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Enzymatic Production of Galactooligosaccharide by *Bullera singularis* β-Galactosidase

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Abstract Galactooligosaccharides (GalOS) were efficiently produced by partially purified β-galactosidase from the yeast strain *Bullera singularis* ATCC 24193. Ammonium sulfate precipitation and ultrafiltration methods were used to prepare the enzyme. The enzyme activity decreased at 50°C and above. A maximum yield of 40% (w/w) GalOS, corresponding to 120 g of GalOS per liter, was obtained from 300 g per liter of lactose solution at 45°C, pH 3.7 when the lactose conversion was 70%. The yield of GalOS did not increase with increasing initial lactose concentration but the total amounts of GalOS did. Volumetric productivity was 4.8 g of GalOS per liter per hour. During this reaction, the by-products, glucose and galactose, were found to inhibit GalOS formation. Reaction products were found to be comprised of disaccharides and trisaccharides according to TLC and HPLC analyses. We propose the structure of the major product, a trisaccharide, to be *o*-β-D-galactopyranosyl-(1-4)-*o*-β-D-galactopyranosyl-(1-4)-β-D-glucose (4'-galactosyl lactose).

Key words: Galactooligosaccharide (GalOS), β-galactosidase, *Bullera singularis*, lactose hydrolysis, functional oligosaccharide

β-D-Galactosidase (EC 3.2.1.23, β-gal) catalyzes both the hydrolysis of β-D-galactosidic linkage and the transgalactosylation (or transgalactosidation) reaction to produce galactooligosaccharides (GalOS). The major industrial application of β-gal is the hydrolysis of lactose to facilitate milk digestibility and to improve the functional properties of dairy products [5, 8]. GalOS, on the other hand, are recognized as the growth-promoting factors of intestinal bifidobacteria and hence, molecules of interest for human health. GalOS are $(galactosyl)_n$-lactose oligomers, where n may vary from 2 to 4. They have useful health effects such as reduction of toxic metabolites and detrimental enzymes, prevention of diarrhea and constipation, protection of liver functions, reduction of serum cholesterol, reduction of blood pressure, and anticancer effects [17, 28]. GalOS are also currently being used as food ingredients and cosmetic additives in several Asian and European countries. The commercial product is a mixture of tetrasaccharide (Gal-Gal-Gal-Glc), trisaccharide (Gal-Gal-Glc), lactose, glucose, and galactose. The structural elucidation of transgalactosylation products shows the prevailing formation of β-(1,6) linkages. The synthesis reaction, however, can also form other linkages, such as β-(1,4) galactosyl linkage [6, 16].

GalOS can be produced enzymatically or by fermentation. Lactose serves as a galactosyl donor and an acceptor to yield di-, tri-, or higher oligosaccharides. The enzymatic process has served advantages over the fermentation; a high substrate concentration can be applied, the reaction condition is simple, the process control is relatively easy, and the purification step can be simplified. The amounts and structures of the oligosaccharides depend on the source of enzymes [24]. The enzymatic syntheses of GalOS from lactose have been reported by many investigators [18-29] and GalOS are being commercially produced by bacterial (*Bacillus circulans*) [14] and fungal (*Aspergillus oryzae* [13]; *Cryptococcus laurentii* [18]) β-gal, using lactose as substrate. However, the productivity is rather low. Therefore, the development of a more efficient and inexpensive method for GalOS production is highly desirable.

Bullera singularis (formerly, *Sporobolomyces singularis*) uses the glucose portion of lactose as energy source and transfers the galactosyl moiety to unused lactose to form GalOS with a maximum yield of 50% [6]. Gorin *et al.* reported the synthesis of GalOS by a crude extract of

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B. singularis disrupted in a modified Hughes press [7]. However, there were no information on the optimization of GalOS production process have been reported. Previously, we reported the culture conditions of *B. singularis* for the optimum production of GalOS [25]. Here, we discuss the optimized GalOS production by the enzyme obtained by ammonium sulfate precipitation and ultrafiltration. The characteristics of production are also presented.

MATERIALS AND METHODS

Materials

Lactose and other carbohydrates were purchased from Difco (Detroit, U.S.A.), Tokyo Chemical Industry (Japan) and Sigma (St. Louis, U.S.A.). Oligosaccharide standards were from Daiwa Kasei KK (Japan) and Sigma. All other biochemical reagents were of analytical grade. *Bullera singularis* ATCC 24193 was obtained from American Type Culture Collection (ATCC).

