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Regioselectivity of the enzymatic transgalactosidation of D- and L-xylose catalysed by β-galactosidases

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Abstract

The regioselectivity of enzymatic transgalactosidation depends on the source of the β-galactosidase used. When the galactosyl acceptor only contains secondary hydroxyl groups, e.g., D- or L-xylose, it is possible to find an enzyme that catalyses preferentially the synthesis of any of the three regioisomers 4-, 3- and 2-*O*-β-D-galactopyranosyl-D-xylose (**1**, **2** and **3**, respectively) or 4-, 3- and 2-*O*-β-D-galactopyranosyl-L-xylose (**4**, **5** and **6**, respectively). Enriched mixtures in **1**, **2** or **3** were obtained using β-galactosidases from *Escherichia coli*, bovine testes or *Aspergillus oryzae*, respectively, by transgalactosidation reaction of *O*-nitrophenyl-β-D-galactopyranoside and D-xylose, and enriched mixtures in **4**, **5** or **6** were obtained in a similar way using β-galactosidases from *Aspergillus oryzae*, lamb small-intestine (intestinal lactase–phloridzin hydrolase) or *Saccharomyces fragilis*, respectively, using L-xylose as acceptor. © 1998 Elsevier Science Ltd.

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1. Introduction

The natural function of glycosidase enzymes is to catalyse the hydrolysis of di-, oligo-, and polysaccharides, as well as non-sugar glycosides. In addition to hydrolysis, it is known the capacity of some glycosidases to catalyse the synthesis of glycosides by the transfer of glycosidic units to other hydroxylated compounds different from water [1]. However, yields and regioselectivities of this transglycosidations are usually low. Nevertheless, the reaction conditions are simple, co-factors are not necessary, some of these enzymes are available with high stability and low cost, and additionally, the reaction is stereospecific in terms of the new glycosidic bond formed. For these reasons, the transglycosidation reaction has attracted the attention of organic chemists due to its potential synthetic applications in the preparation of diverse glycosides [2–5].

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[1] *In memorium* of Jose Alonso who left us at the beginning of this work.

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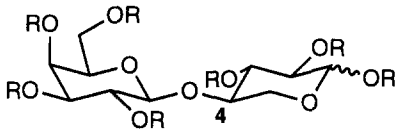
Concerning the control of the regioselectivity of the glycosidase catalysed synthesis of disaccharides, in addition to some data regarding the reaction conditions [6], it has been observed that changes in the configuration [7] or substitution [8,9] at the anomeric carbon and other structural modifications [10] of the sugar acceptor induce significant changes in the ratio of the regioisomers obtained. Besides, the regioselectivity of these reactions is very dependent on the source of the enzyme. For example, it has been known for some time that β-galactosidase from *Escherichia coli* forms preferently β-(1 → 6) bonds when glucose is used as acceptor [11,12] or in the presence of GlcNAc as acceptor, the enzyme from bovine testes gives mainly the β-(1 → 3) regioisomer [13,14], while the β-galactosidase from *Diplococcus pneumoniae* is very selective in the formation of β-(1 → 4) bonds [15].

Previous work in our group with β-galactosidase from *E. coli*, have shown that the yield and regioselectivity of the galactosidation of β-D-xylopyranosides, that only contain secondary hydroxyl groups, are strongly dependent on the anomeric substituent of the xylopyranoside [8,9]. Following these studies of enzymatic transgalactosidation but using free xylose as acceptor, we have described recently the enzymatic synthesis of a mixture of β-galactopyranosyl-D-xyloses enriched in the β-(1 → 4) regioisomer **1** (Scheme 1) [16].

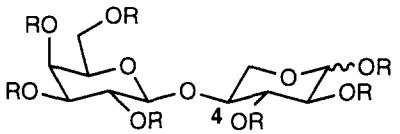
Now we have studied enzymes from different sources regarding to the regioselectivity of transgalactosidation towards secondary hydroxyl groups using free D- and L-xylose as acceptors in order to set up simple enzymatic synthesis of disaccharides **1–6** (Scheme 1).

2. Results and discussion

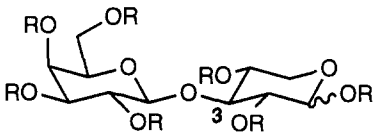
The transgalactosidation reaction was studied using an excess of D- or L-Xyl and *O*-nitrophenyl-β-D-galactopyranoside (ONPG) as galactosyl donor in the presence of six β-galactosidases selected primarily by their availability, source diversity and known transglycosidation properties: a bacterial β-galactosidase from *E. coli* [11], one fungal enzyme from *Aspergillus oryzae* [11], one from yeast Saccharomyces *(Kluyveromyces) fragilis* [17], and three



