. coli*. In yellow, residues 263–273, which present a high RMSF in water. In red, residues 714–722, which present a moderate RMSF, in water too.

Since structural differences were not found between simulations, a detailed analysis of ligand disposition was done (Table 3). Distance from catalytic glutamic residues to the acceptor is usually found to be correlated with regioselectivity and activity changes.[24] In the present work the distance between C1 of the modified glutamic residue and O6 of NAG (*N*-acetylglucosamine) was measured during the simulation time, and it was found to be shorter and have fewer fluctuations in the case of the mixture, mainly at the beginning of the simulation (Fig. 3). This difference could explain the product enhancement obtained through a better disposition in the active site of NAG that may be caused by a favourable NAG-solvent interaction.

Table 3
Distance from C1 of modified residue Glu-537 to O6 of NAG

Solvent (concn)	Average distance (Å)	Standard deviation (Å)	Maximum distance (Å)
5 (2.0 M)	4.33	0.56	6.9
Water	4.50	1.01	8.7

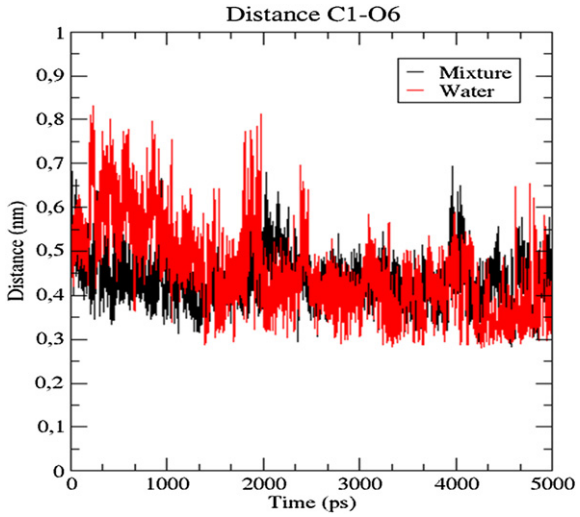


Fig. 3. Distance between C1 carbon of modified residue Glu-537 and O6 oxygen of NAG.

3. Conclusions

To sum up, an enzymatic activity boost, keeping total regioselectivity, was found in the synthetic behaviour of *E. coli* β-galactosidase in glycerol-based solvents. Yields of up to 97% of Gal-β-(1→6)-GlcNac were obtained with total substrate conversion. These reactions take place without noticeable hydrolytic activity and with total regioselectivity, representing a considerable improvement over the use of aqueous buffer or conventional organic solvents. These results reinforce the concept of driving enzymatic syntheses to the desired product simply by adjusting the reaction medium with small amounts of green solvents. Molecular modelling studies point to a better disposition of the substrate in the active centre, with origin in solvation effects, as a possible explanation for the behaviour observed.

4. Experimental section

4.1. General

Commercially available β-galactosidase from *E. coli* and other chemicals were obtained from Sigma.

UV–vis spectra were recorded on a UV-2401 PC Shimadzu. HPLC Agilent 1100 with UV–vis detector using Mediterraneasea 18 15 cm×0.46 5 mm column (Teknokroma) with water/acetonitrile (75:25) as a mobile phase at a flow of 0.7 mL/min. HPLC Jasco with light scattering detector using Lichrosorb NH_2 5 mm 25×0.47

column (Teknokroma) with acetonitrile/water (80:20) as a mobile phase at a flow of 1.0 mL/min. NMR spectra were recorded on Bruker 500 MHz spectrometers. The structure of the enzymatically synthesized disaccharides was assigned by proton–proton shift correlations, carbon–proton shift correlation.

4.2. Solvents

Glycerol-based solvents were synthesized using the same procedures previously described.[11] The alcoholysis of either epichlorohydrin (symmetrical derivatives) or the appropriate glycidol ether (unsymmetrical derivatives) gave the corresponding 1,3-dialkoxy-2-propanols. O-methylation of some selected targets leads to the corresponding 1,2,3-trialkoxypropanes. All the solvents were purified by distillation and used in high pure form. The complete listing of the solvents used in this work is shown in Fig. 1.

4.3. Enzyme activity assay

Protein concentration was determined by the Bradford[25] method using bovine serum albumin as the standard. Hydrolytic activity was determined spectrophotometrically by quantification of *p*NP (*p*-nitrophenol) liberated from correspondent *p*-NP-β-Gal (*p*-nitrophenyl-β-D-galactopyranoside) in Tris/HCl buffer 10 mM pH 7.3. A sample of enzyme solution (20 μL) was added to 80 μL of Tris/HCl buffer to containing 5 mM *p*NP-β-Gal. The reaction mixture was incubated for 3 min at 37 °C. Absorbance was measured at 410 nm. One enzymatic unit was defined as the amount of protein that hydrolyses 1 μmol of *p*NP-β-Gal per minute under the conditions described before.

