0273-2289/98/7502–3—0269$10.50

Synthesis of Oligosaccharides Catalyzed by Thermostable β-Glucosidase from *Pyrococcus furiosus*

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Received January 21, 1998; Accepted June 12, 1998

ABSTRACT

The thermostable β-glucosidase from *Pyrococcus furiosus* has been shown to produce tri- and tetrasaccharides with lactose as a substrate (0.73–1.44 mol/kg) at elevated temperatures (75–95°C). The enzyme has a broad pH optimum (5.0–7.0), and was inhibited more by glucose than by galactose. An increase in the initial lactose concentrations led to a proportional increase of the ratio between the initial oligosaccharide production rate and the initial lactose conversion rate. Because of inhibition by Maillard components, the lowest temperature studied (75°C) is the best for oligosaccharide synthesis. The oligosaccharide yield obtained with this thermostable enzyme is independent of the initial lactose concentration and temperature and a factor 1.4 higher than reported for mesophilic enzymes.

Index Entries: *Pyrococcus furiosus;* β-glucosidase; thermostable enzyme; oligosaccharide synthesis.

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INTRODUCTION

Oligosaccharides are polymeric carbohydrates consisting of 2–10 monomers. The ingestion of these oligosaccharides encourages the proliferation of, e.g., Bifidobacteria in the intestine, which is considered to be beneficial to human health *(1,2)*. There is a growing interest in the synthesis of oligosaccharides for application in functional foods; not only in Japan, but also in Europe, food manufacturers are producing and applying oligosaccharides *(3)*.

Enzymatic synthesis of oligosaccharides is possible with glycosidases because these enzymes, besides hydrolase activity, also show transferase activity. Hence, oligosaccharides are formed during the hydrolysis of disaccharides *(3)*. During transglycosylation, a glycosyl moiety is transferred from a donor to an acceptor molecule:

$$\underset{\textit{(Donor)}}{\text{Glycosyl–O–R}} + \text{Enzyme} \Leftrightarrows \text{Glycosyl–Enzyme} + \text{R–OH}$$

$$\text{Glycosy–Enzyme} + \underset{\textit{(Acceptor)}}{\text{R'–OH}} \Leftrightarrows \underset{\textit{(Product)}}{\text{Glycosyl–O–R'}} + \text{Enzyme}$$

This mechanism was first described for lactose hydrolysis by Wallenfels and Malhotra *(4)*. Using lactose as donor, the acceptor can be a water molecule (hydrolysis), lactose, or a trisaccharide (both transglycosylation). This results in the products galactose or a tri- or tetrasaccharide, respectively. The ratio between hydrolase and transferase activity is important for maximum oligosaccharide yield.

Lactose is an industrial disaccharide that is widely used for food applications. It has been reported that high initial lactose concentrations increase the oligosaccharide yields *(5,6)*. Higher initial lactose concentrations are possible at higher temperatures, and this requires a thermostable and thermoactive biocatalyst. Therefore, β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus (7)* was used in this study. The enzyme purified from *P. furiosus* exhibits high β-galactosidase activity and remarkable thermostability, with a half-life of 85 h at 100°C and 13 h at 110°C. In order to produce larger quantities of enzyme, the corresponding gene has been identified and overexpressed in *Escherichia coli*. The β-glucosidase produced by *E. coli* exhibits similar kinetic properties and a comparable stability as the β-glucosidase purified from *P. furiosus (8)*. Recently, this β-glucosidase has been used in transglycosylation reactions with various structurally different aglycones *(9)*. The free and immobilized enzyme showed a broad substrate selectivity by accepting primary and tertiary organic alcohols.

The aim of this research is to investigate the oligosaccharide synthesis with the thermostable β-glucosidase from *P. furiosus*, with specific attention to the effect of enzyme concentration, pH, glucose and galactose concentration, initial lactose concentration, and temperature.

MATERIALS AND METHODS

Materials

Lactose was from Sigma (St. Louis, MO) and the other chemicals were from Merck (Darmstadt, Germany). β-Glucosidase from *P. furiosus* (13 or 15 ONPG-units/μL) was prepared from an *E. coli* lysate and heated to denature proteins other than the enzyme as described previously *(8)*. Other chemicals were used without further purification.

