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The effects of organic solvents on the synthesis of galactose disaccharides using β-galactosidases

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Abstract

The use of β-galactosidases from five different sources in syntheses through transglycosylation leads to 3-*O*-methyl *allo*lactose, *N*-acetyllactosamine, *N*-acetyl*allo*lactosamine and Gal(β1-6)Gal. The yield and distribution of products depends on the source of the enzyme and on the reaction conditions, viz. the nature of added organic co-solvent. Yields of 3-*O*-methyl *allo*lactose (47%), Gal(β1-6)Gal (6%), *N*-acetyl*allo*lactosamine (30%) were obtained in aqueous buffer with β-galactosidase from *E. coli*. The same reactions occurred at much lower rates in the presence of water-miscible organic solvents. With β-galactosidases from *K. fragilis* and *A. oryzae*, however, the synthesis of the above disaccharides occurred only in the presence of organic solvent (> 60% v/v triethyl phosphate, trimethyl phosphate or tetraglyme) but not in aqueous buffer solution. β-galactosidases from *D. pneumoniae* and *B. circulans* in systems incorporating organic solvent produced 3-*O*-methyl *allo*lactose and *N*-acetyllactosamine in yields of 30–40%. The direct separation of oligosaccharides on a preparative scale can be achieved by Ca^{2+}-ligand exchange chromatography. Ultrafiltration was also used for the efficient recycling of enzymes. © 1997 Elsevier Science Ltd.

Keywords: β-Galactosidases; Disaccharide; Organic solvent; Enzymatic synthesis

1. Introduction

The realisation of the importance of substances containing β-D-Gal in biological systems has led to the demand for the production of these substances in sufficient amounts for applications and fundamental studies [1]. The use of enzymes as an alternative to chemical methods for the synthesis of glycosidic linkages has seen a revival of interest because of the potential advantages of simplicity (no blocking groups), anomeric stereoselectivity and cost [2–5]. Of the various approaches kinetic transglycosylation catalysed by glycosidases has seen a number of applications to the synthesis of β-galactosides [6–27], yields of 2–30% are generally reported [5]. A particular tactic, and the subject of this work, is to carry out

Abbreviations: ONPGal, *o*-nitrophenyl-β-D-galactopyranoside; PNPGal, *p*-nitrophenyl-β-D-galactopyranoside; ACN, acetonitrile; DEG, diethyleneglycol; MEA, 2-methoxyethyl acetate; TEG, triethyleneglycol; TEP, triethyl phosphate; TMP, trimethyl phosphate; TSP, sodium 3(trimethylsilyl)propionate-d_4

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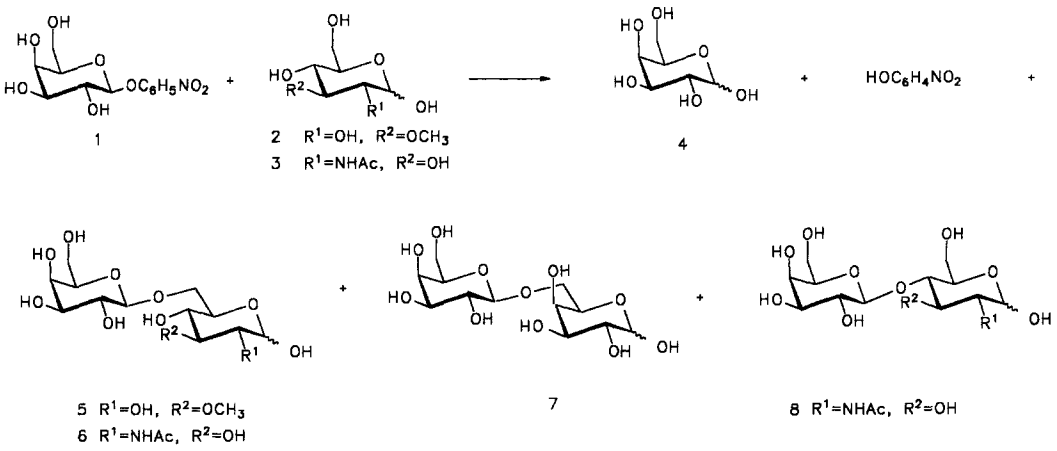
reactions in media of reduced water content with the aim of increasing the yield by reducing the extent of competing hydrolysis (kinetic transglycosylation) or altering the position of equilibrium (reverse hydrolysis) [28], or enabling the dissolution of hydrophobic acceptors such as sterols [18] or aromatic alcohols [29]. The difficulty with this approach in the case of oligosaccharide synthesis is that the substrates require solvents of high polarity and that such solvents often cause enzyme deactivation [30]. Reductions in yield [17,31] and increased reaction times [32] have been reported, and it has been observed that the effect of added solvent is generally not beneficial [4,5]. Nevertheless successful applications have been reported in e.g. 50% acetonitrile [19], 70% acetone [18], 75% acetonitrile [18], 42% DMF [13], 23% DMF [16], 60% diethyleneglycol diethylether [20] and 90% acetonitrile [28]. In fact there are a number of correlations which can be used to predict the denaturing tendency of water-miscible organic solvents, but serious and unpredictable deviations can occur [33,34]. In general the choice of solvent composition for applications of glycosidases has been empirical or based on experiment [18,20] rather than theory. In order to define and extend the range of conditions which might usefully be applied to synthesis using glycosidases we have measured the activities of three different β-galactosidases in a number of homogeneous water-solvent mixtures [35]. The results suggested that in particular cases enzyme activity is maintained in the presence of tetraglyme, trimethyl phosphate, triethyl phosphate or acetonitrile, and therefore that these solvents might be usefully incorporated in synthesis. Here we report on the effects of such solvents on syntheses catalysed by five β-galactosidases using *o*-nitrophenyl galactoside as donor and 3-*O*-methyl Glc or GlcNAc as acceptors (see Scheme 1). 3-*O*-methyl-4-*O*-β-D-galactosyl-D-glucose is a potential substrate for intestinal lactase for the diagnosis of hyperlactasia [36,37].

