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# Towards regioselective synthesis of oligosaccharides by use of α-glucosidases with different substrate specificity

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**Abstract**

α-Glucosidase from two microbial sources, *Bacillus stearothermophilus* and Brewer's yeast, has been used to catalyze transglycosylation reactions and a comparative study was carried out to determine the regioselectivity of this reaction. Bacterial α-glucosidase exhibited higher transfer activity with maltose and was able to synthesize tri- and tetrasaccharides in high yield (27%). In the case of yeast enzyme, only trisaccharides were synthesized in lower yield. Structure analysis of transglycosylation products by means of GC–MS and NMR spectroscopy revealed a correlation between the hydrolytic substrate specificity and the regioselectivity of transglycosylation reaction. Higher substrate specificity of bacterial enzyme, however, influenced its transglucosylation activity toward other saccharide acceptors. 

*Keywords:* α-Glucosidase; Transglycosylation; Regioselectivity; Synthesis of glucooligosaccharides

## 1. Introduction

Enzymatic methods of synthesis of oligosaccharides and their derivatives can be considered as very promising alternatives to chemical synthesis, since chemical synthesis of oligosaccharides is usually complicated by the formation of unwanted anomers. Two types of enzymes have already been used for oligosaccharide synthesis: glycosyltransferases and glycosidases [1].

Many glycosidases that were primarily known to catalyze hydrolysis of oligosaccharides were also found to catalyze transglycosylation reaction [2–4]. Their ability to catalyze the stereospecific formation of glycosidic bonds [5], their availability and possibility of using simple substrates make glycosidases preferable to glycosyltransferases. However, the regioselectivity of these enzymes still remains poor. In many cases, it has been noticed that glycosidases from different sources catalyze the synthesis of different linkages [6–8]. Therefore, several attempts have been made to establish a correlation between the substrate specificity of the hydrolytic reaction and the regioselectivity during transglycosylation [8,9]. This would enable a possible regioselectivity with enzymes of known hydrolytic specificity to be predicted.

The present paper deals with α-glucosidases (EC 3.2.1.20 α-D-glucoside glucohydrolases) from two microbial sources, *Bacillus stearothermophilus* and Brewer's yeast, with different substrate specificity, their effect on

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the regioselectivity of the transglycosylation and on the reaction course with acceptors.

## 2. Results and discussion

*Hydrolytic activity of α-glucosidases on various substrates.*—α-Glucosidases are *exo*-glycosidases, which catalyze the splitting of glucosyl residues from the nonreducing end of short oligosaccharide substrates to liberate D-glucose. However, enzymes from various microorganisms differ mostly with respect to their substrate specificities towards naturally occurring di- and oligosaccharides. Due to these variations, enzymes from both *B. stearothermophilus* and Brewer's yeast were first characterized by their hydrolytic activity against different substrates. The Michaelis constant ($K_m$) and the maximal rate ($V_{max}$) values are summarized in Table 1, together with relative $V_{max}$, which represents the relative values of the cleavage rates of different α-glucosidic bonds in each substrate. With bacterial enzyme, decrease of the rate of maltose, maltotriose and maltotetraose hydrolysis was observed at high substrate concentration. Such behavior has already been described for some α-glucosidases [10]. We assume that the decrease was not caused by substrate or product (D-glucose) inhibition — experimentally proved — but is the result of continuation of transglycosylation in which the produced D-glucose is consumed. According to the results in Table 1, bacterial α-glucosidase is much more specific with respect to the nature of the substrate and the type of linkage than that from yeast. Bacterial enzyme is a highly specific α-(1→4) glucosidase with highest activity for maltose and higher maltooligosaccharides. The enzyme was slightly active for α-(1→3) and α-(1→6) linkages in nigerose and isomaltose. Such high specificity of this enzyme is in agreement with results of Suzuki et al. [11]. The other enzyme is a typical example of yeast α-glucosidase with broad aglycon specificity [12]. It shows higher activity for aryl α-D-glucoside (PNPG) and sucrose than for maltose. In comparison with bacterial enzyme, it also had much higher activity for α-(1→3) and α-(1→6) linkages.

These two enzymes were chosen in order to compare the effect of different hydrolytic specificities on the regioselectivity of transglycosylation reaction, since they were good representatives of α-glucosidases with different hydrolytic properties. Bacterial enzyme had a rather narrow hydrolysis range directed to-

