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Immobilised β-galactosidases and their use in galactoside synthesis

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Abstract

The immobilisation of the commercially available β-galactosidases from *Aspergillus oryzae* and *Kluyveromyces fragilis* has been investigated and optimised. The enzymes were adsorbed on phenol-formaldehyde resins of the Duolite type and subsequently stabilised by crosslinking. The *K. fragilis* enzyme required $MgCl_2$ to maintain its activity. Immobilisation yields from 23% (*K. fragilis* on Duolite A-7) to 54% (*A. oryzae* on Duolite S-761) were obtained. The preparations of *A. oryzae* β-galactosidase catalysed the condensation of galactose and glycerol without addition of water, whereas the native enzyme was inactive. A number of galactatosyl donors were subjected to glycerolysis in the presence of immobilised *A. oryzae* and *K. fragilis* β-galactosidase and the effect of the leaving group and the water content of the reaction medium were investigated. *A. oryzae* β-galactosidase on Duolite S-761 was active without additional water, whereas *K. fragilis* β-galactosidase required a substantial amount of aqueous buffer in the reaction medium.

Keywords: Aspergillus oryzae; *Kluyvermyces fragilis*; Galactose; Glycoside

1. Introduction

Alkyl glycosides are ubiquitous in nature, particularly in plants. Alcohols, phenols and mercaptans exist in plants mainly as their glycosides and are, as such, precursors of natural aromas. Some glycosides also exhibit antimicrobial activity [1]. Alkyl glycosides have great potential as aroma precursors, nature-identical emulsifiers and biosurfactants but their application is hampered by the lack of efficient synthesis methodologies. The acid catalysed synthesis of alkyl glycosides (Fischer alkylation) generally results in a mixture of isomers in which, in the case of glucose or galactose, the α-pyranoside predominates [2]. Chemical methods for the synthesis of anomerically pure glycosides involve protection, activation and deprotection steps and are inherently circuitous [3]. In contrast, anomerically pure alkyl glycosides can be synthesised in one step from a carbohydrate precursor by the use of glycosidases, enzymes which in nature catalyze the hydrolysis of glycosidic bonds, in a reversed hydrolysis or alcoholysis mode.

The synthetic efficiency of such schemes is hampered, however, by the large amount of water that glycosidases need for activity in comparison with, e.g., lipases [4–8]. In reversed hydrolysis—the equilibrium controlled conden-

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sation of a monosaccharide and an alcohol (Reaction 1)—water shifts the equilibrium towards the left. In kinetically controlled transglycosylation (Reaction 2), water causes parasitic hydrolysis of the reactant as well as secondary hydrolysis of the product (see also Fig. 1).

$$\text{Glycosyl-OH} + R^1OH \rightleftharpoons \text{Glycosyl-O}R^1 + H_2O$$

$$\text{Glycosyl-O}R^1 + R^2OH \longrightarrow \text{Glycosyl-O}R^2 + R^1OH$$

In the analogous lipase catalysed esterification of carbohydrates, this problem was solved by the use of a stable, properly immobilised lipase at a water activity of approx. a_w 4×10^{-3} [9–11], whereas the β-glucosidase from almonds required a_w 0.4–0.8 [4–8]. With few exceptions [8,12,13], glycosidases have been used in a synthesis role as 'free' suspensions of lyophilisate, but we reasoned that immobilisation would significantly contribute to the stability of the catalyst at low a_w. Accordingly we have investigated and optimised the immobilisation of the commercially available β-galactosidases (EC 3.2.1.23) from *Kluyveromyces fragilis* and *Aspergillus oryzae* on macroporous phenol-formaldehyde resins; these preparations have been used in a subsequent study introduced below.

The nature of the glycosyl donor exerts an important effect on the yield because it should monopolise the biocatalyst for true kinetic control and in this way suppress the secondary hydrolysis of the product. Galactosyl donors that have been used in published procedures include lactose [14–21], methyl-β-D-galactoside [22], phenyl-β-D-galactoside [4,23,24], *o*-nitrophenyl-β-D-galactoside [25,26] and *p*-nitrophenyl-β-D-galactoside [27] but a full study comparing the glycosyl donors generally used in transglycosylations has never been made.