Preparation of β-Galactosidase Solution

B. singularis ATCC 24193 was subcultured weekly in a lactose-containing medium at 25°C. The culture broth of *B. singularis* was prepared as previously described [25]. The culture medium was centrifuged at 7,000×g and resuspended in 100 mM phosphate buffer (pH 6.0). The suspension was ground with the equal volume of zirconium beads using Bead Beater (Biospec Products, U.S.A.) at full speed, centrifuged for 20 min at 15,000×g at 4°C, and then passed through a 0.45 μm membrane filter to remove fine debris. Ammonium sulfate was added to the supernatant to precipitate protein according to the precipitation table (20~80% cut). The precipitate was recovered by centrifugation at 16,000×g for 30 min and resuspended in the same amount of the buffer. The supernatant was finally concentrated with an 8010 ultrafiltration unit (Amicon, U.S.A.) equipped with an XM-50 membrane (Amicon, U.S.A.) having a molecular weight cut-off of 50,000 Da.

Oligosaccharide Synthesis

GalOS synthesis was performed in a screw-cap flask in the pH range of 3.5 to 6 at 40~45°C in the presence of purified β-gal. We added 0.5 ml of enzyme solution (0.8 U/ml) in 100 mM phosphate buffer (pH 6.0) to 9.5 ml lactose solution containing different lactose concentrations (5~30%) and the mixture was incubated in a water bath. The reaction was stopped by heating at 100°C for 10 min. The mixture taken from the flask was filtered through a 0.45 μm membrane to remove inactivated enzymes and insoluble particles.

Assay of Carbohydrates

All saccharides produced from the reaction were analyzed with Waters high-performance liquid chromatography (HPLC) systems (Balford, MA, U.S.A.) fitted with a Phenomenex IB-Sil NH_2 column (250×4.6 mm) maintained at an ambient temperature. A mixture of acetonitrile-water (3:1) was used as the mobile phase with a flow rate of 1.2 ml/min. Under these conditions a significant amount of disaccharides and trisaccharides were detected while tetrasaccharides were less than 1% (w/w).

Thin-layer chromatography (TLC) was performed on a Merck TLC plate (kiegelgel 60) with the solvent system of *n*-butanol-pyridine-water (6:2.5:1.5). Carbohydrates on the plate were detected by heating at 120°C for 15 min after spraying with anisaldehyde-sulphuric acid solution.

Lactose conversion and GalOS yield were calculated according to the following formulae;

$$\text{Lactose conversion (\%)} = 100\ (\%) \times (\text{initial lactose} - \text{residual lactose})/\text{initial laetose}$$

$$\text{GalOS yield (\%)} = 100\ (\%) \times \text{GalOS produced}/\text{total saccharides}$$

Assay of β-Galactosidase Activity

β-Galactosidase activity was estimated using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate [31]. A mixture containing 0.25 ml of 5 mM ONPG in 100 mM phosphate buffer (pH 6.0) and 0.25 ml of enzyme solution was incubated, at 40°C for 60 min. The reaction was stopped by the addition of 2.0 ml of 10% (w/v) Na_2CO_3 to the reaction mixture and the *o*-nitrophenol released was determined by measuring the absorbance at 420 nm with an HP 8452A spectrophotometer (Hewlett Packard, U.S.A.). One unit (U) of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of *o*-nitrophenol per min under the conditions employed.

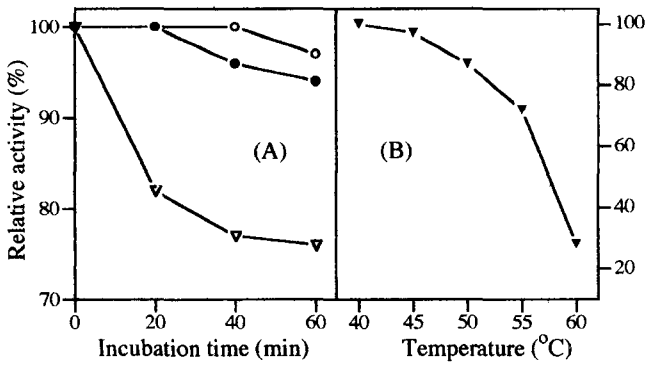

Fig. 1. Effect of high temperature on the activity of *B. singularis* β-galactosidase.