Table 1
Rate parameters for the hydrolysis of various substrates by α-glucosidases from *B. stearothermophilus* and Brewer's yeast

| | *B. stearothermophilus* | | | Brewer's yeast | | |
|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $V_{max}$ [a] | (%) [b] | $K_m$ (mM) | $V_{max}$ [a] | (%) [b] |
| Maltose (α-D-Glc-(1→4)-D-Glc) | 7.2 | 152 | 100 | 19.6 | 25 | 100 |
| Isomaltose (α-D-Glc-(1→6)-D-Glc) | n.d. [d] | [e] | | 34.6 | 6 | 27 |
| Nigerose (α-D-Glc-(1→3)-D-Glc) | n.d. | [e] | | 62.5 | 18 | 72 |
| Trehalose (α-D-Glc-(1→1)-α-D-Glc) | | 0 | 0 | | 0 | 0 |
| Sucrose (α-D-Glc-(1→2)-β-D-Fru) | 9.8 | 18 | 12 | 23.8 | 30 | 132 |
| PNPG (*p*-nitrophenyl α-D-glucopyranoside) | | 71 | 47 | 3.2 | 70 | 280 |
| Maltotriose (α-D-Glc-(1→4)-α-D-Glc-(1→4)-D-Glc) | 2.1 | 147 | 97 | 7.3 | 17 | 68 |
| Maltotetraose (α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc-(1→4)-D-Glc) | 2.5 | 109 | 72 | n.d. | [e] | |
| Starch | 38.4 [c] | 82 | 54 | | 0 | 0 |
| Glycogen | | 0 | 0 | | 0 | 0 |
| Cellobiose (β-D-Glc-(1→4)-D-Glc) | | 0 | 0 | | 0 | 0 |

[a] μmol of D-glucose released from the nonreducing end/min per mg of protein.
[b] Numbers indicate $V_{max}$ relative to maltose.
[c] mg/mL.
[d] n.d., not determined.
[e] The enzyme was slightly active on these substrates at higher concentrations.

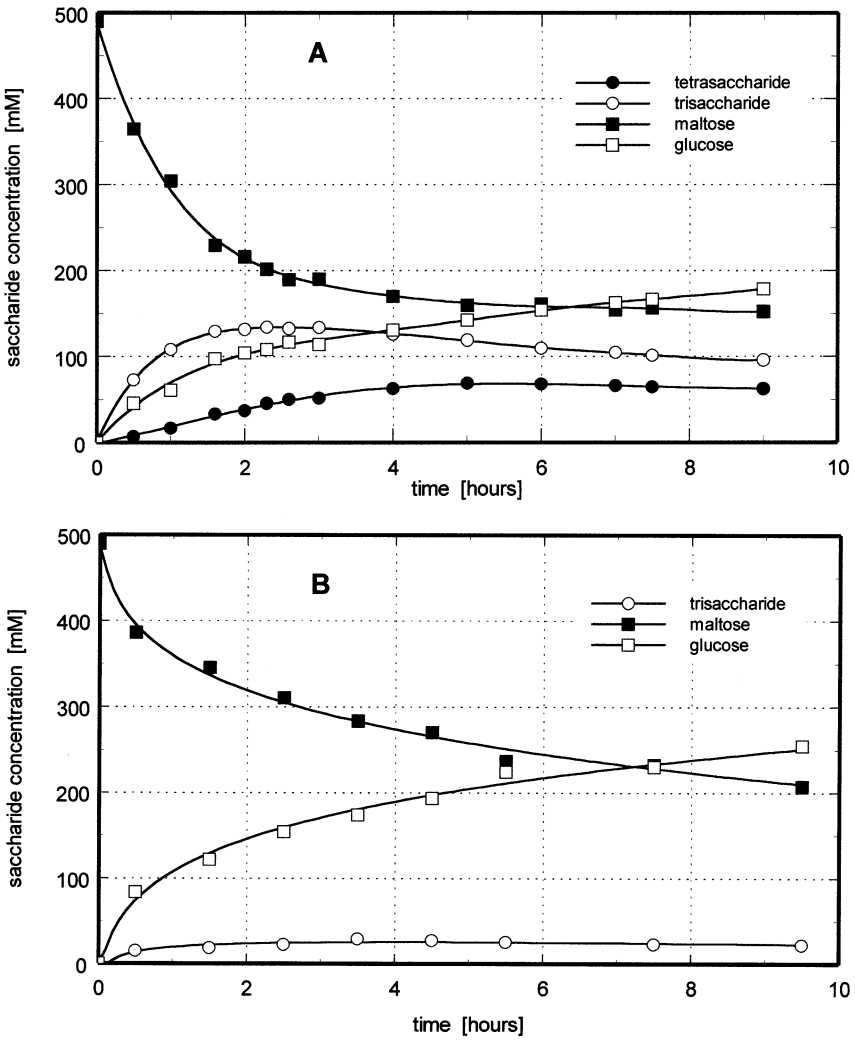


Fig. 1. Transglycosylation activity of α-glucosidase in 15% (w/w) maltose. (A) *B. stearothermophilus* (0.1 M phosphate buffer pH 7.5 at 45 °C); (B) Brewer's yeast (0.1 M phosphate buffer pH 6.8 at 30 °C).

wards one linkage, while the hydrolysis range of yeast enzyme was rather broad.