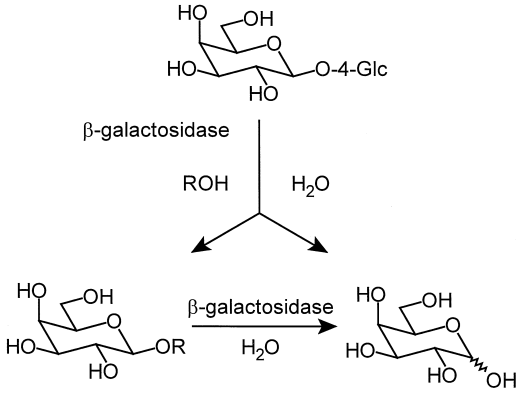

Fig. 1. Transgalactosylation and competing hydrolysis.

To clarify this point we have studied the use of the immobilised β-galactosidases described above in the glycerolysis [5] of a number of galactosides to compare the effects of the leaving group and the water content in the medium. The 1-*O*-β-D-galactopyranosylglycerol product is a useful intermediate for the synthesis of its 3-*O*-fatty acid esters and related emulsion- and liposome-forming galactolipids.

2. Experimental

2.1. Genereral methods

Kluyveromyces fragilis β-galactosidase (solution) was kindly donated by Novo Nordisk (Bagsværd, Denmark). *Aspergillus oryzae* β-galactosidase (lyophilised powder, 4 U/mg), phenyl-β-D-galactoside and methyl-β-D-galactoside were purchased from Sigma. Partially hydrated Duolite A-7 and Duolite S-761 (16–50 mesh particle size) were from Supelco and were used as received. *p*-Nitrophenyl-β-D-galactoside and *o*-nitrophenyl-β-D-galactoside were obtained from Aldrich, lactose monohydrate from Merck, galactose and 1,5-pentanedial (25% (w/w) solution in water) from Acros. Lactulose was received from Solvay-Duphar (Weesp, the Netherlands) as a gift.

Gas chromatography (GC) was performed on a Hewlett-Packard 5890 chromatograph, equipped with a 50 m $\times$ 0.32 mm CP-Sil 5CB column. The carrier gas was nitrogen. Temperature program: 60°C (5 min) to 280°C (10°C/min). Peaks were detected using FID and were integrated on a HP 3396A integrator. Samples (40 μl) were periodically taken from

the reaction mixture and diluted with *N,N*-dimethylformamide (0.5 ml). From this solution 40 μl was taken and treated with 0.5 ml of a stock solution of trimethylsilyllating reagent (104 ml pyridine, 26 ml *N,N*-bis(trimethylsilyl)trifluoroacetamide and 13 ml trimethylsilylchloride). The concentrations of reactants and product in the samples were calculated using independently measured response factors relative to glycerol, which was used as internal standard.

Analytical TLC was carried out on precoated Merck 60 F_{254} aluminium plates. The eluent was a mixture of MeOH and CH_2Cl_2 (2/8, (v/v)). Spots were detected by spraying with 10% (v/v) H_2SO_4 in MeOH and heating on a hot plate.

^1H and ^{13}C NMR spectra were recorded using a Varian-VXR 400S spectrometer. The spectra were analysed using Attached Proton Test (APT) and by comparing them with assigned spectra in the literature.

2.2. β-galactosidase immobilisation

Duolite A-7 (15 g, dry weight 10 g) or Duolite S-761 (16 g, dry weight 10 g) was soaked overnight in 0.1 M sodium acetate buffer pH 4.5 containing 0.1 M NaCl (buffer I). After the carrier had been filtered off and rinsed 4 times with 25 ml 0.1 M sodium acetate buffer pH 4.5 (buffer II), a solution of *Aspergillus oryzae* β-galactosidase (2 g, 4 U/mg) in buffer II (40 ml), was added. 0.01 M $MgCl_2$ was included in the buffer solutions as indicated in Table 1. The mixture was shaken for 4 h at room temperature and decanted. The supernatant was assayed for galactosidase activity as described below. The adsorbed enzyme was cross-linked with 1% 1,5-pentanedial (2.5 h) or dimethyl adipimidate (1 h) in buffer II (40 ml) at 0°C; subsequently the enzyme preparation was washed 5 times with 25 ml buffer II. *Kluyveromyces fragilis* β-galactosidase (3 g, 3.6 U/mg) was treated in the same way, except that 0.1 M sodium phosphate buffer pH 6.5 was used instead of acetate buffer. 0.1 M lactose was included during immobilisation and cross-linking as indicated in Table 1; after cross-linking it was removed by exhaustive washing with buffer.

