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Diastereoselective synthesis of (*R*)-(alkyl)-β-D-galactopyranoside by using β-galactosidase (*Aspergillus oryzae*) in low-water media

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Abstract—A β-galactosidase (from *Aspergillus oryzae*) preparation viz. EPRP (enzyme precipitated and rinsed with propanol), obtained by the removal of bulk water by precipitation with *n*-propanol, showed higher biological activity than the lyophilized powder. FT-IR study confirmed that EPRP had retained the α-helical content of the native structure better than the lyophilized form. Use of this formulation of β-galactosidase under low water conditions (temperature 55 °C, reaction time of 4 h) gave enantioselectivity, $E > 1000$ for the stereoselective synthesis of (*R*)-(1-phenylethyl)-β-D-galactopyranoside, starting from racemic 1-phenylethanol and D-galactose. For racemic 2-octanol also, EPRP worked better. Under similar conditions, (*R*)-(2-octyl)-β-D-galactopyranoside was formed with an enantioselectivity, $E = 38$.

The products arising out of the combination of carbohydrates and other specific molecules are collectively known as glycoconjugates.[1,2] In view of their importance in many biological processes and as starting materials for many drugs, synthesis of glycoconjugates has attracted considerable attention. The chemical synthesis is generally a multi-step procedure with low yield and contamination with unwanted enantiomer. The use of glycosidases is an attractive alternative and can be carried out in two ways. The frequently used way is to carry out a transglycosylation where an activated glycosyl donor is used.[3–5] As pointed out by Vic and Crout, this is both expensive as well as gives too many side products.[4] The second approach is to carry out reverse hydrolysis in low-water media. Synthesis of alkyl-β-D-glycosides from D-glucose or from D-galactose by reverse hydrolysis using β-glycosidases (glucosidase or galactosidase) has been reported with alcohols which lack chiral center.[2,4,5] However, the use of reverse hydrolysis in enzymatic enantioselective glycosylation with a racemic mixture of a chiral alcohol giving a diastereomeric product pair in low-water media has been reported with limited success. Vic et al. synthesized (*S*)-1-phenylethyl-β-D-glucoside from D-glucose using almond-β-D-glucosidase with a diastereomeric excess of 42% (reported *R*:*S* glucoside ratio was 0.4:1.0).[6] The use of enzymes in low-water media is now widely known.[7–9] Unfortunately, as is now also well established, the catalytic efficiency of the enzymes in such low-water media is quite low as compared to their performance in aqueous media.[8–10] In order to improve their catalytic performance in non-aqueous media many approaches have been described.[9,10]

Recently, Bridiau et al.[1] have looked at the optimization of β-galactosidase catalyzed selective galactosylation of a number of aromatic compounds. The % conversions ranged from 9.5% to 96%.[1] The worst conversion was obtained with 1-phenylethanol as the acceptor. Also the resolution of (*R*,*S*)-1-phenylethanol has been extensively investigated to evaluate and validate different enzyme based approaches for kinetic resolution.[11,12] This is because enantiopure 1-phenylethanol is a chiral synthon for synthesis of industrially important compounds like primeveroside.[13] Hence resolution of 1-phenylethanol was taken up in the present work.

It is a well known practice to 'pH tune' the enzyme powder before using it in non-aqueous media.[7,9] This consists of dissolving the enzyme in a buffer at optimum pH and lyophilizing the solution. Table 1 shows that pH tuned lyophilized powder[14] as a catalyst produced 23% conversion[15] to (*R*)-(1-phenylethyl)-β-D-galactopyranoside (compound **3**, Scheme 1) with 93% de_P.[3,16]

Keywords: Glycoconjugates; Galactoside synthesis; β-Galactosidase; Reverse hydrolysis; Low-water media; Diastereoselective synthesis.
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Table 1. Galactosylation of 1-phenylethanol with different formulations of β-galactosidase