Batch Experiments

In a typical batch experiment, 0.04 mol lactose was added to 20 mL 0.02 *M* sodium phosphate buffer (pH 5.0). The bottles were shaken by an end-over-end incubator (110 rpm) at 75°C. When the lactose was dissolved, 1500 U enzyme were added. The enzyme concentration was expressed in U/g solution and the initial lactose concentration in mol/kg solution.

The reaction was followed for approx 50 h. Samples (80 μL) were taken at regular time intervals, and the reaction was stopped by cooling on ice for 15 min. The samples were treated with $Pb(NO_3)_2$ (concentration in the sample, 0.1 mol/L), and stored in the freezer to accelerate precipitation (minimum time, 60 min). Before analysis, the samples were centrifuged 10 min at 13000 rpm and diluted.

Initial lactose conversion rate was expressed in mmol lactose/kg solution/min and was determined over 10% initial lactose decrease. The initial oligosaccharide production rate was expressed in mmol oligosaccharides (sum of tri- and tetrasaccharides)/kg solution/min. The relative oligosaccharide yield was defined as the experimental oligosaccharide yield (mol oligosaccharides/mol initial lactose) divided by the theoretical maximum oligosaccharide yield (0.5 mol oligosaccharides/mol lactose).

High-Performance Liquid Chromatography Analysis

The samples were analyzed by HPLC, using a RSO Oligosaccharide Column (Phenomenex, Bester, Amstelveen, the Netherlands) at 80°C. The column was eluted with double-distilled water (filtered through a regenerated cellulose membrane of 0.45 μm, and purged with helium gas) at a flow rate of 0.3 mL/min. The eluent was monitored by means of a refractive index detector. Lactose, glucose, galactose, and higher saccharides, such as tri- and tetrasaccharides, were detected.

RESULTS AND DISCUSSION

Time-Course Lactose Conversion and Oligosaccharide Synthesis

Figure 1 shows the lactose conversion and oligosaccharide synthesis by the *P. furiosus* β-glucosidase in a typical experiment. Lactose was hydrolyzed to glucose and galactose, and, in addition, tri- and tetrasaccharides were produced. The synthesis of pentasaccharides was not observed under these conditions.

Enzyme Concentration

The β-glucosidase concentration was varied to determine if the enzyme activity was linear with its concentration. Figure 2 shows that the initial lactose conversion rate and the initial oligosaccharide production rate were linear with the enzyme concentration. The ratio (initial oligosaccharide production rate/initial lactose conversion rate) was also linear with the enzyme concentration, and showed a minor decrease with the enzyme concentration. This suggests a dependence of the ratio on the enzyme concentration. Further experiments were carried out with an enzyme concentration of 75 U/g.

pH

Figure 3 shows that the initial lactose conversion rate was independent of the pH from 5.0–7.0, and was lower at pH 4.0. Kengen et al. *(7)* found an optimum pH at 5.0 for the hydrolysis of GlcpβNp in a sodium citrate buffer at 80°C. The ratio (initial oligosaccharide production rate/initial lactose conversion rate) did not depend on the pH in this range (data not shown). No decrease in the relative oligosaccharide yield was measured within the time range of the experiments, except for pH 4.0. Hence, for the other experiments, the yield after 45 h was taken. The relative oligosaccharide yield after 45 h was highest at pH 6.0 (Fig. 3).

It was observed that Maillard reactions occurred during the experiments. This is the reaction between a carbonyl group of a reducing sugar with a free, uncharged amine group of an amino acid or protein *(10)*. The yellow color caused by these reactions intensified with increased pH. It is known that Maillard reactions are suppressed at low pH *(10)*, and hence further experiments were performed at pH 5.0.