The enzyme preparations were stored fully hydrated at +6°C; activities etc. are per gram of fully hydrated preparation. The activity of the immobilised enzyme preparations (Table 1) was determined by measurement of the rate of lactose hydrolysis in the appropriate buffer at the time that 20% or less lactose was hydrolysed at 40°C. One Unit (U) hydrolyses 1 μmol lactose in 1 min at 40°C.

Table 1
Immobilisation of the β-galactosidases from *Kluyveromyces fragilis* and *Aspergillus oryzae*[a]

Enzyme source	Carrier (Duolite type)	$MgCl_2$ (M)	Lactose (M)	Enzyme adsorbed (%)[b]	Cross-linker	Activity (U/g)	Yield[c] (%)	Efficiency[d] (%)
K. fragilis	A-7	–	–	n.d.	1,5-pentanedial	6	1	n.d.
K. fragilis	A-7	0.01	–	71	1,5-pentanedial	89	23	32
K. fragilis	S-761	0.01	–	36	1,5-pentanedial	11	4	11
A. oryzae	A-7	0.01	–	74	1,5-pentanedial	71	25	34
A. oryzae	S-761	–	–	94	1,5-pentanedial	116	46	49
A. oryzae	S-761	0.01	–	92	1,5-pentanedial	118	44	48
A. oryzae	S-761	0.01	0.1	92	1,5-pentanedial	167	53	58
A. oryzae	S-761	0.01	–	92	DMA	157	54	59
A. oryzae	S-761	0.01	0.1	92	DMA	150	50	54

[a] For procedure see experimental part.
[b] Calculated from the residual activity in the supernatant.
[c] Yield = $Units_{found}/Units_{in} \times 100\%$.
[d] Efficiency = $Units_{found}/Units_{adsorbed} \times 100\%$.

2.3. Condensation of galactose and glycerol

Galactose (0.3 mmol) was dissolved in glycerol (12 mmol) in a 10 ml vessel. *A. oryzae* β-galactosidase on Duolite S-761 or *K. fragilis* β-galactosidase on Duolite A-7 (40 U) were added and the mixture was shaken on a rotary table with a frequency of 3 s^{-1} at 40°C. At indicated time intervals, 40 μl samples were taken and analysed by GC as described above.

^{1}H NMR [28] and ^{13}C NMR [29] of 1-*O*-β-D-galactopyranosyl glycerol (D_2O, *tert*-butyl alcohol as reference) were in accordance with data in the literature.

2.4. Transgalactosylation with glycerol

The glycosyl donor—lactose, lactulose, methyl-β-D-galactoside, phenyl-β-D-galactoside, *o*-nitrophenyl-β-D-galactoside or *p*-nitrophenyl-β-D-galactoside—(0.3 mmol) was dissolved in glycerol (12 mmol) in a 10 ml vessel. *A. oryzae* β-galactosidase on Duolite S-761 or *K. fragilis* β-galactosidase on Duolite A-7 (30 U) and various amounts of 0.1 M sodium acetate buffer pH 4.5 which included 0.01 M $MgCl_2$ or 0.1 M sodium phosphate buffer pH 6.5 which included 0.01 M $MgCl_2$, respectively, were shaken on a rotary table with a frequency of 3 s^{-1} at 40°C. At indicated time intervals, 40 μl samples were taken and analysed by GC as described above.

3. Results and discussion

3.1. Immobilisation of β-galactosidases

Immobilisation and cross-linking of glycosidases is a standard technique to increase the life-time and the thermostability of the catalyst in industrial hydrolytic transformations. We wished to extend this technique to low-water media and, for that reason, were restricted to rigid carrier materials that retain their structural integrity under such conditions. We selected two macroporous phenol-formaldehyde resins: Duolite A-7, a weak anion exchanger and Duolite S-761, a neutral resin with methylol functionality. Both types of carrier have previously been successfully applied to the immobilisation of *A. niger* [30,31] and *A. oryzae* [32] β-galactosidase.

