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Enzymatic synthesis of *N*-acetyllactosamine in aqueous-organic reaction media

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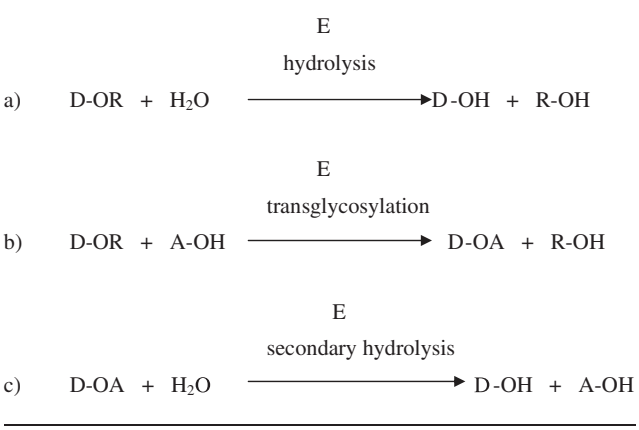
β-Galactosidase from *Bacillus circulans* was used for the biocatalytic transfer of D-galactose (D-Gal) from *o*-nitrophenyl-β-D-galactoside (ONPG) to the O-4 position of 2-acetamido-2-deoxy-D-glucopyranose (D-GlcNAc) forming the disaccharide *N*-acetyllactosamine (LacNAc, β-D-Gal-(1 → 4)-D-GlcNAc). In order to investigate the potential of this biocatalytic synthesis, first the optimal reactant ratio in an aqueous buffer system was determined. On the basis of these standard conditions we then performed reactions in aqueous-organic media applying organic cosolvents of different structure and polarity in various amounts. In this way we received in some cases appreciably better results than without organic cosolvent. The highest obtainable disaccharide yield was 50% in a mixture of 20% (v/v) cyclohexane/ 80% buffer versus 35% in buffer solution alone.

1. Introduction

Because of the growing demand for substances containing defined saccharide structures, further development of advanced methods of sugar synthesis is very important. Enzymatic syntheses can be an alternative or complementary to known chemical methods which often need several laborious steps in synthesis and separation, mostly resulting in low overall yields. With the help of suitable enzymes, glycoside syntheses become less time and material consuming. Industrially used glycosidases like e.g. β-galactosidases for lactose hydrolysis in milk are available in large amounts and at low prices. Besides their hydrolytic activity, they also perform recombinations of sugar moieties by transglycosylation from a donor to an acceptor component in selected reaction media (Scheme 1). To improve the latter activity (step b) and to minimize the former (steps a and c) by medium-engineering is a challenging task in current research [1].

Scheme 1



Due to the flexibility of their acceptor binding site, glycosidases of different sources are suitable for the synthesis of interesting glycosidic core structures such as *N*-acetyllactosamine (LacNAc, **3**) in several oligo- and polysaccharides, N-bound glycoproteins and glycolipids. Compound **3** functions as growth promotor for different bacteria [2]. It is also a part of human blood group determinants and a fragment of cellular adhesion molecules (sialyl-Lewis^x) [3–5]. Several approaches to a favourable synthesis of this disaccharide have been taken comprising chemical as well as enzymatic methodology (see refs. cited in [6]) and literature [7–13]. For an enzymatic process the regioselectivity of the β-galactosidase is an essential factor for its usability. Whereas the enzyme from *E. coli* forms β1–6-linkages exclusively [7], one of the most suitable β-galactosidases for the transfer of D-Gal to the 4-OH in D-GlcNAc **2** is that from *Bacillus circulans* [11]. In this paper we want to report on our studies on the use of this enzyme for an improved synthesis of **3** by choosing various aqueous-organic reaction media.

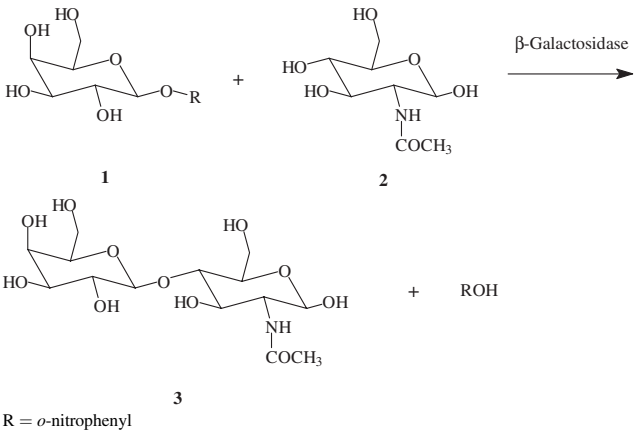
2. Investigations, results and discussion

The β-galactosidase-catalysed formation of **3** is depicted in Scheme 2. The reaction is performed by the catalytic transfer of the galactose moiety from the donor ONPG **1** to the acceptor D-GlcNAc **2**.