2. Experimental

Materials.—Chemicals were purchased from Sigma and Aldrich (Dorset, UK) and were used without further purification, unless otherwise stated. Dry solvents were prepared by standard methods [38]. ONPGal [39], 3-*O*-methyl Glc [40] and GlcNAc [41] were synthesised by methods described in the literature. β-galactosidases from *E. coli* (grade VIII 95% purity), *Bacillus circulans* and *Diplococcus pneumoniae* were purchased from Sigma, Daiwa Kasei and Boehringer Mannheim; and β-galactosidases from *Kluyveromyces fragilis* ('Lactozyme 3000L, Type HP-G, High Purity in Glycerol') and *Aspergillus oryzae* ('Takamin Brand Fungal Lactase') were gifts from Novo Nordisk and Solvay Enzymes respectively. A 50 mM imidazole/HCl buffer containing 1 mM $MgCl_2$, 145 mM NaCl and 5 mM dithiothreitol at pH 7.0 (buffer A), pH 6.5 (buffer B) and 50 mM NaOAc/acetic acid buffer pH 4.5 (buffer C), were used for *E. coli*, *K. fragilis* and *A. oryzae* respectively. β-Galactosidase from *K. fragilis* was dialyzed against buffer B overnight at 4 °C. β-Galactosidase from *A. oryzae* containing starch was purified with isopropanol [42] and the carbohydrate content was determined by phenol-sulphuric acid assay.

General methods.—One unit of enzyme activity is that amount which will liberate 1 μmol of *o*-nitrophenol from ONPGal per minute under the given



Scheme 1. The formation of disaccharides with β-galactosidase.

conditions. ^{1}H and ^{13}C NMR spectra were recorded in D_2O either on a Bruker WH-400 spectrometer (^{1}H, 400.13 MHz; ^{13}C, 100.0 MHz) or a Bruker AMX 600 spectrometer (^{1}H, 600.14 MHz; ^{13}C, 150.92 MHz) with TSP as reference. Peak assignments were based on COSY and DEPT-experiments. Optical rotations were measured on a Perkin–Elmer 241 automatic polarimeter. FABMS was performed on a VG ZAB-SE instrument. An HPLC equipped with a JASCO PU-980 Intelligent HPLC pump and a differential refractometer (Waters Associates R403) was used with a Shodex Asahipak NH2P-50 column (4.6 mm∅ × 250 mm) and Dionex equipment (Pulsed amperometic detector, CarboPac PA1, or PA100 column, 4 × 250 mm, elution with water–NaOH) was used for analytical scale, or a cation exchange column (8 × 500 mm, Dowex-50W X4, 200–400 mesh, Ca^{2+} form) for preparative scale liquid chromatography. *o*-Nitrophenol was extracted from reaction mixtures using *t*-butyl methyl ether in a continuous liquid–liquid extractor. Ultrafiltration was carried out in an Amicon apparatus with a YM 10 membrane (MW cut-off > 10 kDa).