*Transglycosylation reaction.*—The transglycosylation reaction was studied in 15% (w/w) maltose solution and results are shown in Fig. 1(A) and (B). Both enzymes were able to synthesize higher oligosaccharides; the reaction courses of transglycosylation, however, were quite different. In the case of bacterial enzyme, trisaccharides were rapidly synthesized up to a maximum value of 134 mM (yield of about 27% on a molar basis). After reaching this maximum, they started to disappear from the reaction mixture; they were either the subject of secondary hydrolysis or behaved as a suitable acceptor for tetrasaccharide synthesis. Tetrasaccharides were still synthesized at that time up to a concentration of 70 mM and they were subjected to secondary hydrolysis. The transglycosylation activity of yeast enzyme was much lower and the enzyme was able to synthesize only trisaccharides up to a maximum concentration of 30 mM (yield of about 6% on a molar basis). This result is probably connected with the hydrolytic substrate specificity of yeast enzyme. If the enzyme has a low ability to hydrolyze a tetrasaccharide, there is high probability that it also has a low ability to synthesize it, as the binding conditions for enzyme–substrate complex will be the same in both directions.

The transglycosylation activity was further examined as a function of the maltose concentration and results are shown in Fig. 2(A) and (B). The maltose concentration had the same effect on both enzymes, i.e., increasing the maltose concentration in the reaction mixture

caused an increase in the maximum trisaccharide and tetrasaccharide concentrations — trisaccharide from 131 to 798 mM, tetrasaccharide from 39 to 283 mM for bacterial enzyme; trisaccharide from 24 to 217 mM for yeast enzyme. Consequently, the time intervals necessary for reaching the above-mentioned maximum concentrations increased too — from 2 to 23 h and from 6 to 47 h, respectively, in the case of the bacterial enzyme and from 9.5 to 23 h in the case of the yeast enzyme.

The highest checked maltose concentration (50% w/w) has been chosen in order to carry out a comparative study of the product structure from both enzymes (tri- and tetrasaccharides) in order to determine the regioselectivity of the transglycosylation reaction.

*Structural analysis.*—To analyze the structure of the products synthesized by α-glucosidase from *B. stearothermophilus*, samples were taken at three time intervals — 2, 6, 20 h — and after 6 and 20 h for yeast α-glucosidase. After purification by gel chromatography on Biogel P2, the oligosaccharides were analyzed as the partially methylated alditol acetates by means of GC–MS. With the GC conditions used in this study, a baseline separation was easily obtained. Four different methylated alditol acetates were found in the mixtures: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol; 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol.

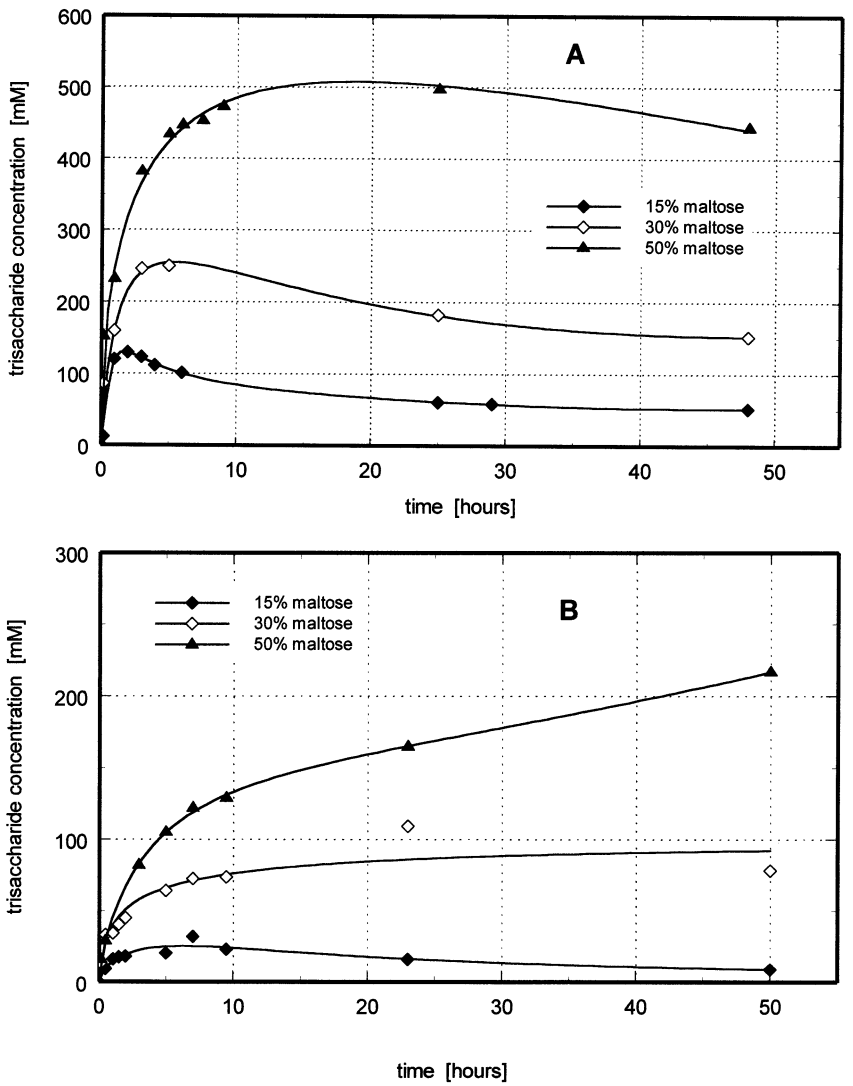


Fig. 2. Effect of increasing maltose concentration on the trisaccharide production in reaction catalyzed by α-glucosidase. (A) *B. stearothermophilus*; (B) Brewer's yeast.