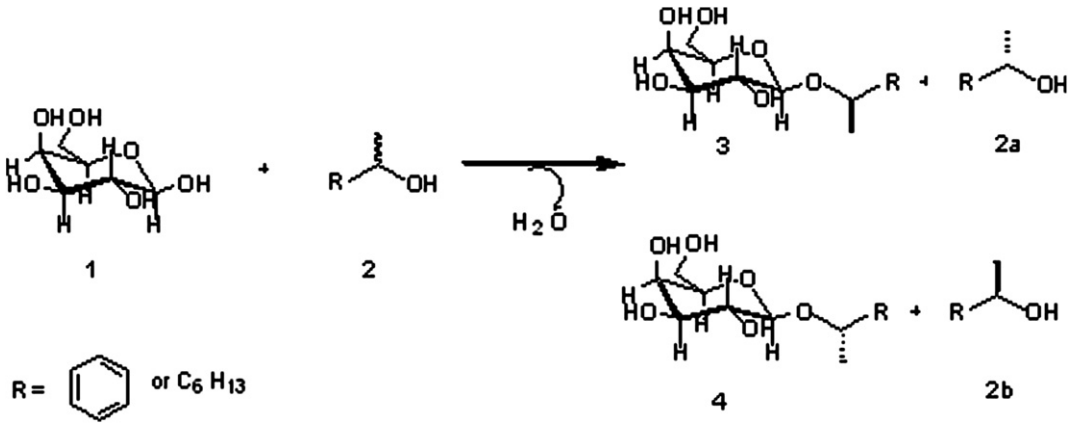
Biocatalyst type	Initial rate (μmol mg^{-1} h^{-1})	C[a] (%) (16 h)	de_P[b] (%)	ee_S[b] (%)	E[c]
Lyophilized	575	23	93	28	36
Colyophilized (with galactose)	700	28	95	36	56
EPRP	1050	42	95	68	80

Different formulations of β-galactosidase[14] were used. The reaction was performed in acetonitrile, dimethyl formamide, water (in 9:0.5:0.5v/v/v ratio). The minimum amount of water (5%, v/v) was required to dissolve the sugar under the reaction condition. Each reaction set was performed twice and the deviations between the pair of readings were within 2%.

[a] C (conversion) = $ee_S/ee_S + de_P$. Further incubation caused a drop in C value: from 16 h to 32 h, C dropped from 23% to 10%, 30% to 13%, and 42% to 25%.

[b] de_P, ee_S stand for diastereomeric excess of the product (Scheme 1, **3** and **4**) and enantiomeric excess of the unreacted substrate (Scheme 1, **2a**,**b**), respectively.

[c] $E = \ln[1 - C(1 + ee_P)]/\ln[1 - C(1 - ee_P)]$, here $de_P = ee_P$. Diastereomeic excess of the product (de_P) can replace ee_P in Chen's equation[23] as there is only a pair of diastereomeric products with β-stereochemistry in the sugar part and differing in the configuration of the aglycon portion due to enantioselection from racemic 1-phenylethanol.



Scheme 1.

Another strategy which has been used for improving the reaction rates in non-aqueous media is the use of sugars as lyoprotectants during lyophilization.[9] Presence of such additives during lyophilization minimizes the structural changes which proteins are known to undergo during lyophilization.[17] In the present instance, as the substrate galactose itself is a sugar, it was a natural choice as a lyoprotectant during lyophilization. Moreover, it has also been pointed out that the presence of a substrate during lyophilization may also minimize structural damage during lyophilization.[18] Thus, it was hoped that for both reasons, lyophilization of the enzymes, with D-galactose, would improve reaction rates. Table 1 shows that the strategy did work. In recent years, the removal of bulk water of enzyme by rinsing/precipitation with an organic solvent rather than lyophilization has emerged as an attractive option to obtain more active biocatalyst preparation for use in low-water media.[19,20] Use of such a formulation called EPRP (enzyme precipitated and rinsed with propanol)[14] yielded even better initial rates and consequently 42% conversion to (*R*)-(1-phenylethyl)-β-D-galactopyranoside (compound **3**, Scheme 1) in 16 h with an improved 95% de (Table 1). FT-IR has emerged as a powerful tool to evaluate the secondary structure of the proteins especially in the context of 'drying' the enzymes.[17] Table 2 shows that precipitated enzyme (EPRP) did have higher α-helix content (21% for EPRP and 10% for freeze-dried form) and a lower β-sheet content (34% for EPRP and 45% for freeze-dried form) than the freeze-dried form. This is in agreement with the known picture about the structural changes undergone by protein during lyophilization.[17] It is believed that lyophilization damages the protein structure by reducing the α-helical content of this protein. The reported α-helical content of this enzyme in the native form is 46%.[21] Thus FT-IR data indicate that removal of bulk water by precipitation with *n*-propanol is less harsh for protein structure as compared to lyophilization. This could explain the higher activity displayed by EPRP. Using this biocatalyst preparation (EPRP),[14]different reaction media were tried (Table 3)