*3-O-methyl-6-O-β-*D*-galactopyranosyl-*D*-glucose* (**5**).—(A) Using β-galactosidase from *E. coli*. To a solution of ONPGal (**1**, 0.1 g, 0.33 mmol) and 3-*O*-methyl Glc (**2**, 0.5 g, 2.6 mmol) in 2 mL of buffer A was added β-galactosidase from *E. coli* (40 units) with stirring. Samples of 0.02 mL were taken at various time intervals over a period of 72 h and diluted with 0.8 mL methanol. Each sample was heated at 100 °C for 5 min, and made up to 1 mL with deionized water. A 25 μL aliquot was examined by HPLC (Dionex). For a semi-preparative scale, the mixture was after 10 h reaction time, continuously extracted for 48 h, while gently heated, with *tert*-butyl methyl ether until most of the 2-nitrophenol had been removed. After freeze-drying, a 30 mg sample of the reaction mixture in water (0.5 mL) was applied to the Ca^{2+} form of a cation exchange chromatography column and eluted with deionized water at a flow rate of 0.2 mL/min at 75 ± 0.5 °C, to give **1** (8.5 mg, 28.3% based on ONPGal added); MS: *m/z* 379.1 $[M + Na]^+$: NMR data are listed in Table 1.

Different mole ratios of donor to acceptor (1:2, 1:4, and 1:8) and various proportions of diglyme [20%, 50%, 60%, 70%. and 80% (v/v)] were also used.

(B) Using β-galactosidase from yeast (*Kluyveromyces fragilis*). β-galactosidase purified from *K. fragilis* (0.05–1.0 mL, 1.6–32 units) was applied in buffer B (total volume 2 mL). Reaction conditions (5 h), and procedures were as above. Organic solvent-buffer cosolvent systems (67% v/v) incorporating tetraglyme, TMP, TEP and acetonitrile were used.

(C) Using β-galactosidase from fungal lactase (*Aspergillus oryzae*). The partly purified enzyme (28 FCC units) was used with the same donor and acceptor in buffer C (2 mL). In this case methoxyethyl acetate (77% v/v) and TMP (77% v/v) were used as cosolvents.

(D) With β-galactosidase from *B. circulans* and *D. pneumoniae*. These enzymes (10U and 1U respectively) were used with the donor and acceptor as above in buffer C with 60% TEP (2 mL).

*6-O-β-*D*-galactopyranosyl-*D*-galactopyranose* (**7**). —Under the conditions described above one other product was found in reactions catalysed by *K. fragilis* and *E. coli*, it was separated by liquid chromatography and identified as **7**.

Table 1
^{13}C NMR chemical shifts (ppm) of D-galactosyl disaccharides **5–8**

Disaccharide	Residue	C-1	C-2	C-3	C-4	C-5	C-6	CH_3	C=O
5	Gal	106.3	73.8	75.6	71.8	78.1	64.0		
	3-*O*-MeGlc-α	95.1	73.5	85.5	71.6	76.3	71.4	63.0	
	3-*O*-MeGlc-β	98.0	73.7	88.2	71.6	77.7	71.6	62.7	
6	Gal	106.2	73.6	75.5	71.5	78.0	63.7		
	GlcNAc-α	93.7	56.9	73.5	72.8	73.5	71.5	25.0	177.6
	GlcNAc-β	97.8	59.5	76.6	72.6	77.8	71.5	24.8	177.4
7	Gal	105.9	71.9	73.5	71.5	77.9	63.7		
	Gal-α	95.1	71.3	72.1	71.3	75.4	71.0		
	Gal-β	99.2	71.7	74.5	71.3	76.5	71.0		
8	Gal	106.2	73.4	75.4	71.0	78.3	61.2		
	GlcNAc-α	93.5	56.5	71.2	81.7	73.0	60.8	25.0	177.6
	GlcNAc-β	97.6	59.3	75.3	81.4	77.8	60.8	24.8	177.4

2-acetamido-2-deoxy-4-O-β-D-galactopyranosyl-D-glucose (**8**).—PNPGal (0.1 g, 0.33 mmol) and GlcNAc (**3**, 0.2 g, 0.99 mmol) in buffer C incorporating 60% TEP (2 mL) with β-galactosidase from *B. circulans* (10 units) or *D. pneumoniae* (20% TEP, 1 unit) at 37 °C for 2 h.