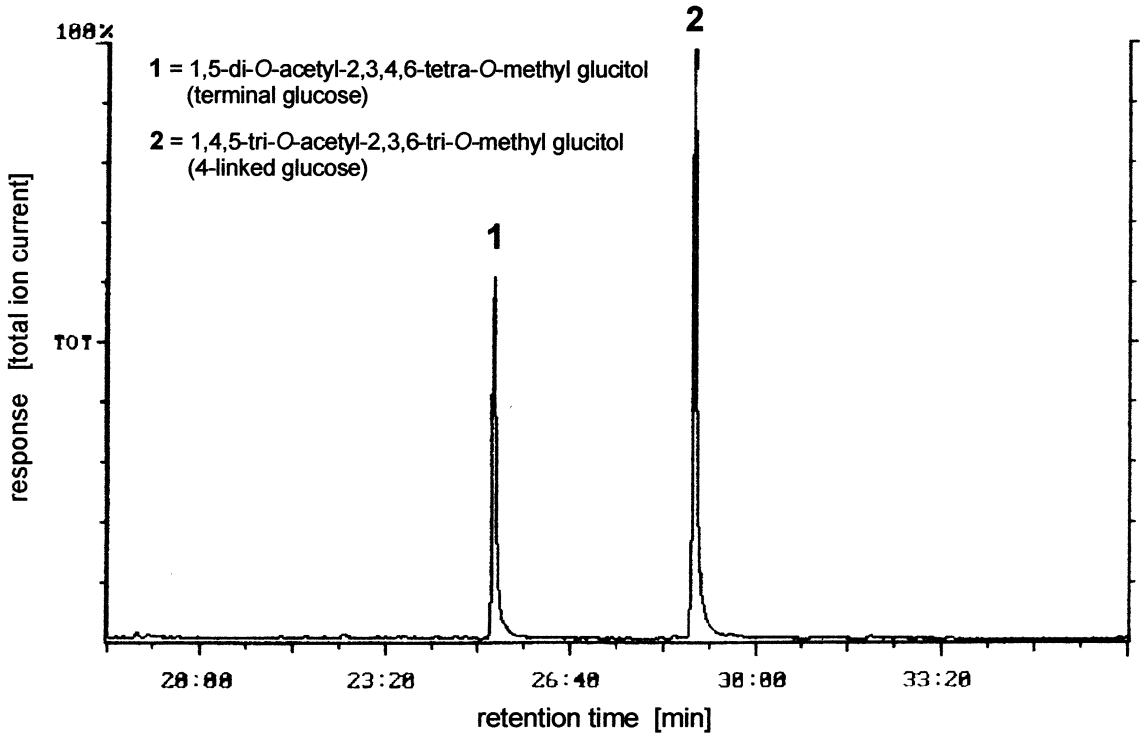


Fig. 3. GC profile of the transglycosylation product (trisaccharide) formed by the α-glucosidase from *B. stearothermophilus* in 50% (w/w) maltose. A sample taken from the reaction mixture (1 mL) after 6 h was purified by gel chromatography on Biogel P2 and the synthesized trisaccharide was analyzed as its partially methylated alditol acetates by means of GC–MS.

Peaks in chromatograms were assigned to individual methylated alditol acetates on the basis of retention time and successful fits to reference library spectra and mass spectra of used standards. Peak areas were used for quantitative calculation. According to these experiments, trisaccharides synthesized by the bacterial enzyme at 2 and 6 h intervals contained exclusively (1→4) linkages. They were assigned as α linkages by enzymatic assay with α- and β-glucosidases. Fig. 3 shows the result of GC analysis of the sample after 6 h. After 20 h of reaction, (1→3) and (1→6) linkages were found in the reaction mixture by GC–MS analysis but still in low proportion (< 5%). All tetrasaccharides synthesized during the reaction contained, according to GC–MS, only α-(1→4) linkages and were identified as maltotetraose. In contrast, trisaccharides produced by α-glucosidase from Brewer's yeast after 6 h contained mainly the α-(1→4) linkage along with a low proportion of α-(1→3) linkage (about 12% as seen from GC). After 20 h the percentage of α-(1→3) linkage increased to 34% and the α-(1→6) linkage also appeared (about 7% as seen from GC). Fig. 4 shows a typical GC analysis of this sample.