(A) A time course of enzyme activity at 40°C (○), 45°C (●), 50°C (▽) in 100 mM phosphate buffer (pH 6.0). (B) Enzyme actvity after 10 min incubation at various temperatures.

RESULTS AND DISCUSSION

Selection of Reaction Temperature

The aqueous enzyme solution was heated for 10 min at various temperatures and residual enzyme activity was measured by ONPG hydrolysis. The enzyme activity was stable up to 45°C, but rapidly decreased above 50°C (Fig. 1). Further GalOS production experiments with *B. singularis* β-gal were therefore performed at 45°C.

Transgalactosylation Reaction

Figure 2 shows the time course of GalOS production in the reaction of 300 g/l lactose with the *B. singularis* β-gal at pH 3.7 and 45°C. The amount of GalOS produced reached the maximum after 30 h. After that, a plateau in the GalOS curves was observed for longer reaction times. This is different from the results reported for β-gal of mesophilic microorganisms, where GalOS eventually decreased as a result of hydrolysis [3, 9, 13, 14, 18, 23, 27]. Instead, the *B. singularis* β-gal gave the reaction pattern of thermophilic microorganisms [2, 15, 26]. About 40 g of GalOS could be obtained from 100 g lactose (40%, w/w). This amount is one of the highest values of GalOS yield ever reported [2, 11-15, 23, 26, 30].

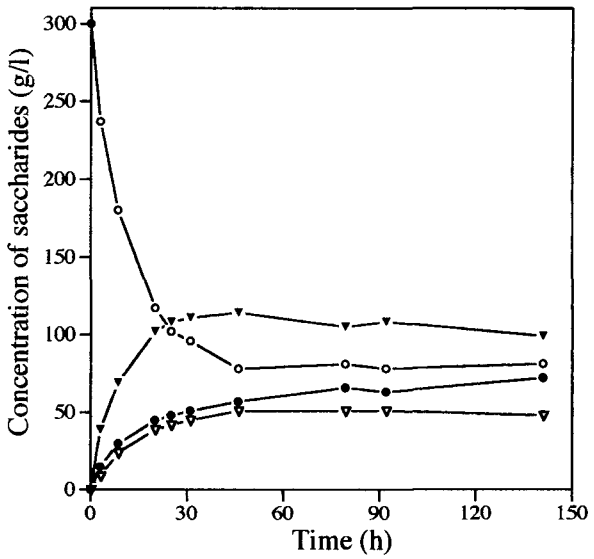

Fig. 2. Time course of GalOS production by β-galactosidase from *B. singularis*.

A mixture containing 0.5 ml of enzyme solution (0.8 U/ml) and 300 g/l of lactose in 10 ml distilled water (pH 3.7) was incubated at 45°C. Saccharides: lactose (○); monosaccharides (glucose plus galactose) (●); disaccharides (▽); trisaccharides (▼).

GalOS production catalyzed by β-gal using lactose, was first demonstrated by Wallenfels [29] and the reaction mechanism responsible was proposed to be trangalactosylation [22, 24]. Prenosil *et al.* reviewed the yield of GalOS produced from lactose by transgalactosylation catalyzed by various β-gal, which ranges between 5% and 45% depending mainly on initial lactose concentration (40~350 g/l) and sources of the enzyme [24]. The representative results of enzymatic batch production of GalOS are summarized in Table 1 including our result. Our system can be considered as an alternative for enzymatic GalOS production due to high yield and simple reaction condition.

Effect of Initial Lactose Concentration

Generally, the yield of oligosaccharides present in the total sugar increased with an increase in the initial lactose concentration. However, *B. singularis* β-gal showed a different behavior. As shown in Fig. 3, the yield of GalOS did not increase as initial lactose concentration increased from 50 to 300 g/l. The limit was the solubility of lactose at 45°C. However, the total amount of GalOS produced increased with the increasing initial concentration of lactose. Thus, the use of high concentrations of lactose was required only to increase volumetric productivity of GalOS (gram-GalOS per reactor volume).

When the GalOS yield is presented as a function of lactose conversion, the yield was not maximal until

Table 1. Batch production of galactooligosaccharides (GalOS) by β-galactosidases from lactose solution[a].