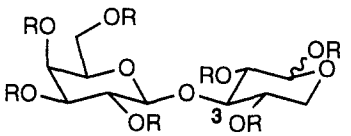
1 R=H
7 R=Ac (α)
8 R=Ac (β)



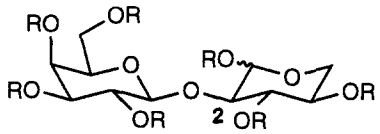
4 R=H
13 R=Ac (α)
14 R=Ac (β)



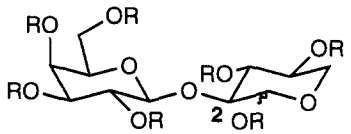
2 R=H
9 R=Ac (α)
10 R=Ac (β)



5 R=H
15 R=Ac (α)
16 R=Ac (β)



3 R=H
11 R=Ac (α)
12 R=Ac (β)



6 R=H
17 R=Ac (α)
18 R=Ac (β)

Scheme 1. Structures of disaccharides **1–6** and their peracetylated derivatives **7–18**.

Table 1
Proton NMR chemical shifts and coupling constants in $CDCl_3$ of the peracetylated disaccharides derived from L-Xyl (**13–18**)

Xylose moiety													
Compound	Chemical shifts (δ)[a]						Coupling constants (Hz)						
	H-1	H-2	H-3	H-4	H-5ax	H-5eq	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5ax}$	$J_{4,5eq}$	$J_{5ax,5eq}$	
13	6.20	4.95	5.40	3.86	3.77	4.10	3.7	10.3	10.3	7.8	–	10.5	
14	5.62	4.98	5.18	3.90	3.51	4.10	8.0	9.2	9.2	10.3	5.1	11.6	
15	6.24	5.06	4.12	4.99	3.63	3.92	3.5	9.3	9.0	10.5	5.0	10.5	
16	5.78	5.03	3.93	4.83	3.53	4.14	5.2	6.2	6.2	6.2	4.1	12.2	
17	6.19	3.87	5.41	4.95	3.66	4.08	3.6	9.6	9.6	11.1	4.0	11.1	
18	5.63	3.73	5.19	4.95	3.47	4.12	7.2	8.5	8.5	9.1	5.3	11.7	
Galactose moiety													
	H-1′	H-2′	H-3′	H-4′	H-5′	H-6a′	H-6b′	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{4',5'}$	$J_{5',6a'}$	$J_{5',6b'}$	$J_{6a',6b'}$
13	4.55	5.15	4.96	5.36	3.86	4.10	4.10	7.9	10.5	3.4	1.1	–	–	–
14	4.57	5.15	4.96	5.37	3.86	4.10	4.10	7.9	10.5	3.3	0.9	5.9	5.9	–
15	4.69	5.16	5.01	5.41	3.95	4.07	4.29	8.0	10.5	3.5	1.0	7.5	6.5	11.5
16	4.65	5.15	4.99	5.38	3.94	4.14	4.14	7.9	10.5	3.3	1.1	6.4	6.4	–
17	4.52	5.08	4.96	5.36	3.90	4.20	4.20	7.7	10.5	3.4	1.1	–	–	–
18	4.61	5.11	4.97	5.36	3.92	4.20	4.20	8.0	10.5	3.4	1.0	6.7	6.7	–

[a]Relative to $CDCl_3$ = 7.24 ppm.

Table 2
GC retention times of the trimethylsilyl derivatives of disaccharides **1–6** and of the corresponding derivatives of mannitol and benzyl-β-D-xylopyranoside (Bn-Xyl) used as chromatographic references

Compound	Mannitol	Bn-Xyl	**1**	**2**	**3**	**4**	**5**	**6**
Retention time (min)	8.33	13.39	22.10 and 22.23	19.67	19.83 and 20.96	20.95	19.54 and 19.83	19.67 and 21.04

Table 3
Maximum yield of disaccharide mixture (**1**, **2** and **3**) derived from D-Xyl obtained with each enzyme expressed as percentage relative to the initial amount of *O*-nitrophenyl-β-D-galactopyranoside and the corresponding regioselectivity expressed as the percentage of each regioisomer relative to the total amount of the mixture. Values in parenthesis correspond to the regioselectivity calculated at the initial points of the curves in Fig. 1

Source of β-galactosidase	Yield (%) of disaccharides	Regioselectivity (%)		
		1	**2**	**3**
Bovine testes	46	6(4)	85(87)	9(9)
Bovine liver	n.d.	n.d.	n.d.	n.d.
Intestinal lactase	36	3(3)	72(80)	25(17)
A. oryzae	37	5(6)	46(43)	49(51)
S. fragilis	17[a]	70(64)	24(30)	6(6)
E. coli	53	81(81)	13(14)	6(5)

[a]A mixture of 1-*O*- and 3-*O*-β-D-galactopyranosyl-D-glycerol was obtained in a 19%.
n.d. non-determined.