Glucose and Galactose Concentration

Deschavanne et al. *(11)* found that most β-galactosidases are inhibited by the monosaccharides produced. To investigate product inhibition with the β-glucosidase from *P. furiosus*, experiments with additional glucose

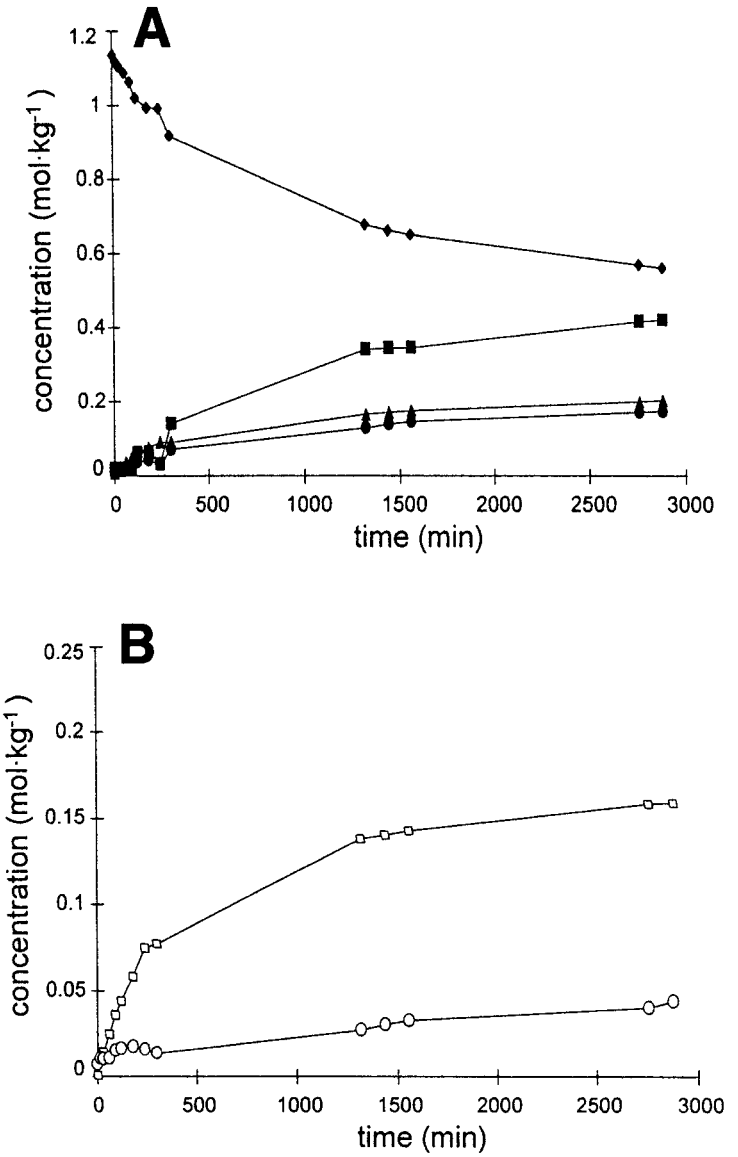

Fig. 1. **(A)** Time-course of lactose conversion (◆, lactose; ■, glucose; ●, galactose, and ▲, oligosaccharide) and **(B)** oligosaccharide synthesis (□, trisaccharide; ○, tetrasaccharide). Experimental conditions: initial lactose concentration, 1.16 mol/kg; enzyme concentration, 75 U/g; sodium phosphate buffer, pH 5.0 at 75°C.

and galactose were done. Figure 4 shows that the enzyme was inhibited significantly by glucose, and to a lesser extent by galactose. Galactose inhibition will be of minor importance, because the galactose concentration is relatively low during oligosaccharide synthesis.