Enzyme source		Reaction condition	Productivity (g/l·h)[b] (GalOS yield, w/w %)	Reference
B. circulans	4.56%	lactose at 40°C and pH 6.0	2.2 (24)	14
C. laurentii	2.5%	lactose at 50°C and pH 5.0	0.35 (28.2)	18
S. rectivirgula	60%	lactose at 70°C and pH 7.0	12.3 (41)	15
T. aquaticus	16%	lactose at 70°C and pH 4.6[c]	2.3 (34.8)	2
S. elvae	20%	lactose at 60°C and pH 5.0	3.25 (39)	20
A. oryzae	38%	lactose at 40°C and pH 4.5	24.3 (32)	11
S. elvae	36%	lactose at 60°C and pH 6.0[d]	6.75 (37.5)	19
S. magnum	20%	lactose at 60°C and pH 5.0	3.0 (36)	21
B. singularis	30%	lactose at 45°C and pH 3.7[e]	4.8 (40)	This work

[a]GalOS produced from the hydrolysis of galactan were excluded in this Table. Lactose concentrations are their initial values in the reactions. [b]Volumetric productivity in batch process is expressed as grams of oligosaccharides per liter per h and GalOS yield is a weight percent of oligosaccharides (tri- and over) based on the total weight of saccharides in the reaction medium. [c]Immobilized enzyme was used. [d]Toluene-treated whole cell biocatalyst was used. [e]Unbuffered lactose solution was used.

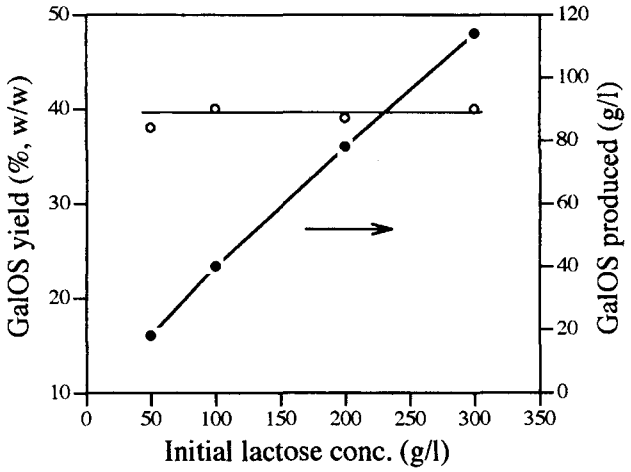

Fig. 3. Effect of initial lactose concentration on GalOS yield (○) and amount (●). Reactions were performed at 45°C.

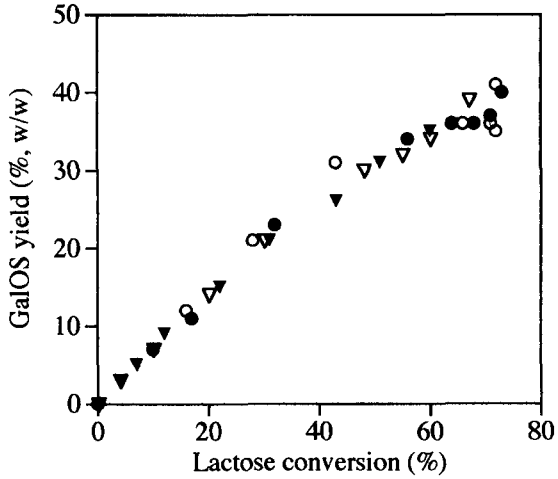

Fig. 4. Relationship between conversion of lactose and amount of GalOS produced by *B. singularis* β-galactosidase.

Initial lactose concentration (g/l): 50 (○); 100 (●); 200 (▽); 300 (▼).

lactose conversion reached 70% (Fig. 4). These findings are contradictory to the results by several investigators reporting a parabolic pattern of GalOS production with conversion [13, 14, 18, 23, 26, 27]. The pattern described here for the *B. singularis* β-gal is unique.

Effect of pH and Temperature on GalOS Production

The effect of pH on the production of GalOS was examined during 140 h of incubation; the amount of GalOS produced and the reaction pattern did not vary in the pH range of 3.5 to 6 (data not shown).