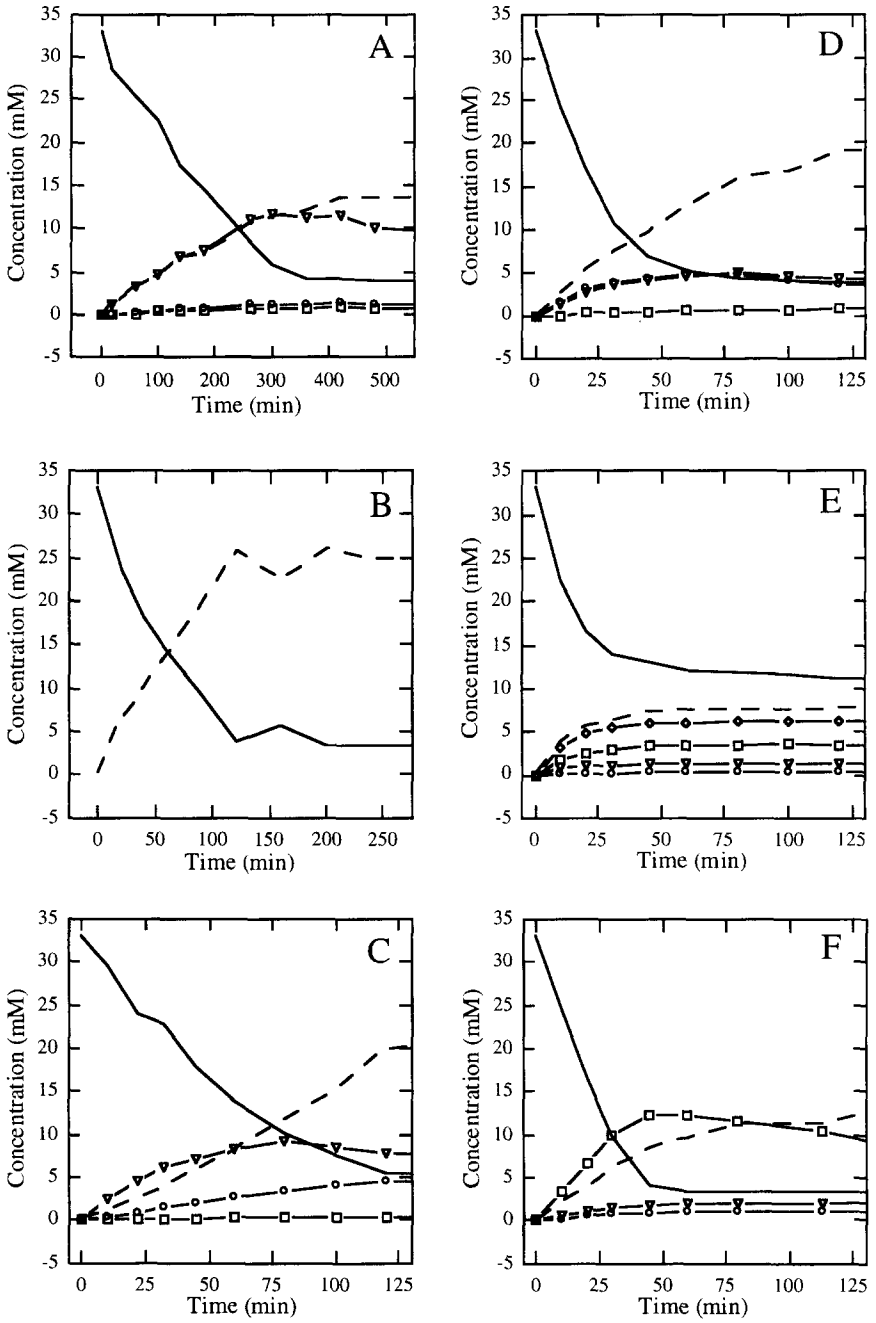


Fig. 1. Time course of disappearance of ONPG (——), and appearance of galactose (· · ·), **1** (□), **2** (▽) and **3** (○) in the enzymatic transgalactosidation of D-Xyl with ONPG using β-galactosidase from: Bovine testes (A), bovine liver (B), lamb small-intestine (C), *A. oryzae* (D), *S. fragilis* (E) and *E. coli* (F). A mixture of 1-*O*- and 3-*O*-β-D-galactopyranosyl-D-glycerol (◇) is formed in the reaction using the enzyme from *S. fragilis* (E).

mammalian β-galactosidases from bovine testes [13,18], from bovine liver [19], and the intestinal lactase [11] (purified from lamb small-intestine [20]). The time course of the reaction was followed by GC analysis of aliquots of the reaction mixture taken at different times.