4.4. General procedure for transglycosylation reactions using β-galactosidase from *E. coli*

A solution of 100 mg (0.17 M) *p*-NP-β-D-Gal (donor) and 550 mg (1.25 M) of *N*-acetylglucosamine (acceptor) in 1 mL of glycerol-based solvent (2 M)-buffer mixture was pre-equilibrated to 30 °C. Afterwards, 155 μmol/min (*U*) of β-galactosidase from *E. coli* were added to the reaction mixture. Reaction was monitored by HPLC UV–vis and final products obtained by HPLC with a light scattering detector (ELSD) were analysed. The reaction was stopped by heating to 100 °C for 5 min. Final reaction mixture obtained was loaded on activated carbon (50% m/m) and Celite (50% m/m) column (50 cm×2 cm, 25 cm column height) eluted with 3 volumes of milliQ water, 3 volumes of 5% ethanol (in water) and 3 volumes of 15% ethanol (in water). Disaccharide enriched fractions were determined by TLC on silica gel 60 (Merck, Darmstadt, Germany), with isopropanol/nitromethane/water (10:9:2) as eluent with detection by spraying the plates with 10% aq H_2SO_4 in methanol and heating. Ten, these fractions were collected in 15% ethanol; they were pooled and analysed by HPLC-ELSD as described above. ^{1}H NMR spectrum (D_2O) was recorded to purified disaccharide on a Bruker 500 MHz spectrometer.

4.5. Molecular Modelling

4.5.1. Protein model. The X-ray structure of the β-galactosidase of *E. coli* was obtained from the Brookhaven Protein Data Bank (PDB ID: 1PX3). The enzyme is composed by four identical units assembled in a tetramer. To simplify the study the monomer A was selected. The structure was relaxed with 2000 steps of Conjugative Gradient (CG) energy minimisation and it was used for docking and molecular dynamics simulations.

4.5.2. Molecular docking. In order to describe the system properly, a two-dockings approach was followed.[24] In a first attempt, *p*-nitrophenol-β-galactopiranose was initially docked into the active centre of the enzyme to determine the position of the molecule to simulate the glycosyl–enzyme intermediate. We selected the best pose according to geometrical (close enough to the nucleophilic residue Glu-537)[26] and energetic criteria (favourable energy) and set up a covalent link between the nucleophilic glutamic residue and the galactose. In the second docking, the glycosyl–enzyme was used to perform a docking with the NAG. The best solution was selected taking only into account energetic criteria, and it was used as the starting structure for the subsequent molecular dynamics studies. Docking procedures were carried out with Autodock 4 software,[27] using a 60×60×60 grid points box around nucleophilic glutamic residue (Glu-537), to embrace all residues implicated in the mechanism, and with a grid spacing of 0.375 Å. A Lamarckian genetic algorithm with the standard parameters was selected.

4.5.3. Solvent parameterisation. The glycerol derivative (**5**) used for the simulation was parameterised for the GROMOS 96 43a1 force field as it was described in our recent work. The mixture of glycerol derivate and water was previously equilibrated 300 ps at 298 K and 1 atm before solvating the system.

4.5.4. Molecular dynamics. Molecular dynamics simulations were set up with the best solution obtained from docking and the glycosyl–enzyme intermediate. Parameters adopted in GROMOS 96 43a1 were used for the protein, while the parameters required for the galactose–glutamic residue were derived in a consistent manner with the force field.[28] NAG parameters were generated by the Dundee PRODRG 2.5 Server.[29] For all systems, an initial minimisation was performed with 500 steps of SD followed by 1500 steps of Polak–Ribiere Conjugate Gradients (CG). The minimized complexes were solvated in different cubic boxes, fulfilling the requirement of a minimum distance of 1.2 nm between any atom of the complex and the faces of the box. In one case, the monomer was solvated with SPC water molecules, to reproduce the buffer conditions, and in the other, a mixture of glycerol derivate and SPC water molecules was used, with the aim to reproduce the conditions for the regioselectivity change. Following, each box was minimized with 500 steps of SD and 3000 steps of CG and equilibrated 100 ps at 298 K and 1 atm (NPT conditions). Finally, a 5 ns production simulation for the water system was carried out while a 20 ns production simulation for the mixture was performed. All MD simulations were set up with GROMACS software suite (v4.0.7).[30]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2011.08.009.

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