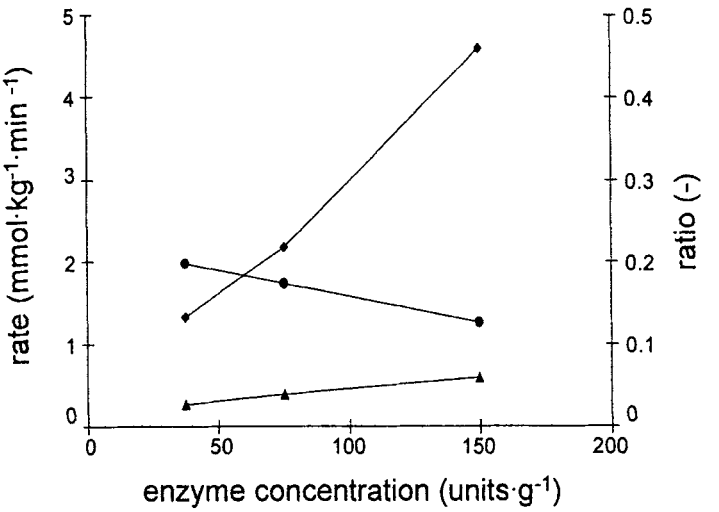

Fig. 2. The initial lactose conversion rate (positive value) (◆), the initial oligosaccharide production rate (▲), and the ratio (initial oligosaccharide production rate/initial lactose conversion rate) (●) as function of the enzyme concentration (lines for guidance). Experimental conditions: initial lactose concentration, 1.16 mol/kg; sodium phosphate buffer, pH 5.0 at 75°C.

Initial Lactose Concentration and Temperature

Table 1 shows the conversion rates and the ratio as functions of temperature and initial lactose concentration. As expected, the initial lactose conversion rate and the oligosaccharide production rate both increased with increasing temperature. The rates did not appear to depend on the initial lactose concentration, but the ratio did increase slightly with the initial lactose concentration. This confirmed the anticipated higher oligosaccharide yields at higher initial lactose concentrations *(5,6)*.

Figure 5A shows the relative oligosaccharide yield as function of the initial lactose concentration at different temperature. The oligosaccharide yield was not very dependent on the initial lactose concentration and temperature. Onishi et al. *(12)* compared the production of oligosaccharides at 30°C by several microorganisms, and found relative yields below 0.33. The highest relative yield measured here for the β-glucosidase from *P. furiosus* is 0.46. This is about the same as reported for the thermostable β-galactosidase from *Sterigmatomyces elviae (13)*. Experiments with excess enzyme amounts did not result in higher yields (Fig. 5B). This suggests enzyme inactivation.

The enzyme inactivation is most likely caused by Maillard products. The enzyme inactivation observed in sugar solutions was high compared

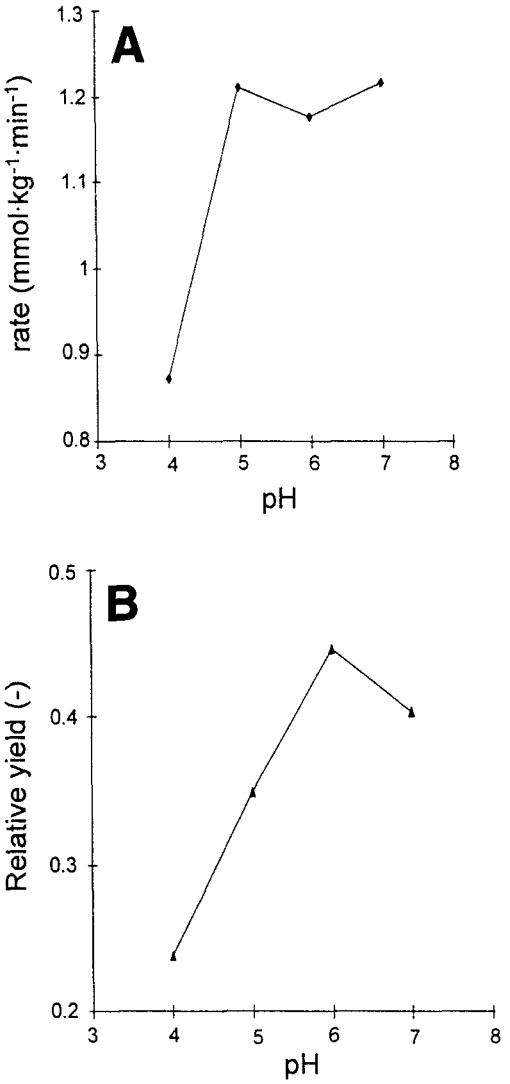

Fig. 3. The initial lactose conversion rate (positive value) (◆), and the relative oligosaccharide yield after 45 h (▲) as function of the pH (lines for guidance). Experimental conditions: initial lactose concentration, 1.16 mol/kg; enzyme concentration, 75 Units/g; 0.02 *M* sodium phosphate buffer at 75°C.