2-acetamido-2-deoxy-6-O-β-D-galactopyranosyl-D-glucose (**6**).—(A) Using *E. coli* β-galactosidase. ONPGal (0.1 g, 0.33 mmol) and GlcNAc (0.56 g, 2.6 mmol) in buffer A (2 mL) were incubated with β-galactosidase from *E. coli* (22 units) at 25 °C. Samples were withdrawn for analysis during 72 h, after which the reaction mixture was separated by HPLC to give, after freeze-drying, **6** (34 mg, 30% based on ONPGal: *m/z* 401 $[M + Na]^+$.

(B) Using *K. fragilis* β-galactosidase. A dialized solution of β-galactosidase from *K. fragilis* (32 units) was used with the same donor and acceptor in 60% (v/v) TMP (2 mL) at 25 °C for 5 h. Reaction conditions and procedures were as used for *E. coli*.

3. Results and discussion

Fig. 1a shows the HPLC chromatogram of a reaction mixture containing ONPGal and 3-*O*-methyl glucose (donor:acceptor ratio 1:8), with *E. coli* β-galactosidase after 50 h. The major product peaks 3-*O*-methyl-*allo*lactose (5.78 min) and Gal(β1-6)Gal (7.58 min) were identified by isolation, NMR and mass spectrometry (see Experimental and Table 1). In particular, the positions of linkage in the disaccharides were readily assigned from the ^{13}C chemical shifts. Other products with longer retention time were formed in very small amounts. Fig. 2 shows the percentages of reaction components present as a function of time. The yield of the major kinetic product, 3-*O*-methyl *allo*lactose, reached a maximum of 47% based on the amount of donor after 6 h and declined thereafter; the concentrations of donor and acceptor showed corresponding decreases during the same period. Thereafter there were slower increases in the concentrations of Gal and 1,6-digalactose as 3-*O*-methyl *allo*lactose was hydrolysed. As anticipated, the yield of this disaccharide was increased by using a higher amount of acceptor, but not usefully beyond an initial ratio to donor of 4:1. A similar result was observed when GlcNAc was the galactosyl acceptor, in this case a maximum yield of 30% of *allo*lactosamine was obtained. The incorporation of organic solvents into reactions catalysed by the *E. coli* enzyme was not beneficial, decreases in reaction rate and reduced yields were observed. Thus when vari-

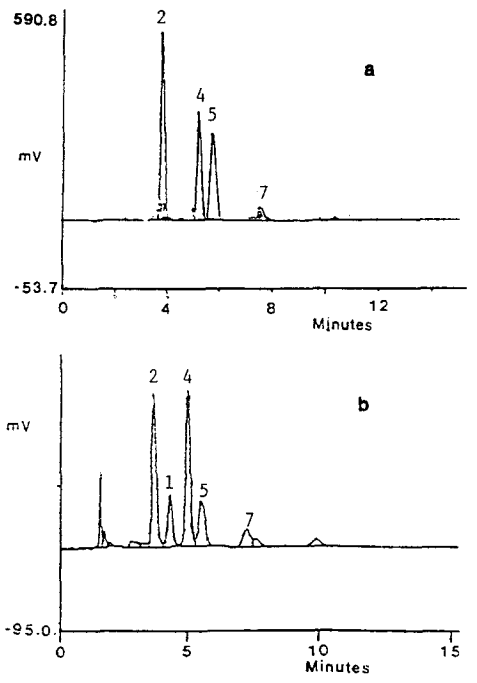


Fig. 1. HLPLC chromatograms of reaction mixtures: (a) ONPGal + 3-*O*-methylglucose (initial mol ratio 1:8) + *E. coli* enzyme in buffer, after 50 h; (b) ONPGal + 3-*O*-methylglucose (initial mol ratio 1:2) + *K. fragilis* enzyme in 67% TEP, after 1 h.