In order to have standards, pure samples of compounds **1–6** were obtained by enzymatic transglycosidation of D- or L-Xyl in a preparative scale, followed by acetylation of the disaccharides products and HPLC separation of each component. The regiochemistry of the glycosidic bond formed was determined from the NMR spectra of the corresponding peracetylated derivatives **7–12** [16] and **13–18** (see Table 1 for ^{1}H NMR shifts). After deacetylation of each anomeric pair, the GC retention times for all six

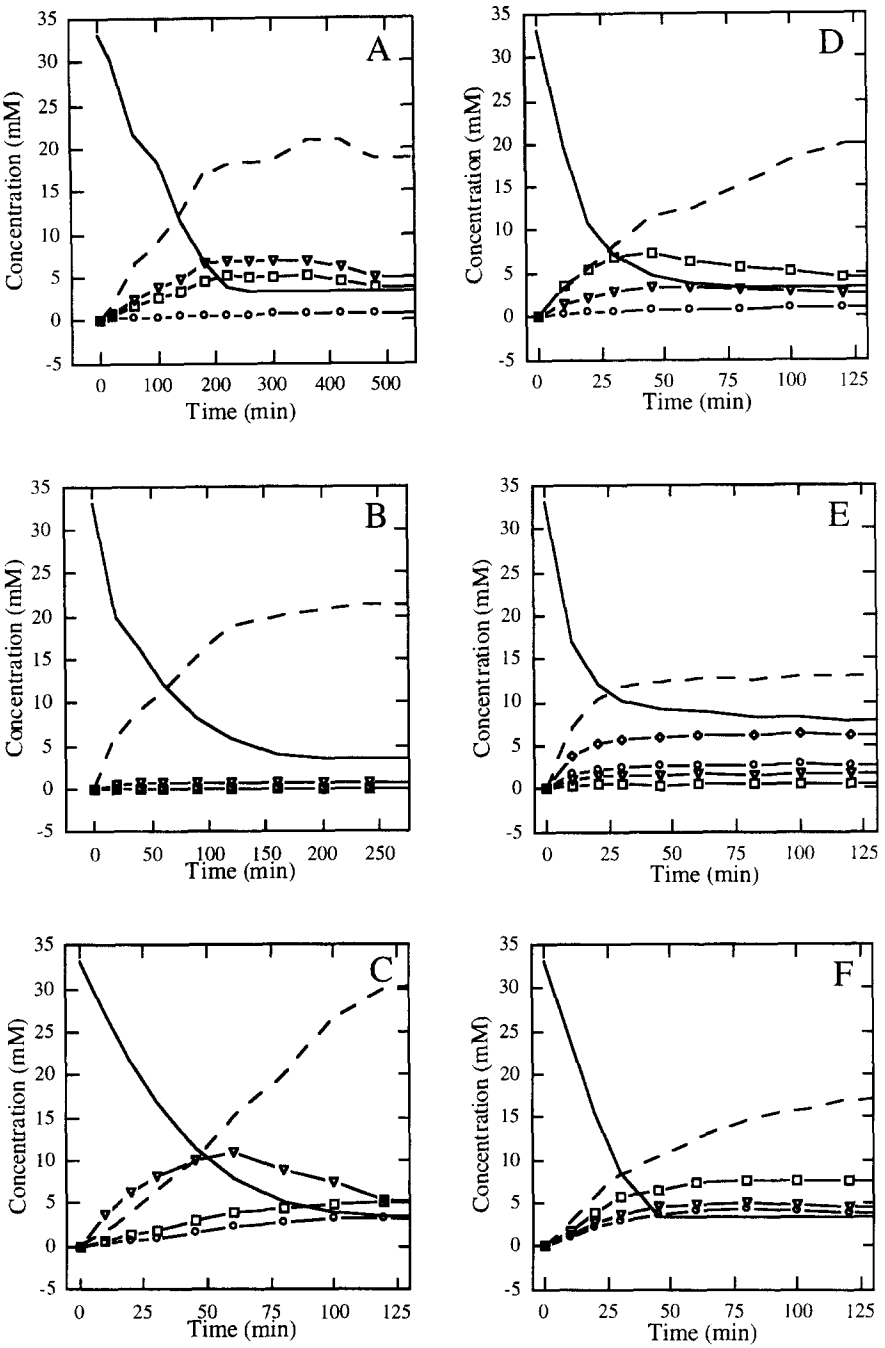


Fig. 2. Time course of disappearance of ONPG (——), and appearance of Gal (· · ·), **4** (□), **5** (▽) and **6** (○) in the enzymatic transgalactosidation of L-Xyl with ONPG using β-galactosidase from: Bovine testes (A), bovine liver (B), lamb small-intestine (C), *A. oryzae* (D), *S. fragilis* (E) and *E. coli* (F). A mixture of 1-*O*- and 3-*O*-β-D-galactopyranosyl-D-glycerol (◇) is formed in the reaction using the enzyme from *S. fragilis* (E).

disaccharides **1–6** were determined (Table 2). In the chromatograms from the reaction aliquots, apart from the peaks assignable to **1–6**, some other non-identified minor peaks were observed. In fact, galactosyl galactosides and higher range oligosaccharides are known to occur in transglycosidation reactions [8]. Additionally, it is not possible to exclude the presence of small quantities of the 1-1 galactosyl xylosides that have been described in reactions catalysed by galactosyl transferase [21].