Although an equimolar ratio of reaction partners seems necessary, it is common practice to use one educt in excess, in order to shift the reaction equilibrium. Thus, we started a reaction series with equimolar amounts (50 mM) of **1** and **2** and changed the acceptor/donor ratio in both directions. The results for reactions in acetate buffer are shown in Fig. 1.

The donor in threefold excess proved to be the best choice giving 35% **3** besides additional transfer products such as

Scheme 2



$(Gal)_2$-ONP and $(Gal)_3$-ONP (NP = *o*-nitrophenyl). This formation of oligomeric galactose derivatives increased with a decreasing acceptor/donor ratio, thereby resulting in lower yields of **3**. Although higher concentrations of the acceptor also slightly favour formation of the desired product, the obtainable yields are less convincing. We therefore maintained this favourable 1 : 3 acceptor/donor ratio in all the further experiments.

In addition, the synthesis outcome is also influenced by the amount of educts. At lower substrate concentrations hydrolysis strongly competes with the transfer reaction, whereas importantly higher concentrations of the sugar derivatives lead to formation of tri- and tetrasaccharides as well as (1 → 6) bonded disaccharides which are difficult to separate. These observations apply to the synthesis of **3** in different aqueous-organic reaction media as well.

As to the choice of cosolvents, we had checked earlier in preliminary experiments the influence of organic solvents of different structure and polarity on the activity and stability of β-galactosidase from *B. circulans*. On the basis of these results the solvents compiled in the Table seemed of interest and were used for the synthesis reaction in different portions with acetate buffer.

With almost half of them it was possible to get better yields of **3** than in buffer alone. The results of the synthesis in different aqueous-organic mixtures are often in good correlation with activity and stability of the enzyme in these media. Although solvent polarity does not seem to be the most decisive factor, mainly less polar cosolvents rank among the more suitable ones, cyclohexane being the best. In general, we may conclude that addition

Table: β-Galactosidase-catalysed synthesis of 3 in aqueous-organic media (yield in %)

Organic cosolvent	Portion of cosolvent (v/v)				Maximum yield (%)
	20%	40%	60%	80%	
Without	–	–	–	–	35
Cyclohexane	50	48	33	32	50
n-Dibutyl ether	45	37	34	17	45
Tetrachloromethane	40	38	41	43	43
2-Butanone	40	41	30	25	41
Methyl *t*-butyl ether	41	20	3	0	41
Ethyl acetate	39	38	35	31	39
Toluene	36	30	22	30	36
1,4-Dioxane	30	20	4	0	30
Tetrahydrofurane	28	23	19	12	28
n-Hexane	21	23	25	28	28
Acetonitrile	27	23	19	17	27
Acetone	27	20	0	0	27
t-Butanol	26	24	16	11	26
Trichloromethane	24	18	23	11	24
Diethylenglycol-dimethylether	19	23	9	0	23

Experimental conditions: 150 mM **1**, 50 mM **2**, 2 ml total volume; acetate buffer (0.1 M, pH 5), β-galactosidase from *B. circulans* in amounts of 5; 10; 5 and 25 U, resp., increasing with the content of cosolvent; reaction time 0,5–5 h; relative standard deviation 5%.

of 20% organic solvent is an advantageous and sufficient amount, whereas higher concentrations rather scarcely or even badly affect the synthetic result. The latter phenomenon is very distinctive for more polar solvents, such as 1,4-dioxane, acetone and diethylenglycol dimethylether, but also valid for e.g. methyl *t*-butyl ether. The better yields of **3** in the presence of 20–40% organic cosolvents can be explained by a more favourable solubility of **1** under these conditions and by diminished hydrolysis. If we used lactose as donor in the presence of aqueous organic mixtures, no significant increase in yield could be reached at comparable substrate concentrations. At higher cosolvent concentrations not just the enzyme activity is reduced, but also a preferential transfer of galactose to **1** and $(Gal)_n$-ONP instead to **2** takes place. This effect is independent of the amount of enzyme and may be caused either by the altered solubility properties of the system or by interference with the acceptor binding site of the β-galactosidase. In biphasic media an extraction of the liberated *o*-nitrophenol into the organic phase could possibly favour the galactosyl transfer. This may equally hold for certain mixtures of buffer with 2-butanone, tetrahydrofurane and acetonitrile, which become heterogeneous in

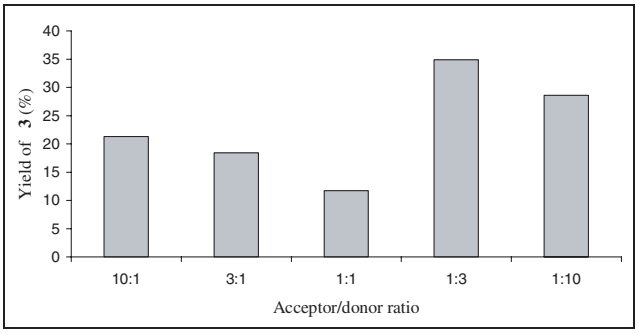


Fig. 1: Influence of the acceptor/donor ratio on the synthesis of **3** catalyzed by β-galactosidase from *B. circulans*. Conditions: concentration of the minor reactant 0,05 M; 2 ml acetate buffer (0,1 M, pH 5); 5 U β-galactosidase; 30 °C; relative standard deviation 5%.