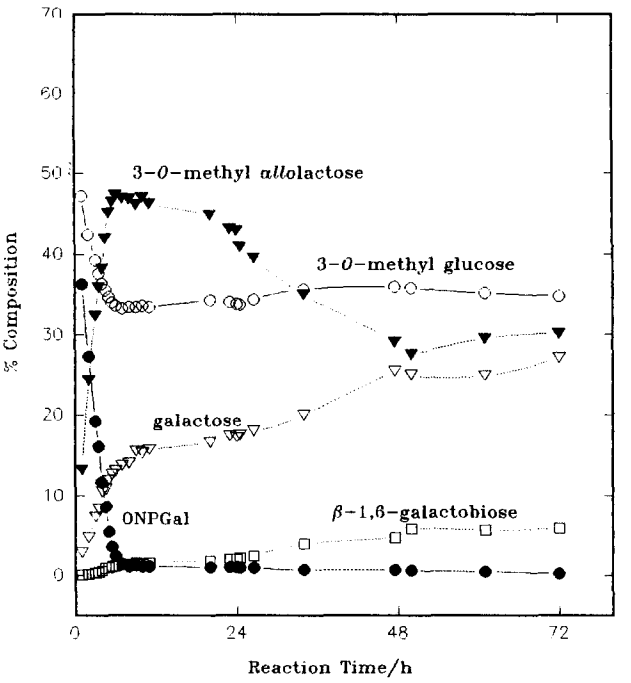


Fig. 2. Plots of % composition vs time for reaction of ONPGal with 3-*O*-methylGlc (initial mol ratio 1:8) catalysed by β-galactosidase from *E. coli*.

ous concentrations of diglyme [20–80% (v/v)] were used to form 3-*O*-methyl *allo*lactose with *E. coli* β-galactosidase, as the concentration of diglyme was increased, the reaction became progressively slower and gave smaller yields of disaccharides. There was some indication that the rate of loss of product through hydrolysis was reduced in the presence of a cosolvent, but no indication that the maximum yield could be improved (see Table 2).

Using an ultrafiltration apparatus, the same product yields were obtained as with the single batch system, in the first run. During subsequent runs, however, the product yields decreased. Even so, the ultrafiltration technique could be used up to 10 times without replacement of the enzyme sample. It is thus a useful method because the enzyme can be used as a recoverable catalyst, allowing further purification of products without heating the enzyme solution and the removal of proteinaceous material.

When β-galactosidase from *K. fragilis* was applied in aqueous buffer, ONPGal was simply hydrolysed by the enzyme, and no transfer to acceptor was observed. Since hydrolytic enzyme activity in aqueous-organic solvent mixtures had been observed in our previous work, TMP, TEP, TEG and acetonitrile in mixtures with the same buffer were used as reaction media and the reaction mixtures analysed. The addition of organic co-solvents did divert the reaction from hydrolysis to synthesis to some extent, a yield of 13.5% of *allo*lactose was obtained in 67% TEP (see Fig. 1b and Table 2). A similar observation was made when the enzyme from *A. oryzae* was used, no disaccharide was obtained in purely aqueous buffer under the conditions used, but 11% could be isolated from a reaction conducted in 77% TMP. The galactosyl transfer properties of the enzymes from *E. coli* and *K. fragilis* were rather different with regard to the formation of 1,6-digalactose; catalysis by the *E. coli* enzyme produced it at a low rate (e.g. 6% after 48 h) presumably by reversion, whereas under catalysis by the *K. fragilis* enzyme it was a kinetic product (6% after < 1 h, 1% after 16 h).

The β-galactosidases from *B. circulans* [25,42–47] and *D. pneumoniae* [48] were examined because of

Table 2
Reactants, conditions and products in enzymic syntheses

Enzyme	Solvent	Acceptor	Donor/acceptor ratio	Reaction time (h)	Product (yield %)
E. coli	buffer A	**2**	1:2	6	**5** (36)
			1:4		(45)
			1:8		(47)
			1:8	48	**7** (5.9)
		3	1:2	1	**6** (23)
			1:4		(28)
			1:8		(30)
			1:12		(26)
			1:16		(22)
	20% DIG	**2**	1:4	55	**5** (44)
	50% DIG			144	(20)
	60% DIG				(12)
	70% DIG				(4.4)
	80% DIG				(2.0)
K. fragilis	buffer B	**2, 3**	1:2	96	nil
	67% TEP	**2**	1:2	< 1	**5** (14), **6** (5.6)
	67% TMP	**2**	1:2	< 1	**5** (7.5), **6** (3.5)
	67% TEG	**2**	1:2	< 1	**5** (4.6), **6** (1.7)
	67% ACT	**2**	1:2	< 1	nil
	60% TMP	**3**	1:2	< 1	**6** (12)
A. oryzae	77% TMP	**2**	1:2	< 1	**5** (11)
	77% MEA	**2**	1:2	< 1	nil
B. circulans	60% TEP	**2**	1:3	2	**5** (40)
		3	1:3	2	**8** (37), **5** (6)
D. pneumonii	60% TEP	**2**	1:3	2	**5** (33)
	20% TEP	**3**	1:3	2	**8** (31)