The time course of the reaction for each enzyme is presented in Figs. 1 and 2 when D- and L-Xyl were the acceptors, respectively. In all cases, the reaction conditions were similar, with a ratio donor:acceptor 1:10, and at the enzyme optimal pH. A first view of the progress curves shows that each enzyme has a

Table 4
Maximum yield of disaccharide mixture (**4**, **5** and **6**) derived from L-Xyl obtained with each enzyme expressed as percentage relative to the initial amount of *O*-nitrophenyl-β-D-galactopyranoside and the corresponding regioselectivity expressed as the percentage of each regioisomer relative to the total amount of the mixture. Values in parenthesis correspond to the regioselectivity calculated at the initial points of the curves in Fig. 2

Source of β-galactosidase	Yield (%) of disaccharides	Regioselectivity (%)		
		4	**5**	**6**
Bovine testes	31	39(37)	55(55)	6(8)
Bovine liver	< 4	n.d.	n.d.	n.d.
Intestinal lactase	39	23(14)	64(77)	13(9)
A. oryzae	45	64(67)	29(27)	7(6)
S. fragilis	19[a]	10(11)	32(35)	58(54)
E. coli	46	43(45)	33(30)	24(25)

[a]A mixture of 1-*O*- and 3-*O*-β-D-galactopyranosyl-D-glycerol was obtained in a 18%.
n.d. non-determined.

particular behaviour. In Tables 3 and 4 the total yields of D- and L-derivatives, respectively, and the regioselectivity for each enzyme taken when the total amount of disaccharides reached its maximum are summarised. Two mammalian enzymes, from bovine testes and lactase, presented significant selectivity to hydroxyl 3 of both D- and L-Xyl (Fig. 1A and C, Fig. 2A and C, respectively). On the contrary, the bovine liver enzyme did not show appreciable transglycosidation to any of the secondary hydroxyl groups of neither D- nor L-Xyl (Fig. 1B, Fig. 2B). In the case of the other two enzymes from eukaryotic origin, the one from *A. oryzae* has a low selectivity favouring, almost equally, the synthesis of the β-(1 → 2) **3** and β-(1 → 3) **2** regioisomers when D-xyl was the acceptor (Table 3, Fig. 1D), while the formation of the β-(1 → 4) regioisomer **4** was preferred with L-Xyl (Table 4, Fig. 2D). The *S. fragilis* enzyme presented a regioselectivity opposite to the *A. oryzae* enzyme, with D-Xyl the β-(1 → 4) regioisomer **1** is obtained in major proportion and the β-(1 → 2) **6** regioisomer is formed preferentially in the presence of L-Xyl. The behaviour of the bacterial *E. coli* β-galactosidase is different to all previous enzymes. It shown the highest regioselectivity towards the formation of the β-(1 → 4) bond with D-Xyl, and a low preference in the formation of **4** with L-Xyl. In all cases, the synthesised products can be also substrates of the enzyme. When the reaction products reach certain concentration the hydrolytic reaction can become predominant. If the reaction time is sufficiently long the yield and regioisomeric ratio can change significantly along the time axis [8] as it can be observed in the intestinal lactase case (Fig. 1C). For comparison, relative initial rates of formation of each regioisomer with each enzyme were calculated from the initial points of the progress curves (Table 3, data in parenthesis). In all cases, the regioselectivities observed at initial times were not very different from the obtained when total disaccharide yield was at the maximum.

The intestinal lactase and bovine testes galactosidase gave the closest results in regioselectivity. Both enzymes showed the highest selectivity towards HO-3 with both D- or L-xyl. This result is in accordance with previous transgalactosidation studies using hexoses as acceptors with either intestinal lactase [11], or bovine testes β-galactosidase [13,14]. With both enzymes, even in the presence of primary hydroxyl groups, the secondary HO-3 is preferred for transgalactosidation. These data may indicate that the presence of hydroxyls at adjacent positions to the OH nucleophile must be an important motif for the recognition of the acceptor by the enzyme. Interestingly, these results also correlate with the preference of these enzymes for galactosyl β-(1 → 3)-glucosides in the hydrolytic reaction [11,13]. For other β-galactosidases, a similar correlation between the hydrolysis and transgalactosidation has been observed [15,22]. On the other hand, the lack of transglycosidation products with the β-galactosidase from bovine liver and D-Xyl could be ascribed to the absence of a primary hydroxyl in the acceptor substrate, since it has been reported that α-methyl glucoside is a good transgalactosidation acceptor for this enzyme, probably forming the β-(1 → 6) disaccharide [19]. The β-galactosidase from *A. oryzae* is a very available enzyme, and has been extensively used in reactions with quite different acceptors [8,22–24]. From our results, there is not an evident common feature that can explain the regioselectivity observed with each Xyl enantiomer, however, it could be the enzyme of choice for the synthesis of 4-*O*-β-D-galactopyrano-