The structure and purity of trisaccharides synthesized by α-glucosidase from *B. stearothermophilus* after 2 and 6 h were confirmed by NMR spectroscopy. $^1$H NMR spectra of natural saccharides suffer from heavy overlaps of proton resonances due to an inherent low dispersion of their chemical shifts. The analysis was carried out by the complete assignment of proton resonances of maltotriose as a standard and the NMR spectra were then compared with spectra of the trisaccharide samples. All resonances in the $^1$H NMR spectra were assigned by using a combination of one- and two-dimensional $^1$H–$^1$H homonuclear experiments such as COSY and TOCSY. While the double-quantum filtered COSY spectrum allowed us to determine three bond correlations, the analysis of the TOCSY spectrum established the whole proton coupling network within a ring of a trisaccharide. The anomeric protons (H-1$^{I}$, H-1$^{II}$ and H-1$^{III}$) are usually the most downfield shifted resonances in the proton NMR spectra of saccharides; therefore, they served as the starting points for the determination of the coupling network of

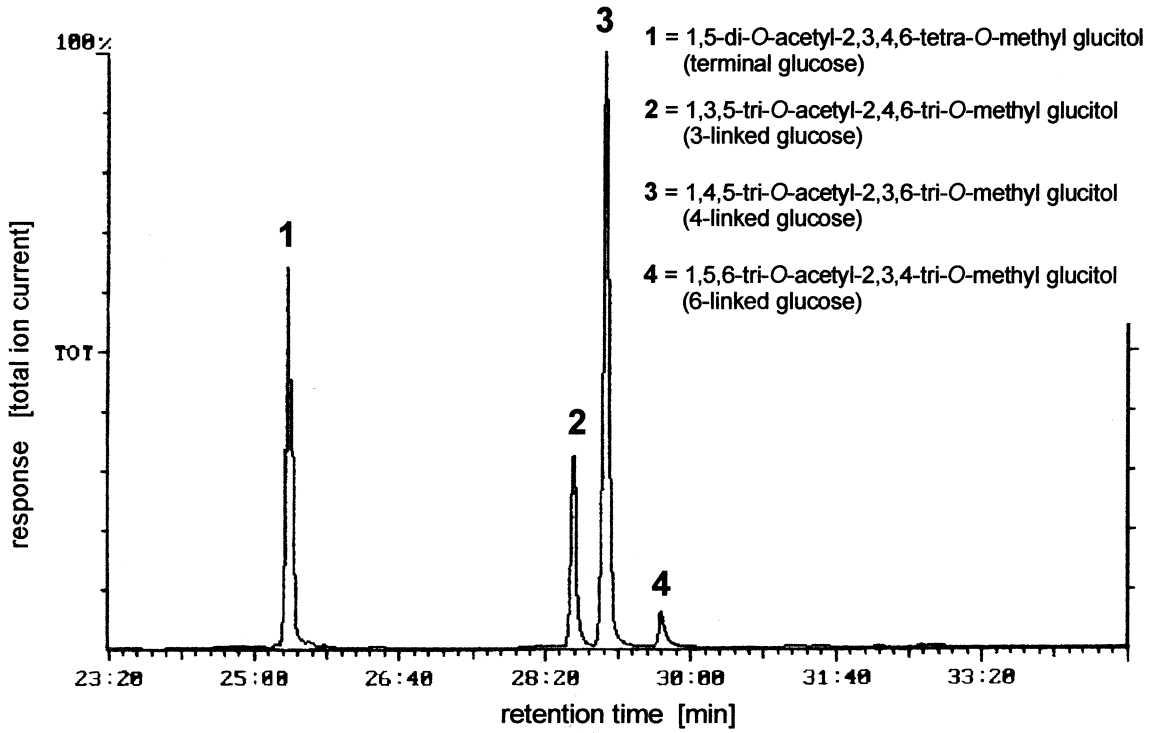


Fig. 4. GC profile of the transglycosylation product (trisaccharide) formed by the α-glucosidase from Brewer's yeast in 50% (w/w) maltose. A sample (1 mL) taken from the reaction mixture after 20 h was purified by gel chromatography on Biogel P2 and the synthesized trisaccharide was analyzed as its partially methylated alditol acetates by means of GC–MS.

a corresponding sugar ring (Fig. 5). After establishing the molecular skeleton, the type of glycosidic linkage in a trisaccharide has been examined using NOESY experiments. The most crucial NOE interactions have been observed between protons H-$1^{II}$ and H-4 and protons H-$1^{III}$ and H-$4^{II}$, respectively, in agreement with α-(1→4) linkages between sugar residues in maltotriose. By comparing the NOESY spectra of maltotriose with the spectrum of a trisaccharide, an identical pattern of NOE cross-peaks has been found, which confirms the α-(1→4) glycosidic linkages.

*Transglycosylation reaction with different acceptors.*—One of the possible pathways of the transglycosylation reaction is the substrate glycosylation, when a glucose residue from the donor is transferred to a substrate (acceptor) molecule. D-Mannose, D-xylose, D-sorbose and D-galactose were used as acceptors and the capability of both enzymes to transfer D-glucose to these acceptors was evaluated. As some of the transglycosylation products (disaccharides) were not completely separated from maltose, their presence in the course of the transglycosylation reaction was determined on the basis of the disappearance of the acceptor saccharide from the reaction mixture

