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Enzymatic Production of Galactooligosaccharides by β-Galactosidase from *Bifidobacterium longum* BCRC 15708

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The production of galactooligosaccharides (GOSs) by transgalactosylation using β-galactosidase from *Bifidobacterium longum* BCRC 15708 was studied. Other than lactose, galactose, and glucose, two types of GOSs, tri- and tetrasaccharides, were formed after β-galactosidase action on 40% lactose. Trisaccharides were the major type of GOS formed. Generally, an increase of the initial lactose concentration in the reaction mixture resulted in a higher GOS production. A maximum yield of 32.5% (w/w) GOSs could be achieved from 40% lactose solution at 45 °C, pH 6.8, when the lactose conversion was 59.4%. The corresponding productivity of GOSs was 13.0 g/(L·h). Transgalactosylation activity of β-galactosidase from a test organism showed a relatively lower sensitivity toward glucose and galactose than that from other organisms. The addition of 5% or 10% glucose or galactose to the reaction mixture did not significantly ($p > 0.05$) reduce the transgalactosylation reaction of β-galactosidase.

KEYWORDS: ***Bifidobacterium longum*; β-galactosidase; galactooligosaccharides; transgalactosylation**

INTRODUCTION

Galactooligosaccharides (GOSs) are (galactosyl)$_n$lactose oligomers, where *n* may vary from 2 to 4, and they are synthesized from lactose by a transgalactosylation reaction, catalyzed by β-galactosidase (EC 3.2.1.23) (*1*). GOSs have many useful health benefits such as the suppression of serum phenol and *p*-cresol levels, prevention of diarrhea and constipation, reduction of serum cholesterol and blood pressure, and prevention of colon cancer (*2*−*5*). Furthermore, GOSs are selectively utilized by bifidobacteria and added to nutritional foods such as yogurt to improve the growth of beneficial intestinal flora (*4*, *6*). Currently, various commercial products with GOSs are available in the market, and they usually contain a mixture of tetrasaccharide, trisaccharide, lactose, glucose, and galactose (*5*).

The linkage between D-galactose units and components in GOSs depends on the source of the enzyme and conditions used in the reactions (*7*). Generally, GOS production by transgalactosylation activity of β-galactosidase increases by increasing the initial concentrations of lactose in the reaction mixture, while the effects of pH on the reaction have been reported to be rather small (*7*−*9*). Moreover, most of the β-galactosidases used in these studies are not approved for food use (*8*, *10*).

Bifidobacterium, a probiotic organism, as well as its β-galactosidase, is generally recognized as safe (GRAS) and has been used in the food system for a long time (*11*). Along with β-galactosidase from *Lactobacillus reuteri* (*12*), we have previously noted that *Bifidobacterium longum* BCRC 15708 may potentially be an industrial strain for the production of β-galactosidase, owing to its GRAS nature, coupled with its high yield of β-galactosidase with corresponding high levels of transgalactosylation activity (*13*). Furthermore, the various properties of β-galactosidase produced by this test organism have been further purified and characterized (*14*). In this study, we further explored the production of GOSs by β-galactosidase from *B. longum* BCRC 15708. The effects of initial lactose concentrations, pH values, reaction temperatures, glucose, and galactose on the transgalactosylation action of this enzyme were examined.

MATERIALS AND METHODS

Microorganism. *B. longum* BCRC 15708 was obtained from the Food Industry Research & Development Institute, Hsinchu, Taiwan, and used as the test organism.

Culture Condition and β-Galactosidase Preparation. The organism was inoculated into MRS broth (Difco, Detroit, MI) supplemented with 0.05% L-cysteine (MRSC broth, Sigma, St. Louis, MO) at 37 °C for 12 h. Cells were harvested by centrifugation (10000*g* for 10 min at 4 °C), then diluted with the same medium to a population of ca. 10^9 CFU/mL, and used as the inoculum.

For β-galactosidase production, a 20% (v/v) inoculum was inoculated into a 5 L jar fermenter (BR 5, Exon Science Inc., Taipei, Taiwan) consisting of a cylindrical culture vessel with a working volume of 3 L. This fermenter was equipped with Exon BR 5 software (BR 5, Exon Science Inc.) to control the temperature, pH, and agitation speed. The culture medium consisted of 4% lactose, 3.5% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.03% L-cysteine. The pH of the medium was maintained at 6.5 by adding sterile 6 N NaOH

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or HCl. The culture temperature and agitation speed were controlled at 37 °C and 100 rpm, respectively.