syl-L-xylose (**4**). The enzyme from *S. fragilis* that appears in early reports describing transgalactosidation reactions [17] gave the best results concerning the regioselective synthesis of the $\beta(1 \rightarrow 2)$ regioisomer **6** among the enzymes tested, although the yield was low. However, it should be noticed that the reaction stopped around 70–80% transformation of ONPG (Fig. 1E, Fig. 2E). Besides, commercial preparations of this enzyme contained a high proportion of glycerol (50%) as stabilising agent. Although the enzyme preparation was diluted 64-fold for the assays, glycerol still competed favourably with both xylose enantiomers in the transgalactosidation reaction. In fact, a mixture of 1-*O*- and 3-*O*-galactopyranosyl-D-glycerol, identified by means of the NMR spectra of their peracetylated derivatives, was the major component among the reaction products (see Fig. 1E, Fig. 2E). A better yield may result if glycerol were removed from the enzyme preparation (e.g., by dialysis). Finally, the bacterial β-galactosidase from *E. coli* has been extensively studied in transgalactosidation reaction [5,8,9,16]. It is well known its high selectivity towards the HO-6 group of glucosides and galactosides [12,25] although this selectivity can be altered drastically depending on the structure of the acceptor [7,9,10]. With D-Xyl which lacks a primary hydroxyl, the *E. coli* β-galactosidase catalysed the synthesis of the $\beta(1 \rightarrow 4)$ regioisomer **1** with the highest selectivity (81%) and efficiency (43% yield of **1**) of all the enzymes tested in this work. On the other hand, the regioselectivity observed with L-Xyl decreased and the $\beta(1 \rightarrow 4)$ regioisomer **4** (43%) is only slightly preferred over the others (**5** 33% and **6** 24%).

The selectivity to any of the secondary OH groups of either D- or L-Xyl is not complete with any of the enzymes studied in this work. However, it is possible to select one of those enzymes in order to prepare enriched mixtures in each one of the six disaccharides. For example, the reactions with *E. coli* and bovine testes enzymes are the most selective and efficient cases for the preparation of **1** (81% selectivity, 43% yield) and **2** (85% selectivity, 39% yield), respectively, **4** and **5** can be obtained with significant regioselectivity (64% in both cases) using *A. oryzae* enzyme and intestinal lactase, respectively, for the preparation of **6** the *S. fragilis* galactosidase (53% selectivity) can be chosen and, finally, **3** is slightly preferred (49% selectivity) when *A. oryzae* galactosidase is used.

The present results indicate that the choice of the enzyme is an easy and direct way to modulate the regioselectivity of the transglycosidation. At this moment, and considering the results from this work and from the literature, there is not a clear way to predict the behaviour of one enzyme in transglycosidation reactions with a given acceptor. Therefore, a first screening of enzymes should be carried out. Once an enzyme is selected for the synthesis of a given product, the improvement in yield and regioselectivity could be accomplished by optimising the reaction factors.

3. Experimental

L-Xyl and D-Xyl were commercial products obtained from Aldrich. *O*-Nitrophenyl-β-D-galactopyranoside (ONPG) and the β-galactosidases from *E. coli*, *A. oryzae*, Bovine liver, Bovine testes, and *S. fragilis* were purchased from Sigma. Lactase-phloridzin hydrolase (LPH) was isolated from lamb small-intestine as described earlier [20].

One unit of activity is the amount of enzyme that liberate one μmol of *o*-nitrophenol from ONPG per min at 37 °C in the corresponding buffer: 50 mM sodium phosphate–1 mM magnesium chloride–0.5 mM β-mercaptoethanol (pH = 7) for *E. coli* galactosidase, 50 mM sodium phosphate–2.5 mM magnesium chloride–0.5 mM β-mercaptoethanol (pH = 7) for *S. fragilis* galactosidase, 100 mM sodium phosphate (pH = 7.3) for bovine liver enzyme, 100 mM sodium acetate–acetic acid (pH = 4.5) for bovine testes galactosidase, 50 mM sodium phosphate (pH = 5.9) for LPH and 100 mM pyridine–26 mM acetic acid (pH = 5.8) for *A. oryzae* galactosidase.