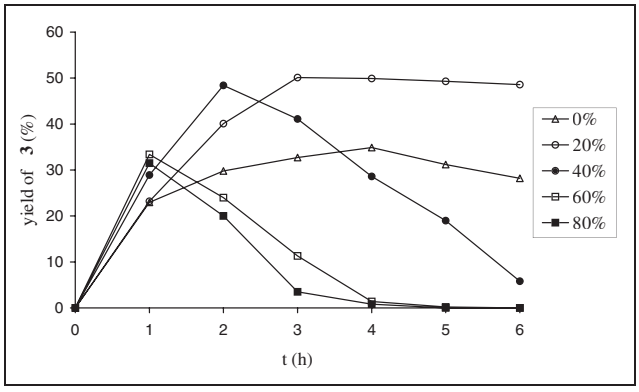


Fig. 2: Kinetics of the synthesis of **3** in biphasic cyclohexane/buffer mixtures. Conditions: 150 mM **1**, 50 mM **2**; mixtures of acetate buffer (0,1 M, pH 5)/cyclohexane with a total volume of 2 ml; 5 U β-galactosidase; 30 °C; relative standard deviation 5%.

the presence of reactants. As hydrophilic substrates together with the enzyme almost entirely are located in the aqueous phase, their increased concentrations will also benefit the synthesis outcome.

Fig. 2 shows the effect of an increasing cyclohexane content on the kinetics of the formation of **3**. Up to a portion of 60% cyclohexane the reaction rate slightly increases. However, after attaining the maximum concentration of about 30% **3** in the presence of 60–80% cyclohexane, the product is rapidly hydrolysed. With 40% cyclohexane the kinetic optimum appears later. The remarkable yield of about 50% **3** at this time also diminishes again by product hydrolysis, if the reaction is not stopped. Optimal conditions are finally established with 20% cyclohexane in the mixture, enabling high transfer rate as well as good product stability.

In summary, we should like to emphasize that β-galactosidase from *B. circulans* is a suitable biocatalyst for transgalactosylation using **1** as donor in different aqueous-organic reaction media with polar as well as non-polar cosolvents. Thus, the synthesis of **3** could be markedly improved in some cases in comparison to reaction in aqueous buffer solution. Only moderate amounts of about 20% cosolvent were necessary. The best result was attainable by addition of cyclohexane. Although the obtained yields remain moderate so far (up to 50%), this kind of medium-engineering provides improvements to the existing methodology.

3. Experimental

3.1. Materials

β-Galactosidase from *B. circulans* was a gift of Daiwa Kasei K. K., Osaka, Japan. **1** was purchased from Bachem and **2** from Sigma. Organic solvents and buffer salts were of analytical grade and used without further purification.

3.2. Enzyme activity measurements

The rate of hydrolysis of the substrate **1** is determined by measuring the absorbance of the delivered *o*-nitrophenol at 420 nm [14]. One Unit (U) is defined as the hydrolysis of 1 μmol **1** per minute under the above conditions.

3.3. Enzymatic transglycosylation reactions

A typical transglycosylation procedure for determination of the best acceptor donor ratio is as follows. To a volume of 1.9 ml acetate buffer (0.1 M, pH 5.0) the necessary amounts of **1** and **2** given in Fig. 1 were added with stirring. For starting the reaction, 5 U β-galactosidase in 0.1 ml acetate buffer were added. The reaction mixture was then stirred at 30 °C and the progress of reaction followed by HPLC; first at 30 min from the beginning and then every hour. Each transgalactosylation reaction was verified two times.

A typical transglycosylation procedure in aqueous-organic solvents is as follows. For a final volume of 2 ml the necessary amount of the organic solvent was added to the acetate buffer (0.1 M, pH 5.0). Then 0.2 mmol **2** and 0.6 mmol **1** were added with stirring. For starting the reaction the amount of β-galactosidase given in the Table was added. The reaction mixture was stirred at 30 °C and the progress of reaction followed as described above. Each transgalactosylation reaction was verified two times.

3.4. Analytical methods

Analytical HPLC was done with a Spectra Series 100 system and a LiChrospher NH_2 column (5 μm), 250 × 4 mm from Merck using an acetonitrile-water eluent (80/20) at a flow rate of 1 ml/min. Analyses were performed using a light scattering detector from S.E.D.E.X (France). Determination of the compounds was performed by a Jasco/VG Platform LC-MS system using the ESI method.

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