The β-galactosidases from *Kluyveromyces fragilis* and *Aspergillus oryzae* were adsorbed on the resins at their optimum pH (6.5 and 4.5, respectively) and subsequently treated with 1,5-pentanedial at 0°C. This latter procedure, which involves the linking of lysine NH_2 groups at the protein surface via intra- and intermolecular imine bonds (Fig. 2), prevents desorption of the catalyst and results in enhanced thermostability [33] (although with a penalty in activity).

From the results (Table 1) it becomes clear that the *K. fragilis* enzyme requires Mg^{2+} to be active. It has previously been reported that metal ions enhance both the activity and the thermostability of *K. fragilis* β-galactosidase [34]. It was much better absorbed on Duolite A-7 than on S-761 and the efficiency—a measure of the loss of activity due to interaction with the carrier as well as to cross-linking—was also much better with the former carrier. The β-galactosidase from *A. oryzae* was immobilised on Duolite A-7 with comparable result as regards yield and efficiency, but Duolite S-761 performed much better with the *A. oryzae* enzyme: adsorption was nearly quantitative and

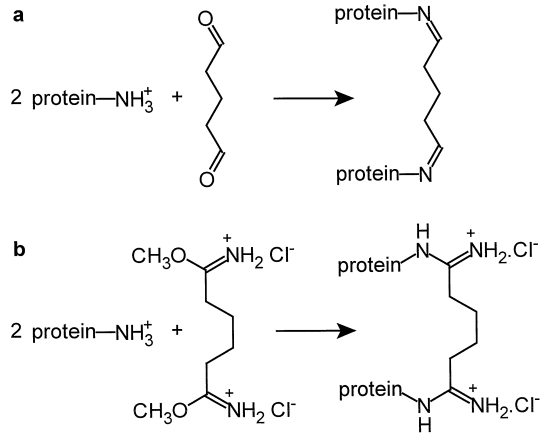

Fig. 2. Crosslinking of a protein, a: with 1,5-pentanedial: b: with dimethyl adipimidate (DMA).

Table 2
Condensation of galactose and glycerol catalysed by *A. oryzae* β-galactosidase[a]

Carrier	Lactose (M)	Cross-linking reagent	Activity (U/g)	Product (%)	Synthesis rate (nmol min^{-1} U^{-1})
none	–	–	4000	0	0.0
Duolite S-761	–	1,5-pentanedial	118	27	1.9
Duolite S-761	0.1	1,5-pentanedial	167	28	1.9
Duolite S-761	–	DMA	157	30	2.1
Duolite S-761	0.1	DMA	151	34	2.4

[a]Reaction conditions: galactose (0.3 mmol), glycerol (12 mmol), β-galactosidase preparation (40 U), 40°C, 18 h.

approx. 50% of the adsorbed activity was retained after cross-linking.

Because it is known that substrate or a competitive inhibitor will protect the active site during immobilisation and cross-linking [35] we performed the immobilisation of *A. oryzae* β-galactosidase on Duolite S-761 in the presence of 0.1 M lactose. Indeed the activity increased to 167 U/g compared to 118 U/g in the absence of lactose (Table 1). A different approach that we briefly investigated is the use of dimethyl adipimidate (DMA) as cross-linking reagent. Di-imido esters were introduced for this purpose by Hartman and Wold [36]. Compared with 1,5-pentanedial these have the advantage that a large number with different spans are available; they are also specific for amino groups and do not alter the charge on the protein surface (Fig. 2). Cross-linking with DMA of *Escherichia coli* β-galactosidase increased its thermostability [37]. When *A. oryzae* β-galactosidase on Duolite S-761 was cross-linked with DMA, the activity of the immobilised enzyme preparation was raised by 30% to 157 U/g; lactose had no effect in this case (Table 1).

3.2. Condensation of galactose and glycerol

In order to study the activity of the immobilised enzyme preparations at low water activity, we performed the reversed hydrolysis of galactose with glycerol in the absence of added water. A slow reaction was observed with the *A. oryzae* β-galactosidase on Duolite S-761 preparations (Table 2). The native enzyme was not active under these conditions, which attests to the stabilising effect of adsorption and cross-linking. The synthesis rates of the immobilised preparations were approx. 0.2% of the rate of lactose hydrolysis with only minor differences on the basis of hydrolytic activity. We ascribe the low rate partially to the ineffectivity of