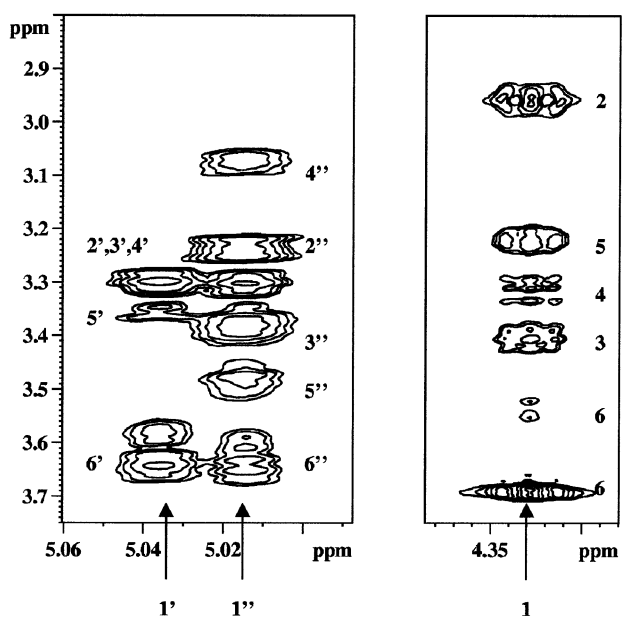


Fig. 5. TOCSY spectrum (500 MHz, $Me_2SO$, 30 °C) of the trisaccharide synthesized by the α-glucosidase from *B. stearothermophilus*, showing the coupling network starting at corresponding anomeric protons H-$1^{I}$, H-$1^{II}$ and H-$1^{III}$.

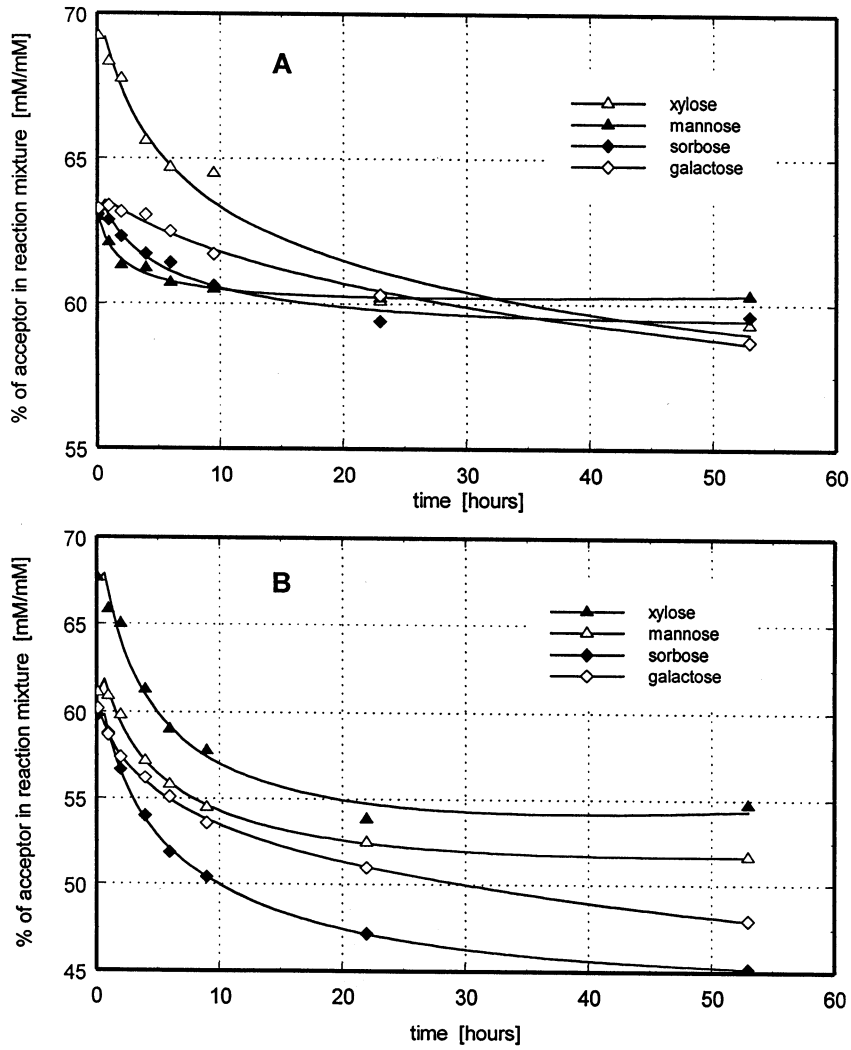


Fig. 6. Time course of consumption of saccharide acceptors from the reaction mixture during transglycosylation reaction in the presence of α-glucosidase. (A) *B. stearothermophilus*; (B) Brewer's yeast. The mixtures of maltose (15% w/w) and one of the acceptors (D-xylose, D-mannose, D-sorbose, D-galactose) were incubated with the enzyme in a 0.1 M phosphate buffer. The reaction was monitored by HPLC.