Table 2. FT-IR analysis[25] of amide I band of β-galactosidase enzyme formulations

Peaks[a] (cm^{-1})	Type	FD (%)	EPRP (%)
1610 ± 1	Side chain residues	14	14
1635 ± 1	β-Sheet	45	34
1654 ± 2	α-Helix	10	21
1670 ± 2	Contribution of turn and β-segments	23	16
1688 ± 2	Turn	11	15

The freeze-dried (FD) or lyophilized and EPRP formulations of the enzyme were analyzed in the solid form and the changes in the secondary structure were recorded (see Figs. 2a and b for the FT-IR spectra in the Supplementary data).

[a] Peaks were identified as mentioned in the earlier studies of β-galactosidase.[21]

Table 3. Effect of the change in the solvent system for galactosylation of 1-phenylethanol with EPRP

Solvent	Initial rate (μmol mg^{-1} h^{-1})	C^a (%) (4 h)	$de_P{}^a$ (%)	$ee_S{}^a$ (%)	E^a
Acetonitrile/DMF/water (9:0.5:0.5, v/v)	1050	15	97	17	78
Acetonitrile/DMSO (9:1, v/v)	1675	22	97	27	86
DMSO	5953	43	98	73	220

Variation in the initial rates and enantioselectivity. EPRP of β-galactosidase[14] was tried with different solvent system. After 4 h the de and ee values were noted in each case and conversion and *E* values were calculated. Each reaction set was performed twice and the deviations between the pair of readings were within 2%.

[a] *C*, de_P, ee_S, *E* bear the same meanings as described in Table 1.

with a reduced reaction time of 4 h. It was found that DMSO could replace both DMF plus water and this improved initial rates as well as conversion (%) without loss of diastereoselectivity.[22] As mentioned in Table 1, $E = 220$ was obtained. Glycosidation reaction is accompanied by production of water as well. The water content in the reaction medium is known to influence the reaction rates in non-aqueous enzymology.[7] Hence the reaction in DMSO was repeated in the presence of the molecular sieves. The effect of the amount of the molecular sieves present in the reaction medium on the initial rate of product formation and de_P (%) is shown in Figure 1. There was some increase in initial rates as well as in de_P (%) with optimum amount of molecular sieves. Reducing water content in organic media is known to improve enantioselectivity.[23] This is presumed to be due to the reduction in conformational flexibility which favors enantioselectivity by the enzyme.[24] The separation of the reactants from the products was done on the HPLC chiral column.[16] Thus, 45% conversion with >99% de under the best conditions was achieved. The enantioselectivity, *E*, was based on the de of the product and the ee of the remaining unreacted (*S*)-enriched 1-phenylethanol. Corresponding to 45% conversion this was >1000.

In order to evaluate possible versatility of this approach, galactosylation of another alcohol was attempted. Matsumura et al. have attempted to prepare enantiomerically pure (*R*)-2-octyl-β-D-galactopyranoside by transgalactosylation. Only 17% yield with 63% ee could be obtained. So 2-octanol was chosen as the second alcohol for galactosylation by reverse hydrolysis. Table 4 shows the variation in the initial rates with enantioselectivity of different formulations of β-galactosidase for the reaction with racemic 2-octanol in 20% (v/v) DMSO in acetonitrile. The medium was chosen from our earlier experience which showed that DMSO as a cosolvent in acetonitrile was a better option to start with. The same trend was observed in this case also. The better values were achieved with EPRP in DMSO (Table 4). Presence of 10% molecular sieves (w/w, reactants) in the reaction medium further improved the initial rate, conversion, and the de_P (Fig. 1b). Under optimized conditions, thus, an improved rate of 1320 μmol mg^{-1} h^{-1} corresponding to 33% conversion to 2-octyl-β-D-galactopyranoside was