For enzyme extraction, the detailed procedure was described in our previous paper (*13*). The culture of *B. longum* BCRC 15708 was centrifuged (10000*g*, 10 min) at 4 °C. The pellets were washed twice with a 0.03 M sodium phosphate buffer (pH 6.8), resuspended in the same buffer, and then subjected to ultrasonic treatment (model 300, Misonix, Farningdale, NY). The extract was centrifuged at 15000*g* at 4 °C for 10 min, and the supernatant obtained was used as the crude enzyme solution.

GOS Formation by β-Galactosidase. GOS formation with β-galactosidase was studied in a screw-cap flask. The reaction mixture (100 mL) containing crude enzyme (2000 U) and 40% lactose (sigma), dissolved in 0.03 M sodium phosphate buffer (pH 6.8, unless otherwise specified), was incubated in a 45 °C water bath. Samples (1 mL) were taken from the flask at appropriate time intervals, terminated by heating at 100 °C for 10 min to inactivate the enzyme, then diluted, and filtered through a 0.45 μm membrane to remove insoluble particles. The amounts of GOSs and other saccharides produced were assayed by high-performance liquid chromatography (HPLC). When the optimal conditions for the transgalactosylation reaction were examined, the reactions were studied at six different initial lactose concentrations (5%, 10%, 20%, 30%, 40%, and 50%), five different pH values (4.8, 5.8, 6.8, 7.8, and 8.8), and five different temperatures (25, 35, 45, 55, and 65 °C) for 10 h. To examine the effects of glucose and galactose on transgalactosylation, the reaction mixture was added with various concentrations (5−20%) of glucose or galactose to the lactose solution.

Determination of β-Galactosidase Activity. The determination of β-galactosidase activity was consistent with the method followed in our previous research (*13*). The reaction mixture was composed of 0.5 mL of the enzyme source and 0.5 mL of 15 mM *o*-nitrophenyl β-D-galactopyranoside (ONPG) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 37 °C, 2.0 mL of 0.1 M sodium carbonate was added to the reaction mixture to stop the reaction. Absorbance was measured at 420 nm with a spectrophotometer (model Helios α, Unicam Co., Cambridge, U.K.). One unit of β-galactosidase activity was defined as the amount of enzyme producing 1 μmol of *o*-nitrophenol/min under the assay condition.

Determination of GOSs, Glucose, Galactose, and Lactose. The amounts of GOSs and other saccharides were determined by HPLC, and the detailed procedure and HPLC system were described in our previous study (*13*). The HPLC system consisted of a degassing system (model DG-2410, Sanwa Tsusho Co., Tokyo, Japan), a pump (880-LC, Jasco Co., Tokyo, Japan), a carbohydrate analysis column (Rezex RNM carbohydrate column, 7.8 × 300 mm, Phenomenex Co., California), a guard column (Rezex RNM carbohydrate column, 7.8 × 50 mm, Phenomenex Co.), a column heater (800-LC, Jasco Co.), a refractive index detector (830-RI, Jasco Co.), and a chromatography data system (SISC Co., California). The eluent was predegassed distilled water at a flow rate of 0.4 mL/min. The column temperature was maintained at 85 °C, and the detector temperature was set at 45 °C.

The lactose conversion and GOS yield were calculated according to the following formulas:

$$\text{lactose conversion (\%)} = \frac{(\text{initial lactose content} - \text{residual lactose content})}{\text{initial lactose content}} \times 100$$

$$\text{GOS yield (\%)} = \frac{\text{GOS produced}}{\text{total saccharides}} \times 100$$

Total saccharides is the sum of the saccharides including glucose, galactose, lactose, and GOSs present in the reaction solution.

Statistical Analysis. In this study, the mean values and the standard deviation were calculated from the data obtained with triplicate trials. These data were then compared by Duncan's multiple range method (*15*).

RESULTS AND DISCUSSION

Formation of GOSs by β-Galactosidase from *B. longum* BCRC 15708. GOSs are produced from lactose by a transgalactosylation reaction, catalyzed by β-galactosidase (*1*, *16*). Moreover, the linkage between D-galactose units and components in the final product depends upon the source of the enzyme (*7*, *17*). Berger et al. (*18*) and Splechtna et al. (*19*), respectively, showed that using *Thermus aquaticus* YT-1 and *L. reuteri* β-galactosidase for transgalactosylation yielded two types of GOSs, tri- and tetrasaccharides. With *Saccharomyces fragilis* β-galactosidase, 12 GOSs were detected on paper chromatograms, while 10 GOSs were formed with the β-galactosidase from *Aspergillus niger* (*17*). β-Galactosidase from *Kluyveromyces lactis* and *Kluyveromyces fragilis* produced mainly trisaccharides (*20*). On the other hand, a considerable amount of tetra- and pentasaccharides were synthesized by the β-galactosidase from *Bacillus circulans* (*7*).