by HPLC. The results are shown in Fig. 6(A) and (B). Based on these data, α-glucosidase from *B. stearothermophilus* exhibited a considerable transfer activity only against D-xylose. In contrast, yeast α-glucosidase was able to transfer D-glucose residues to all of the tested acceptors — the yield was around 12% on a molar basis. In the case of D-xylose acceptor, the heterooligosaccharide product was completely resolved under the used HPLC conditions and its formation is shown in Fig. 7. According to the results in Fig. 7, the product was in both cases formed very rapidly. However, after reaching the maximum concentration, the heterooligosaccharide was not further hydrolyzed under these reaction times and conditions, as it was observed in experiments using maltose alone. In order to explain this different reaction course, the heterooligosaccharide synthesized by the bacterial α-glucosidase was isolated and its effect on enzyme activity was examined. These experiments have shown that the product of xylose glucosylation — 4-*O*-α-D-glucosyl-D-xylose as based on GC–MS analysis — was a substrate for bacterial α-glucosidase — the $K_m$ value was determined as 152 mM — and had no inhibition effect on the enzyme. A higher value of the Michaelis constant for this substrate could offer the explanation of such a course. The enzyme exhibited lower affinity for glucosyl-xylose than for the other sacchar-

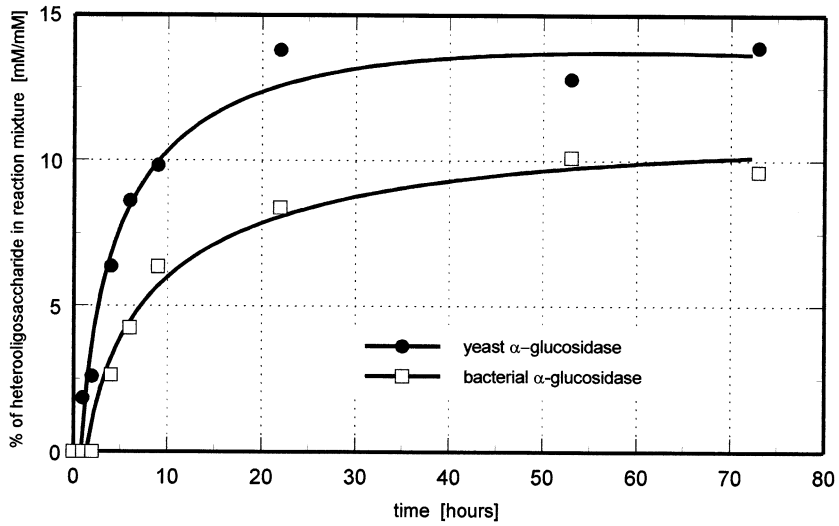


Fig. 7. Time course of formation of D-glucosyl-D-xylose disaccharide. The mixture of maltose (15% w/w) and D-xylose acceptor (15% w/w) was incubated in the presence of α-glucosidase in a 0.1 M phosphate buffer and the reaction was monitored by HPLC.

ides present in the mixture — maltose and maltotriose — and therefore glucosyl-xylose was not preferentially used as substrate for hydrolysis or acceptor for glycosylation.

## 3. Conclusions

Our results imply that information on the hydrolytic substrate specificity of glycosidases can be very valuable in order to predict the regioselectivity of transglycosylation reactions. A specific α-glucosidase from *B. stearothermophilus*, which hydrolyzes preferentially the α-(1→4) linkage, showed a preferential transfer to C-4 of maltose and thus maltotriose was exclusively formed during the first stage — 6 h — of the reaction. In contrast, yeast α-glucosidase with broader substrate specificity catalyzed the formation of α-(1→3) and α-(1→6) linkages along with α-(1→4). These results suggest that specific glycosidases are regioselective during the transglycosylation reaction and could be used for the selective synthesis of desired linkages.

Other possible features of specific glycosidases can be also deduced from the experiment where four different saccharides were employed as acceptors in the transglycosylation reaction. We assume that due to high specificity, bacterial α-glucosidase did not exhibit transfer activity to most of the tested acceptors. In contrast, yeast α-glucosidase transferred D-glucose to all of them and the yield of transglycosylation corresponded to the yield of product when only maltose was used. The reason for the transfer activity of bacterial α-glucosidase against D-xylose could be attributed to the structure of the acceptor. D-Glucose and D-xylose have the same configuration at C-1, 2, 3 and 4 and therefore no steric interference can be expected during the transfer reaction in contrast with the other tested acceptors. It is interesting to note that during the reaction with D-xylose, hydrolysis of the transglycosylation product was not observed as in previous experiments with maltose. In our opinion, this could be attributed to low enzyme affinity for this substrate as compared with other saccharides present in the reaction mixture.

In summary, these results suggest that the hydrolytic specificity of a selected α-glucosidase is connected with the regioselectivity of the transglycosylation reaction. The selection of enzymes with suitable and narrow hydrolytic specificity could eventually overcome the main problem of glycosidases in oligosaccharide preparation, which is the formation of isomeric mixtures. However, it is still necessary to study in more detail the behavior of such enzymes toward other saccharide or non-saccharide acceptors before their use in glycosylation reactions.

## 4. Experimental

*Materials.*—Saccharides and *p*-nitrophenyl α-D-glucopyranoside (PNPG) were purchased from Sigma (USA). Reagents used for the structural analysis of products were purchased from Fluka and E. Merck (Germany). α-Glucosidases from *B. stearothermophilus* and Brewer's yeast were commercial products (Sigma, USA).