to water *(7)*, in which the half-life was 85 h at 100°C and 13 h at 110°C. Inactivation caused by Maillard products (and inhibition by glucose) might be avoided in a continuous operating process. The stability of the β-glucosidase may also be further increased by immobilization *(9)*. This would result in higher oligosaccharide yields.

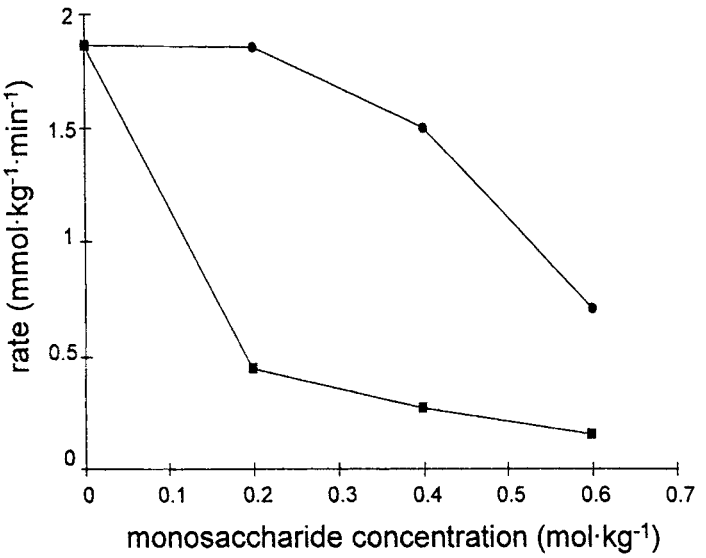

Fig. 4. The initial lactose conversion rate (positive value) as function of the glucose (■) and galactose concentration (●) (lines for guidance). Experimental conditions: initial lactose concentration, 0.40 mol/kg; enzyme concentration, 75 U/g; sodium phosphate buffer, pH 5.0 at 75°C.

Table 1
Initial Lactose Conversion Rate (Positive Value), Initial Oligosaccharide Production Rate, and Ratio (Initial Oligosaccharide Production Rate/Initial Lactose Conversion Rate) as Function of Initial Lactose Concentration and Temperature

Initial lactose (mol/kg)	Initial lactose conversion rate (mmol/kg/min)			Initial oligosaccharide production rate (mmol/kg/min)			Ratio (−)		
	75°C	85°C	95°C	75°C	85°C	95°C	75°C	85°C	95°C
0.73	1.6	—	—	0.32	—	—	0.20	—	—
0.93	1.4	2.8	3.4	0.31	0.68	1.1	0.23	0.24	0.32
1.01	1.6	—	—	0.40	—	—	0.25	—	—
1.09	1.1	—	—	0.30	—	—	0.28	—	—
1.16	0.89	1.9	3.3	0.45	0.65	1.3	0.50	0.35	0.39
1.23	—	2.0	—	—	0.71	—	—	0.36	—
1.29	—	2.1	—	—	0.79	—	—	0.37	—
1.37	—	1.5	2.4	—	0.65	0.79	—	0.42	0.33
1.44	—	—	2.2	—	—	0.94	—	—	0.43

Experimental conditions: enzyme concentration, 75 U/g, sodium phosphate buffer, pH 5.0.