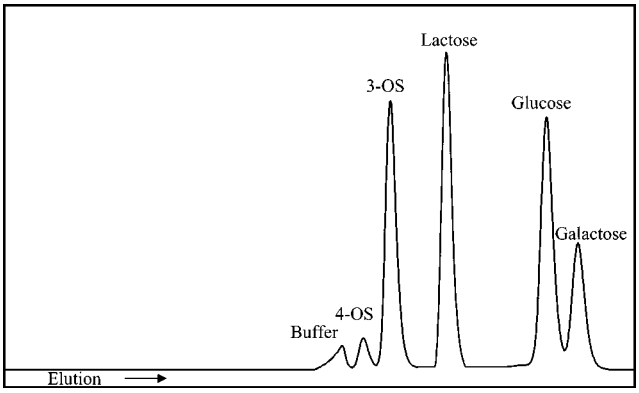

Figure 1. HPLC elution pattern of the reaction products formed after 10 h of transgalactosylation by β-galactosidase from *B. longum* BCRC 15708. The reaction was performed at 45 °C and pH 6.8 with an initial lactose concentration of 40% in the reaction solution containing 2000 U of β-galactosidase. Elution time (min): 11.5 for sodium phosphate buffer, 12.4 for tetrasaccharide (4-OS), 13.4 for trisaccharide (3-OS), 15.3 for lactose, 18.9 for glucose, and 20.0 for galactose.

Figure 1 shows a typical HPLC chromatogram of sugars with β-galactosidase from *B. longum* BCRC 15708 in the reaction mixture after 10 h of observation. Only two types of GOSs, tri- and tetrasaccharides, were noted. Concentrations of the former and latter were 12.7% and 1.2% (w/v), respectively. Our results were similar to those observed for β-galactosidase of *A. oryzae* (*8*), *Bullera singularis* (*21*), *T. aquaticus* YT-1 (*18*), and *L. reuteri* (*19*). They all demonstrated that trisaccharides were the main GOSs synthesized by β-galactosidase. Additionally, Tzortzis et al. (*22*) reported that four types of GOSs were produced within the transgalactosylation reaction by using the whole cells of *B. bifidum* NCIMB 41171, while trisaccharides were still the main product. It should be noted, however, that the analytical system employed in this study could not differentiate, e.g., different di- or trisaccharides. Since the main hydrolysis products of the reaction, glucose and galactose, could also serve as acceptors of the galactosyl moiety, it cannot be ruled out that various non-lactose disaccharides are formed during the transgalactosylation reaction, especially during the later phase of this reaction when considerable concentrations of glucose and galactose are present in the reaction mixture.

Figure 2 shows a time course of GOS production and lactose hydrolysis catalyzed by the *B. longum* BCRC 15708 β-galactosidase at pH 6.8 and 45 °C. Initially, a rapid reduction in lactose concentration was accompanied by a high rate of GOS formation. The maximal amount of GOSs, ca. 30.1% (w/w) of total sugars in the reaction mixtures, was reached after 10 h of incubation. Meanwhile, the content of glucose in the reaction

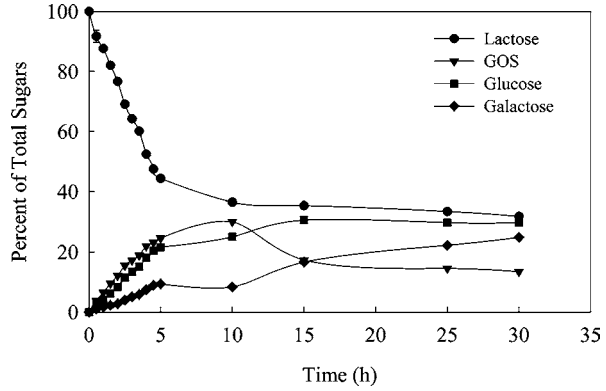

Figure 2. Time course of the lactose hydrolysis and the GOS formation catalyzed by β-galactosidase from *B. longum* BCRC 15708. The reaction was performed at 45 °C and pH 6.8 with an initial lactose concentration of 40%. Bars indicate standard deviations.