GC was performed on a capillary column SE-54 (15 m) using a Hewlett Packard capillary gas chromatograph. A flow rate of 1 mL/min of nitrogen and a flame detector were utilised (temperature program: initial temperature 160 °C; initial time 2 min; rate 5 °C/min; final temperature 250 °C). HPLC was performed on a semipreparative normal phase column μPorasil (Waters) using a HPLC Waters System with refraction index detector.

*4-, 3- and 2-*O-β-D-*galactopyranosyl-*D-*xylose* (**1**, **2** *and* **3**).—The same procedure as described earlier [16,26] was applied to obtain **1** as the major product. Compounds **2** and **3** were prepared in a similar way but using the β-galactosidase from *A. oryzae* that gave a disaccharide mixture enriched in **2** and **3** (**1**:**2**:**3** 1:9:10). Spectroscopic and physical data of the products **1**, **2**, and **3** were identical to those described.

4-, 3- and 2-O-β-D-galactopyranosyl-L-xylose (**4**, **5**, *and* **6**).—(A) Using β-galactosidase from *E. coli*. To a solution of ONPG (1 g, 32 mM) and L-Xyl (5 g, 320 mM) in buffer (pH = 7.0) was added β-galactosidase from *E. coli* (0.4 mg, 320 U/mg), and the mixture was incubated at 37 °C for 50 min. The reaction was stopped by heating for 10 min at 100 °C. The solution was concentrated and fractionated on a carbon/celite column [27] (elution gradient water → 85:15 water–ethanol) or a Bio-Gel P2 column (water as eluent), thus, separating the mixture of disaccharides **4**, **5** and **6** (445 mg, 2:1.2:1 proportion, 43% relative to initial ONPG) from D-Gal and the excess of L-Xyl. The fraction containing **4**, **5** and **6** was acetylated with Ac_2O–pyridine to give a mixture of **13**, **14**, **15**, **16**, **17** and **18** that were subsequently purified by semipreparative normal phase HPLC chromatography. In a first step using 55:45 hexane–EtOAc as eluent **15**, **16** and a mixture of **13**, **14**, **17** and **18** were obtained. When 1:4 toluene–ethyl ether was applied to this last fraction, **13** and **14** could be separated and different fractions enriched in either **17** or **18** were obtained. See Table 1 for ^{1}H NMR data.

Deacetylation of each anomeric pair **13–14**, **15–16** and **17–18** gave pure **4**, **5** and **6**, respectively.

Compound **4**: ^{1}H NMR (500 MHz, D_2O, δ 4.76): δ 5.17, 4.57 (2 d, 1 H, $J_{1,2}$ 3.7 and 7.9 Hz, H-1α,1β), 4.56 (d, 1 H, $J_{1',2'}$ 7.6 Hz, H-1′α,1′β), 4.16 (dd, $J_{5eq,4}$ 5.5 Hz, $J_{5eq,5ax}$ 11.9 Hz, H-5eqβ), 3.92 (dd, 1 H, $J_{4',3'}$ 3.5 Hz, $J_{4',5'}$ 1.1 Hz, H-4′α,β), 3.78–3.67 (m, 5 H), 3.65 (dd, 1 H, $J_{3',2'}$ 9.8 Hz, $J_{3',4'}$ 3.5 Hz, H-3′α,β), 3.60 (t, $J_{3,2} = J_{3,4}$ 9.3 Hz, H-3β), 3.56 (dd, $J_{2,1}$ 3.7 Hz, $J_{2,3}$ 9.5 Hz, H-2α), 3.53 (dd, 1 H, $J_{2',1'}$ 7.6 Hz, $J_{2',3'}$ 9.8 Hz, H-2′α,β), 3.37 (dd, $J_{5ax,4}$ 10.4 Hz, $J_{5ax,5eq}$ 11.9 Hz, H-5axβ), 3.26 (dd, $J_{2,3}$ 9.3 Hz, $J_{2,1}$ 7.9 Hz, H-2β); ^{13}C NMR (200 MHz, D_2O): δ 105.2 (C-1′α,β), 97.7 (C-1β), 93.2 (C-1α), 80.0, 79.9 (C-4α,β), 76.4, 76.0, 75.2, 73.9, 73.0, 72.5, 72.3, 69.8, 65.9, 62.2, 61.6. Anal. Calcd. for $C_{11}H_{20}O_{10} \cdot H_2O$: C, 40.00; H, 6.71. Found: C, 40.10; H, 6.54.