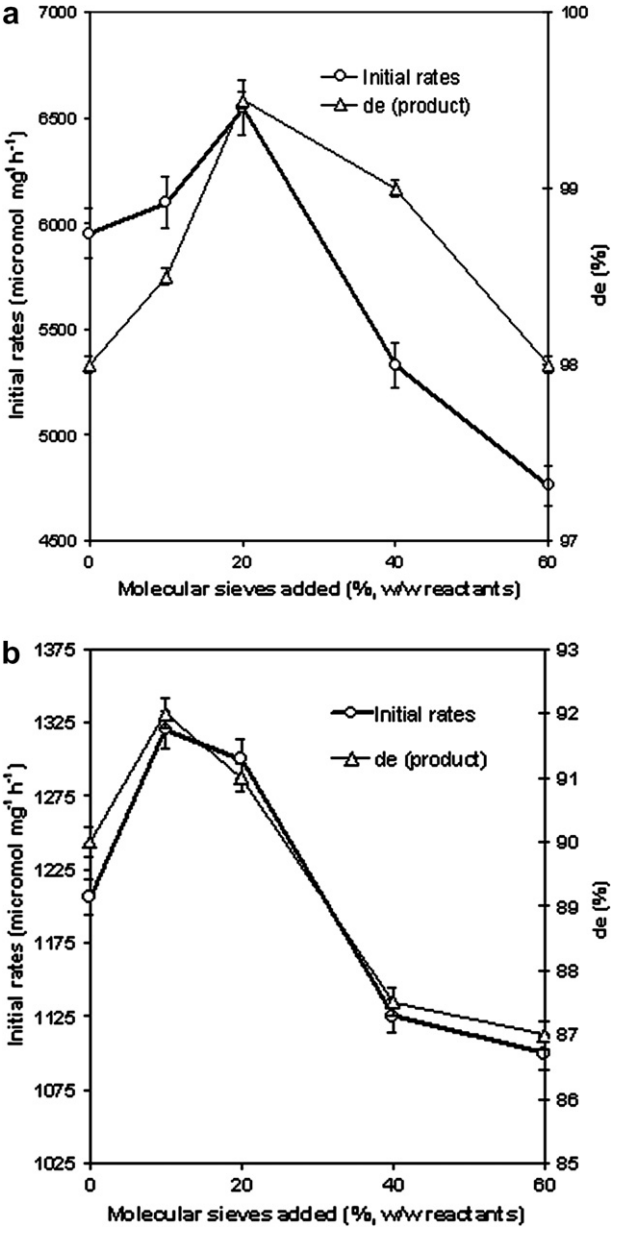


Figure 1. Change in the initial rates with EPRP and de (product) with the varied amount of molecular sieves in reaction media: Activated molecular sieves (3 Å) were added in varied amounts (10–60%, w/w reactants) in the reaction medium DMSO, incubated at 55 °C at 200 rpm in an orbital shaker, and initial rates were studied in each case. (a) Synthesis of (*R*)-1-phenylethyl-β-D-galactopyranoside: Diastereomeric excess (de) for the product was determined after 4 h. The maximum de value corresponds to 45% conversion with 20% (w/w, reactants) molecular sieves. The reactions were done in duplicates and the deviations between the pair of readings were within 2%. (b) Synthesis of (*R*)-2-octyl-β-D-galactopyranoside: Diastereomeric excess (de) for the product was determined after 10 h. The maximum de value corresponds to 33% conversion with 10% (w/w, reactants) molecular sieves. The reactions were done in duplicates and the deviations between the pair of readings were within 2%.

obtained within 10 h with a 92% de_P (continuing the reaction beyond 10 h gave a lower de_P).

To conclude, for galactosylation of racemic 1-phenylethanol, a conversion of 45% with a $de_P > 99\%$ ($E > 1000$)

Table 4. Synthesis of (*R*)-2-octyl-β-D-galactopyranoside in 20% DMSO (v/v) in acetonitrile

Biocatalyst type	Initial rate (μmol mg^{-1} h^{-1})	C^a (%) (10 h)	$de_P{}^a$ (%)	$ee_S{}^a$ (%)	E^a
Lyophiliized	534	17	72	14	7
Co-lyophilized	735	21	78	20	10
EPRP	936	27	88	32	21
EPRP[b]	1206	30	90	38	28

Variation of initial rates and enantioselectivity of the different formulations of β-galactosidase. Each reaction set was performed twice and the deviations between the pair of readings were within 2%.
[a] *C*, de_P, ee_S, *E* bear the sam meanings as described in Table 1.
[b] The solvent used in this case for the reaction was 100% DMSO.