reports that they show regioselectivity in galactosyl transfer for the formation of a (1,4)- rather than a (1,6)-linkage. In accord with this expectation, with GlcNAc as acceptor with the *B. circulans* enzyme, *N*-acetyllactosamine (37% yield) was formed exclusively after 1 h; thereafter the formation of a minor amount of *N*-acetyl*allo*lactosamine was observed. This result was also obtained with lactose as galactosyl donor, *N*-acetyllactosamine was obtained in a yield of 23.2%; prolonged incubation reduced the yield in favour of the *allo* isomer. However, with 3-*O*-methyl glucose as acceptor in 60% TEP, the 1,6-linkage was preferentially formed. A recent study of galactosyl transfer catalysed by the *B. circulans* enzyme concluded that selectivity towards C-4 of the acceptor required the presence of a 2-acetamido substituent [49]. When *D. pneumoniae* β-galactosidase was used as the catalyst in 20% TEP *N*-acetyllactosamine was obtained a yield of 33%, a significant improvement on that (12%) reported [46] in 20% Me_2SO. In our previous work we observed that the activities of *E. coli*, *A. oryzae* and *K. fragilis* β-galactosidases were lost rather rapidly in Me_2SO [35]. We conclude that a suitable choice of organic solvent can lead to an improved yield of the desired product. Recent results obtained with β-galactosidases from *Streptococcus* 66446K in 20% TEP and *Bifidobacterium bifidum* in 60% TEP support this contention [50].

From these experiments it appears that the main effect and potential advantage of introducing organic cosolvents in the synthesis of oligosaccharides by enzymic transglycosylation is a change in the proportion of condensation products rather than a change in regioselectivity e.g. in the cases of *A. oryzae* and *K. fragilis*. The results given above indicate that this applies in most cases, but to a limited extent. However the proportion of products at any given time will also depend on the nature of organic solvent present. This conclusion was also reached by Laroute et al. [51] who examined the glucoamylase-catalysed glucose condensation in 25 water-miscible and 9 water-immiscible solvents. The relative proportion of isomaltose to maltose formed (after a fixed time) varied from 0.56 (total yield 2.8% in 1,3-butanol) to 7.3 (total yield 29%, in diethylene glycol diethyl ether). The most important determinant of the nature and relative yields of products is the source of the enzyme. In the present work, the enzyme from *E. coli* produced 3-*O*-methyl *allo*lactose in aqueous buffer, but in the presence of organic solvents this reaction was very slow. This behaviour was completely reversed for *K. fragilis* β-galactosidase: disaccharides were produced relatively rapidly when organic cosolvent was present.

It is interesting to note that solvents which confer a high retention of hydrolytic stability on the enzyme e.g. acetonitrile (67% v/v) for *K. fragilis* or MEA (77% v/v) for *A. oryzae* [35] did not necessarily aid the synthesis of disaccharides. On the other hand, in TMP (67% v/v) and TEP (67% v/v), media in which the activity of β-galactosidase from both these sources was reasonably stable, the degree of disaccharide synthesis was high. The reason for this behaviour is not immediately obvious, and it appears that solvents can have different effects on different reactions catalysed by the same enzyme.

The ratio of donor to acceptor is important in determining the yields of desired products. In general, the higher the ratio of acceptor to donor, the higher the yield of condensation products. Absolute substrate concentrations affect both the yields of products and their distribution over the incubation time. These results indicate that the optimum amount of enzyme is sensitive to solvent conditions, substrate concentrations and donor:acceptor ratios and it is essential to determine these conditions in order to increase synthesis and reduce hydrolysis. In general, an increased amount of enzyme is required for synthesis of oligosaccharides if organic cosolvent systems rather than aqueous buffer are used. The suitable donor to acceptor ratio, the amount of enzyme and the amount of organic solvent have to be determined by trials.

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