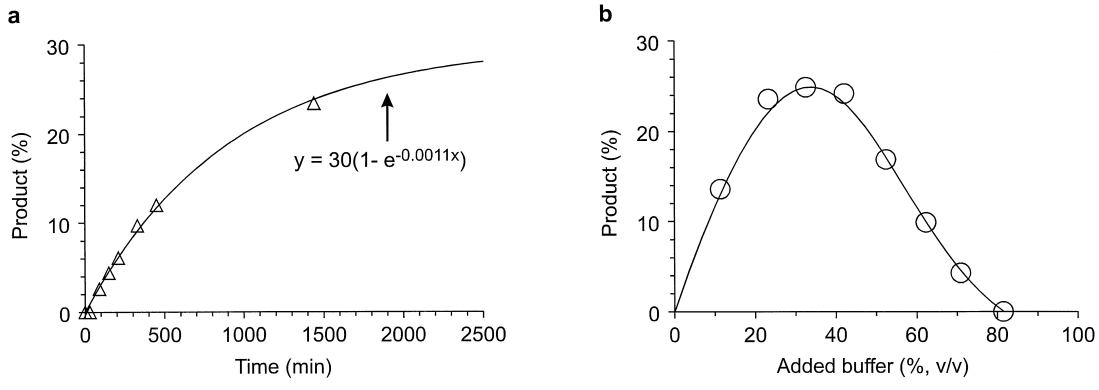

Fig. 3. Condensation of galactose and glycerol catalysed by *K. fragilis* β-galactosidase on Duolite A-7. Reaction conditions: 0.3 mmol galactose, 12 mmol glycerol, 40 U β-galactosidase, pH 6.5, 40°C. a: plot of product formation against time (50% buffer); b: plot of product formation against % added buffer pH 6.5 (reaction time 24 h).

galactose as a galactosyl donor (vide infra), but also to the low water activity. It is significant in this respect that *K. fragilis* β-galactosidase required at least 15% water in the medium for catalytic activity (see Fig. 3b).

3.3. Effect of the galactosyl donor in transgalactosylation

The immobilised β-galactosidase preparations of *A. oryzae* on Duolite S-761 and *K. fragilis* on Duolite A-7 (both crosslinked with 1,5-pentanedial) were used in the glycerolysis of a number of galactosyl donors (see Table 3). 1-*O*-β-D-Galactopyranosyl glycerol was mainly formed as a mixture of two diastereomers, but a small amount of 2-*O*-β-D-galactopyranosyl glycerol was also present. This pattern—a preference for primary alcohol functions without enantioselection at the β-C atom of the acceptor—has been reported previously [18,19].

3.3.1. Effect of water

With the *A. oryzae* enzyme the reactions already took place without additional aqueous buffer, similar to the reversed hydrolysis reaction described above. Upon the addition of 50% (v/v) of aqueous buffer the initial rate increased by a factor of 3 (methyl-β-D-galactopyranoside) to over 30 (4-nitrophenyl-β-D-galactopyranoside). The relatively large increase in rate shown by the phenyl galactosides could be caused by destabilisation of these non-polar reactants in water-rich medium, but this point obviously requires further study.

3.3.2. Effects of the catalyst

At a buffer concentration of 50% both catalysts have a useful activity, which makes it possible to compare their characteristics. In reversed hydrolysis the synthesis rate of the *A. oryzae* β-galactosidase soon levelled off (data not shown) whereas in the same reaction *K. fragilis* β-galactosidase showed a first order progress towards an estimated equilibrium conversion of approx 30% (Fig. 3a). In transgalactosidation the *A. oryzae* enzyme shows normal kinetics, i.e., the product yields passed through a maximum after which secondary hydrolysis predominates (see Fig. 4). In contrast, with the *K. fragilis* β-galactosidase the reaction progressed towards a maximum product yield; subsequently the reaction seems to come to a standstill without significant secondary hydrolysis. The reason for this behaviour is obscure, but in view of the results in reversed hydrolysis (Fig.