Oligosaccharide formation was not significantly affected between 40° and 45°C when the initial lactose concentration was 300 g/l. In addition, salts such as sodium and potassium seemed to have no effect on GalOS production (data not shown). Meanwhile, the activity of β-gal of *Streptococcus thermophilus* was

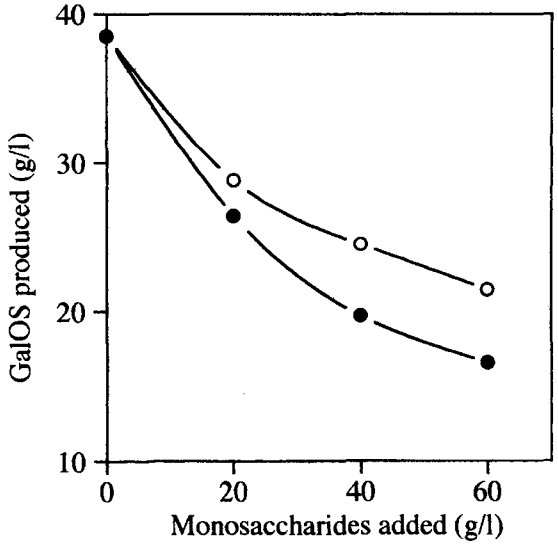

Fig. 5. Effect of monosaccharide concentration on GalOS production.

Mixture containing 0.5 ml of enzyme solution and 100 g/ml of lactose in 100 mM potassium phosphate buffer (pH 6.0) and the indicated concentration of glucose in total volume of 10 ml were incubated at 45°C for 50 h. Monosaccharides: galactose (○); glucose (●).

reported to be independent of change in pH and the presence of the inhibitor Na^+ [26].

Inhibition by Glucose and Galactose

The effect of monosaccharides (glucose and galactose) on GalOS production was examined (Fig. 5). The amount of GalOS production decreased as the amount of monosaccharides increased, which indicated that glucose and galactose inhibited GalOS production.

Galactose is generally considered as a competitive β-gal inhibitor, competing with lactose for the active site of the enzyme [24]. The greater part of the galactosyl moiety is transferred to adjacent lactose due to the high transgalactosylation activity of *B. singularis* β-gal. Thus, glucose can inhibit transgalactosylation more strongly than galactose. Therefore, GalOS productivity could be improved by removing glucose from the reaction mixture. In order to remove glucose, Onishi *et al.* introduced a cell culture following the enzymatic reaction. This method resulted in the yield 64% of GalOS from 36% lactose [19]. Yun and Song used glucose oxidase to produce pure and high-content fructooligosaccharide [32].

Identification of GalOS Produced

The major product, a trisaccharide (in Fig. 2), had the same retention time (6.3 min) as authentic *o*-β-D-galactopyranosyl-(1-4)-*o*-β-D-galactopyranosyl-(1-4)-*o*-β-D-glucose (4'-galactosyl lactose) on HPLC analysis (data not shown).

Gorin *et al.* reported that *B. singularis*, by transgalactosylation, produced a trisaccharide 4'-galactosyl lactose as a main product and a tetrasaccharide with the same configuration as a minor one [6]. In light of these earlier results, the main transgalactosyl product in the present work could be identified as 4'-galactosyl lactose. Elucidation of the disaccharide structure is on the way. The structure of GalOS was the same as that of the trisaccharide formed by *C. laurentii* [16], *B. circulans* [14], and *Sterigmatomyces elviae* [12]. Another trisaccharide, *o*-β-D-galactopyranosyl-(1-6)-*o*-β-D-galactopyranosyl-(1-4)-*o*-β-D-glucose (6'-galactosyl lactose), was reportedly formed by *A. oryzae* [27], *S. lactis* [3], and *P. chrysogenum* [1]. Yet another, *o*-β-D-galactopyranosyl-(1-3)-*o*-β-D-galactopyranosyl-(1-4)-*o*-β-D-glucose (3'-galactosyl lactose), was formed by *A. oryzae* [11] and by *Bifidobacterium bifidum* [4].

CONCLUSIONS

The β-galactosidase from *B. singularis* was able to produce GalOS in high yields during the hydrolysis of lactose. Conversion of lactose (300 g/l) to oligosaccharide gave a yield of 40%, corresponding to 120 g/l. The GalOS yield remained the same with increasing initial lactose concentrations but a higher lactose concentration increased GalOS productivity. The oligosaccharides consisted of disaccharides and trisaccharides. The *B. singularis* β-galactosidase provides exceptional advantages, not being affected by environmental factors such as temperature, pH, presence of salts, and initial lactose concentration. To improve the yield suitable for the commercial production of GalOS, a process considering glucose removal and enzyme immobilization may be applicable.

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