in 4 h is a considerable improvement as compared to 9.5% conversion reported by Bridiau et al.[1] and 39% conversion with a de_P (reported as ee) of 98% (corresponding $E = 188$) reported by Matsumura et al.[3] For galactosylation of racemic 2-octanol, again EPRP was found to be better than the other formulations of β-galactosidase (Table 4). Under optimized conditions, 33% conversion was achieved within 10 h with a fairly high 92% de_P which is a considerable improvement over the earlier reported value of 63% de_P.[3] The present work outlines an optimization strategy which combines the use of a highly active biocatalyst with a suitable reaction medium for the diastereoselective galactosylation of secondary alcohols.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.11.006.

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14. *Preparation of different formulations of the biocatalyst for low-water media*: *Lyophilized enzyme preparation*: β-Galactosidase powder (5 mg) was dissolved in 200 μL of 100 mM phosphate buffer (pH 7.3),[3] frozen at −23 °C, and was lyophilized for 24 h. In another case, the same amount of enzyme was dissolved along with galactose (18.6 mg) and was lyophilized for 24 h.
EPRP (*enzyme precipitated and rinsed with propanol*): 25 μL of enzyme solution (20 mg/100 μL of 100 mM phosphate buffer, pH 7.3) was precipitated in 200 μL of ice cold dry *n*-propanol, centrifuged, and the recovered pellet was repeatedly rinsed with *n*-propanol before using for the reaction.
15. β-*Galactosylation with 1-phenylethanol*: In screw-capped vials, D-galactose (18.5 mg) and 25 μL of racemic 1-phenylethanol (Merck, Germany) were dissolved in a solution of acetonitrile, DMF, water (in 9:0.5:0.5, v/v ratio) so that the final volume of the reaction volume was 1.0 mL. After pre-incubation at 55 °C for 15 min, 5 mg (10%, w/w reactants) enzyme preparation was added. As an alternative reaction medium, anhydrous DMSO (water content <0.005%, v/v, Acros Organics, USA) was tried instead of DMF and water. The reaction was carried out at 55 °C at 200 rpm in an orbital shaker. β-*Galactosylation with 2-octanol*: D-Galactose (18.5 mg) and 35 μL of racemic 2-octanol (Merck, Germany) were dissolved in a solution of acetonitrile, DMSO (in 4: 1, v/v ratio) and the reaction was performed as mentioned earlier.
16. *HPLC analysis and determination of the diastereomeric excess* (*de*) *of the product*: The product analysis was done with chiral column [Chiracel OD RH; eluents: (i) hexane/isopropanol/ethanol = 75:23:2 (v/v) at a flow rate of 0.5 mL/min by DAD-UV (217 nm) for 1-phenylethanol system, (ii) acetonitrile/water = 9:1 (v/v) at a flow rate of 0.25 mL/min by RID for 2-octanol system] fitted in Agilent 1100 series HPLC system. The peaks (retention time) were identified as (*S*)-1-phenylethyl-β-D-galactopyranoside (4.43 min), (*R*)-1-phenylethyl-β-D-galactopyranoside (4.68 min), (*R*)-1-phenylethanol (4.90 min), (*S*)-1-phenylethanol (5.06 min), and (*S*)-2-octyl-β-D-galactopyranoside (8.75 min), (*R*)-2-octyl-β-D-galactopyranoside (9.20 min), (*R*)-2-octanol (10.04 min), (*S*)-2-octanol (10.3 min). Finally, the enantioselectivity was calculated by Chen's equation.[23]
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25. *FT-IR analysis of the different formulations of* β-*galactosidase*: The spectra were recorded on Nicolet™ FT-IR 6700 spectrophotometer with Smart Miracle™ ATR accessories having zinc selenide crystal. Different formulations of β-galactosidase, freeze-dried (FD), and EPRP, were taken directly on the zinc selenide crystal plate and an average of 200 scan was taken with a resolution of 2 cm^{-1}. The spectra were analyzed over 1600–1700 cm^{-1} (amide-I band).[21] The data collection and Fourier Self Deconvoluation (FSD) were done by using OMNIC 2.1 software. The peaks were resolved and were fitted with Gaussian curves. From the best fit, the peak areas were noted.