Table 3
Glycerolysis of galactosyl donors catalysed by the β-galactosidases from *A. oryzae* and *K. fragilis*[a]

Emzyme preparation:	*Aspergillus oryzae*/Duolite S-761				*Kluyveromyces fragilis*/Duolite A-7		
Buffer added (%, v/v):	0	50			50		
Donor	Initial rate (nmol min^{-1} U^{-1})	Initial rate (nmol min^{-1} U^{-1})	Product (%)	Time (min)	Initial rate (nmol min^{-1} U^{-1})	Product (%)	Time (min)
Galactose	n.d.	3.5	19	1440	2.9	23	1440
Lactose	14	85	70	180	26	69	350
Lactulose	24	172	69	90	50	65	240
β-Gal-OMe	62	185	70	60	29	57	360
β-Gal-OPh	50	508	100	45	49	92	330
β-Gal-OPh-*p*-NO_2	41	1330	82	30	94	75	150
β-Gal-OPh-*o*-NO_2	20	390	99	45	57	96	360

[a]Reaction conditions: galactosyl donor, 0.3 mmol, glycerol, 12 mmol, aqueous buffer pH 4.5 or 6.5, galactosidase 30 U, 40°C.

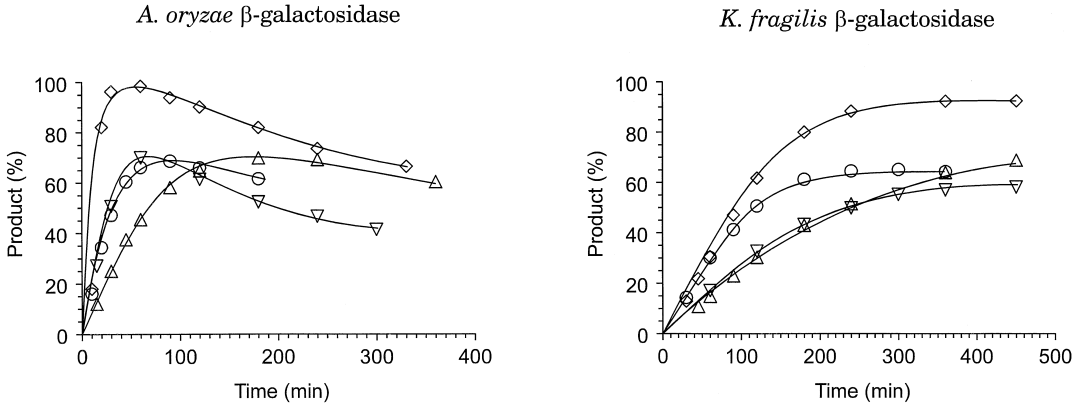

Fig. 4. Time-course of the glycerolysis of lactose (△), lactulose (○), methyl β-D-galactopyranoside (▽), phenyl β-D-galactopyranoside (◇). Reaction conditions: galactosyl donor, 0.3 mmol, glycerol, 12 mmol, aqueous buffer pH 4.5 or 6.5, 50% (v/v), galactosidase 30 U, 40°C.

3a), inactivation of the catalyst or product inhibition does not seem likely.

In general the *A. oryzae* β-galactosidase gives higher yields than the one from *K. fragilis* which is also less active on a per-Unit basis. The latter enzyme is remarkably less sensitive to the nature of the leaving group.

3.3.3. Reactant effects

The *A. oryzae* enzyme converted lactose 25 times as fast as galactose, as judged by the initial rate; for the *K. fragilis* enzyme this factor amounted to 9. This observation attests to the relative inefficiency of reversed hydrolysis compared with transgalactosylation. The latter procedure also results in a much higher product yield (70 vs. approx 20%) due to kinetic control. Lactulose—which is produced on an industrial scale from lactose by alkaline isomerisation [38]— proved to be an efficient galactosyl donor that reacted twice as fast as lactose at the same overall synthesis/hydrolysis ratio. Remarkably, lactulose has not previously been employed in transgalactosylation, although its hydrolysis by β-galactosidases has been investigated [39]. Especially the three β-phenyl galactosides reacted rapidly and gave high product yields, although the effect of the nitro group on the course of the reaction is rather small. The high reaction rate with the phenol leaving group obviously renders kinetic control very efficient. These results also indicate that the kinetic synthesis/hydrolysis ratio of the transglycosylation reaction is high. Consequently, secondary hydrolysis of the product, which comprises a slow hydrolysis in multiple reaction cycles that are each subject to the same synthesis/hydrolysis ratio, is the main restrictive influence on the yield.

4. Concluding remarks

We have shown that the β-galactosidase from *Aspergillus oryzae* can be fixed efficiently to a macroporous carrier. The resulting preparation was active in the glycerolysis of a number of galactosyl donors without a need for additional water, whereas the native enzyme was inactive under the same conditions.

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