*Enzyme assay.*—The α-glucosidase activity was measured using maltose as a substrate. One unit of activity was defined as the amount of the enzyme that produces 1 μmol of D-glucose/min at pH 6.8 and 37 °C. The enzyme was incubated at 37 °C with 4% (w/w) substrate solution in 0.1 M sodium phosphate buffer pH 6.8. The reaction was terminated by adding 10% $Na_2CO_3$ (w/v) and the liberated D-glucose was assayed by the glucose oxidase–peroxidase method using the Oxochrom Test (Lachema, Czech Republic).

*Kinetic analysis.*—Kinetic parameters for various substrates were measured using 12 different concentrations of substrates ranging from 0.2 to 5 times the $K_m$ value under the same reaction conditions as stated for maltose. Special care was taken to ensure that initial reaction rates were measured. Kinetic data were processed using a nonlinear regression analysis program based on the Michaelis–Menten equation. To examine the effect of glucosyl-D-xylose on α-glucosidase activity, two sets of reactions were carried out. To analyze a possible inhibition effect of this compound, the enzyme was incubated in the mixture of maltose (0.2 M) and one of the ten various concentrations of 4-*O*-α-glucosyl-D-xylose (ranging from 0.5 to 0.4 M) for 30 min. After stopping the reaction, the amount of liberated D-glucose was measured using the glucose oxidase–peroxidase method and compared with the reaction running without transglycosylation product. The other set of reactions was conducted under the same conditions with the only exception being that maltose was omitted from the mixture. This set of reactions was used for the calculation of the $K_m$ value for this substrate.

*Transglycosylation reaction.*—Experiments on the transglycosylation activity of α-glucosidase were carried out under the following conditions: 0.1 M potassium phosphate buffer pH 7.5, 45 °C for the bacterial enzyme and 0.1 M sodium phosphate buffer pH 6.8, 30 °C for the yeast α-glucosidase. The enzyme (0.5 U/mL) was incubated with 1 mL of maltose solution at various concentrations (15, 30, and 50% w/w) or with maltose (15% w/w) as glycosyl donor and other monosaccharides (15% w/w) as acceptor. Samples were taken at various time intervals from the reaction mixture and were heated for 3 min at 100 °C to denature the enzyme. After filtration, 20 μL aliquots were analyzed by HPLC.

*High-performance liquid chromatography (HPLC).*—Oligosaccharide synthesis was monitored by HPLC (Waters, USA). All reaction products were identified and quantified on Supelcogel Ca column with elution by deionized water at a flow rate of 0.5 mL/min at 80 °C. Elution was monitored by a differential refractometer. Product quantification was based on comparison of peak areas with those of standard sugars and D-galactose as internal standard.

*Structural analysis of reaction products.*—To obtain transglycosylation products for structural analysis, α-glucosidase was incubated in 50% (w/w) maltose solution. Aliquots (1 mL) were taken at different time intervals. The products were purified by gel filtration on a column (2.5 × 120 cm) of Biogel P-2 (fine grade) eluted with degassed, deionized water at 0.25 mL/min. Carbohydrates were detected by refraction index. All fractions containing carbohydrates were collected and lyophilized prior to structural analysis. For the reaction where xylose was employed as acceptor, the reaction mixture (15% maltose and 15% D-xylose) was separated by HPLC. Fractions containing the produced heterooligosaccharide (glucosyl-xylose) were collected and lyophilized. Product structures were analyzed by methylation analysis using the methylation protocol of Ciucanu and Kerek [13] and the hydrolysis, reduction and acetylation protocol of Harris et al. [14]. Partially methylated alditol acetates were analyzed by GC–MS.

GC–MS experiments were performed on a MAGNUM GC–MS ion trap system (Finnigan MAT, USA) equipped with heated inlet option (Spectronex AG, Basel, Switzerland).

The system used a Varian SPI injector (held at 125 °C and programmed to 250 °C at a rate of 125 °C/min) and a DB-5 ms capillary column (JW Scientic, USA) 30.0 m × 0.25 mm × 0.25 μm thickness. The carrier gas (He 99.996%) velocity was 33.1 cm/s (at 60 °C). The GC oven was maintained at 100 °C for 0.10 min, increased at 3 °C/min to a maximum of 250 °C and held for 5 min. Samples were introduced as 0.5 μL aliquots with a Hamilton syringe. The transfer line was held at 250 °C and ion manifold at 200 °C. The ion trap was tuned using the default software setting (Magnum 2.4., Finnigan MAT) to obtain suitable mass calibration, filament emission current, multiplier voltage and automatic gain control (AGC) settings. The GC–MS ion abundance test using *p*-1-bromo-4-fluorobenzene and bis(pentafluorophenyl)phenylphosphine was performed weekly to check the GC–MS system performance.

*NMR spectroscopy*.—All NMR data were acquired on a Bruker AVANCE DRX 500 spectrometer operating at 500.132 MHz for $^1$H NMR. Spectra were run at 33 °C in 99.95 % $Me_2SO$-$d_6$ (E. Merck). The double-quantum filtered COSY [15], TOCSY (DIPSI-2) [16] and phase-sensitive NOESY [17] experiments were collected as 256 × 1K complex points and processed into matrices of 1 × 1K complex points. The NOESY spectrum was acquired with a mixing time of 500 ms.

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