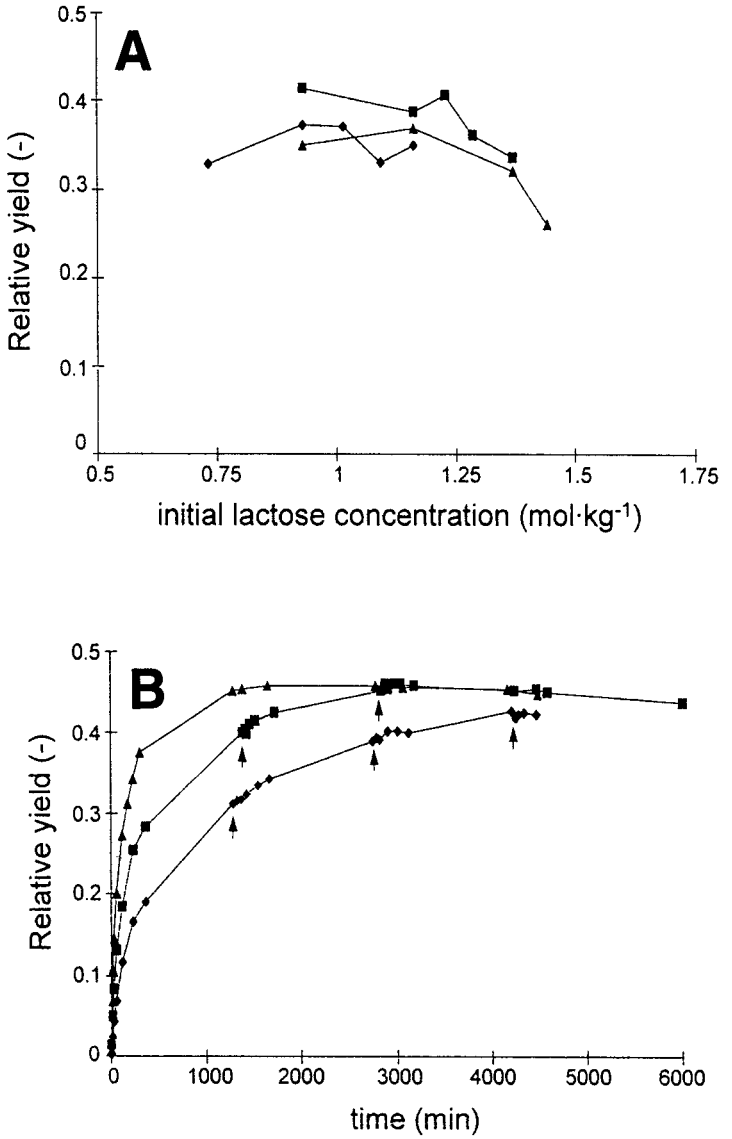

Fig. 5. **(A)** Relative oligosaccharide yield as function of the initial lactose concentration at different temperatures and **(B)** time-course of relative oligosaccharide yield at different temperatures (◆, 75°C; ■, 85°C; ▲, 95°C) (lines for guidance). Experimental conditions in (A): enzyme concentration, 75 U/g; sodium phosphate buffer, pH 5.0. Experimental conditions in (B): initial lactose concentration, 1.16 mol/kg; enzyme concentration, 75 U/g, except at 95°C: 150 U/g (arrows indicate addition of 750 U enzyme); sodium phosphate buffer, pH 5.0.

CONCLUSION

Synthesis of oligosaccharides by the *P. furiosus* β-glucosidase was observed under all reaction conditions studied in this research. With an enzyme concentration of 75 U/g, the enzyme activity is linear with the enzyme concentration, and lactose conversion is accomplished in an acceptable time. The *P. furiosus* enzyme has a broad pH optimum, which allows operation at a low pH (5.0), in order to diminish the production of Maillard components. The production of inhibiting Maillard components is more pronounced at higher temperatures, and therefore oligosaccharide synthesis appears to be best at the lowest temperature (75°C) studied. Increased initial lactose concentrations resulted in increased oligosaccharide concentrations, but did not affect the oligosaccharide yields. However, the oligosaccharide yield obtained with the *P. furiosus* β-glucosidase was higher than reported for mesophilic enzymes by a factor 1.4.

ACKNOWLEDGMENTS

This work was partly supported by the Association of Biotechnological Schools (ABON), Borculo Whey Products, and the European Union FAIR contract CT-96-1048.

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