Compound **5**: ^{1}H NMR (400 MHz, D_2O, δ 4.76): δ 5.21, 4.6 (2 d, 1 H, $J_{1,2}$ 3.6 and 7.9 Hz, H-1α,1β), 4.67, 4.65 (2 d, 1 H, $J_{1',2'}$ 7.8 and 7.9 Hz, H-1′α,1′β), 3.92 (dd, $J_{5eq,4}$ 5.6 Hz, $J_{5eq,5ax}$ 11.1 Hz, H-5eqβ), 3.90 (d, 1 H, $J_{4',3'}$ 3.3 Hz, H-4′α,β), 3.89–3.69 (m, 6 H), 3.66, 3.65 (2 dd, 1 H, $J_{3',2'}$ 10.0 Hz, $J_{3',4'}$ 3.3 Hz, H-3′α,3′β), 3.61 (dd, $J_{2,1}$ 3.6 Hz, $J_{2,3}$ 9.2 Hz, H-2α), 3.57, 3.56 (2dd, 1H, $J_{2',1'}$ 7.9 Hz, $J_{2',3'}$ 10.0 Hz, H-2′α,2′β), 3.33 (t, $J_{5ax,4} = J_{5ax,5eq}$ 11.1 Hz, H-5axβ), 3.31 (dd, $J_{2,1}$ 7.9 Hz, $J_{2,3}$ 9.0 Hz, H-2β). ^{13}C NMR (200 MHz, D_2O): δ 103.7, 103.5 (C-1′α,β), 96.9 (C-1β), 92.5 (C-1α), 84.5 (C-3β), 82.1 (C-3α), 75.8, 73.1, 71.7, 70.6, 69.3, 69.2, 69.1, 65.3, 61.5, 61.3. Anal. Calcd. for $C_{11}H_{20}O_{10} \cdot H_2O$: C, 40.00; H, 6.71. Found: C, 39.55; H, 5.92.

Compound **6**: ^{1}H NMR (500 MHz, D_2O, δ 4.7): 5.35, 4.73 (2 d, 1 H, $J_{1,2}$ 3.0 and 7.8 Hz, H-1α,1β), 4.69, 4.50 (2 d, 1 H, $J_{1',2'}$ 7.3 and 7.8 Hz, H-1′α,1′β), 3.93 (dd, $J_{5eq,4}$ 5.9 Hz, $J_{5eq,5ax}$ 10.8 Hz, H-5eqβ), 3.91 (d, $J_{4',3'}$ 3.9 Hz, H-4′α,β), 3.83–3.64 (m, 7 H), 3.61–3.47 (m, 3 H), 3.33 (t, $J_{5ax,4} = J_{5ax,5eq}$ 10.8 Hz, H-5axβ). ^{13}C NMR (200 MHz, D_2O): δ 103.5, 103.1 (C-1′α,β), 97.6 (C-1β), 91.7 (C-1α), 81.4 (C-2β), 79.7 (C-3α). Anal. Calcd. for $C_{11}H_{20}O_{10} \cdot H_2O$: C, 40.00; H, 6.71. Found: C, 39.89; H, 6.55.

(B) Using β-galactosidase from *A. oryzae*. ONPG (1 g, 32 mM) and L-Xyl (5 g, 320 mM) were incubated in buffer (pH = 5.8). The reaction procedures were the same as with *E. coli*. The yield of disaccharides was 39% (404 mg) relative to initial ONPG, obtaining **4**, **5** and **6** in 16:5:1 proportion.

(C) Using β-galactosidase from *S. fragilis*. Following the same procedure as before, with 5.6 mg of the enzyme (7 U/mg), 309 mg (30%) of a mixture of **4**, **5** and **6** (1:3:5) was obtained. In this case, a mixture of 1-*O*- and 3-*O*-galactopyranosyl-D-glycerol, identified by NMR, was isolated after the carbon/celite column. This mixture came from the reaction of ONPG and D-glycerol present in 50% in the commercial enzyme preparation as a stabilising agent.

Transglycosidation reaction analysis.—The following general procedure was applied at least in duplicate with the six enzymes assayed: To a solution of *o*-nitrophenyl-β-D-galactopyranoside (32 mM) and D- or L-xylose (320 mM) in 125 μL of the corresponding buffer, was added β-galactosidase. The amount of enzyme was adjusted to obtain 0.2–0.7 μmol of ONPG transformation per min per mL under the reaction conditions. The mixture was left at 37 °C during 2–8 h (owing to the high value of the bovine testes β-galactosidase, this enzyme was used at a lower concentration and its reaction was followed during a larger period of time). At different time intervals, 10 μL aliquots were taken, frozen, lyophilised, silylated and analysed by GC. The incubation mixture contained 0.4 mM mannitol as internal standard for the GC analysis.

GC analysis: Each aliquot residue was dissolved in pyridine (30 μL) containing 1 mM of benzyl xylopyranoside as reference. Trimethylsilyl imidazole (30 μL) was added, the mixture was kept at 60 °C for 30 min and then injected in the GLC system. The amount

of each product was measured using calibration curves previously obtained in the same conditions. The yields were calculated at the point when the formation of the disaccharides was the highest.

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