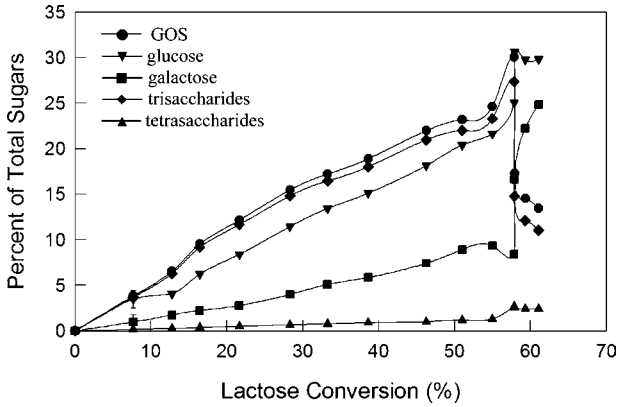

Figure 3. Formation and degradation of GOSs during lactose conversion by β-galactosidase from *B. longum* BCRC 15708. The reaction was performed at 45 °C and pH 6.8 with an initial lactose concentration of 40%. Bars indicate standard deviations.

mixture was found to be much higher than that of galactose, indicating the involvement of galactose in GOS formation. As the reaction time was extended beyond 10 h, the GOS concentration decreased, while an increase in the contents of glucose and galactose in the reaction mixture was observed. These observations were similar to those reported for β-galactosidase from other microorganisms, where GOSs eventually decreased as a result of hydrolysis (*8*, *21*−*23*). This demonstrated that transgalactosylation dominated early in the reaction, producing GOSs in a high yield, while hydrolytic activity of β-galactosidase takes over as the reaction further proceeds.

Further looking into the formation and degradation of GOSs during lactose conversion, it was noted that, before the maximum lactose conversion was reached at 57.8%, there was an increase in the concentrations of GOSs, glucose, and galactose, while the amounts of GOSs decreased and formation of monosaccharide (glucose and galactose) increased as lactose conversion further proceeded (**Figure 3**). As shown in **Figure 3**, β-galactosidase of *B. longum* BCRC 15708 showed a maximum GOS yield of 30.1% (w/w) with a lactose conversion of 57.8%, while β-galactosidase from *A. oryzae* and *B. bifidum* was reported to exhibit a maximum GOS yield of 26.6−29.0% (w/w), which was obtained by a 50−60% conversion of lactose (*8*, *22*).

Effects of Lactose Concentrations on GOS Production. Various investigators reported that the initial lactose concentration in the reaction mixture is the most significant factor

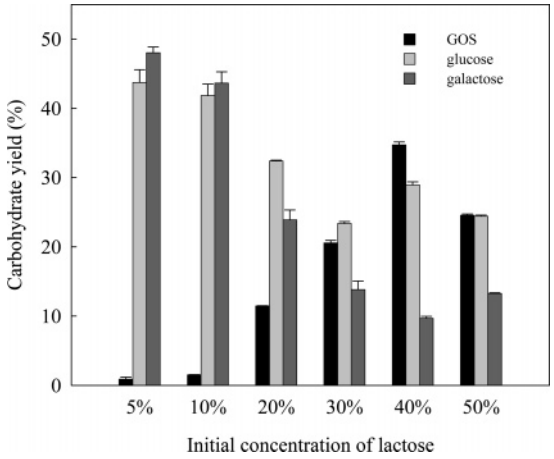

Figure 4. Effect of the initial lactose concentration on the GOS production catalyzed by β-galactosidase from *B. longum* BCRC 15708. The reaction was performed at 45 °C and pH 6.8 for 10 h. Bars indicate standard deviations.

affecting GOS formation (*8*, *7*, *24*, *25*). **Figure 4** shows the carbohydrate yields of GOSs, glucose, and galactose in the reaction mixture after 10 h of catalysis of the reaction by β-galactosidase of *B. longum* BCRC 15708. It was found that the production of GOSs increased with increasing initial lactose concentration from 5% to 40%. A maximum GOS production was reached when the initial lactose concentration was 40%, and further increases in lactose concentration resulted in the reduction of GOS production. It was also noted that the hydrolysis reaction dominates in reaction solutions containing a lower lactose concentration (5−30%), while GOS formation dominated in reaction mixtures having a higher lactose concentration (40−50%). Transgalactosylation is a process in which β-galactosidase hydrolyzes lactose, and instead of transferring the galactose moiety to the hydroxyl group of water, it transfers the galactose moiety of lactose to a hydroxylated compound, which could be galactose, lactose, or galactose-containing oligosaccharides (*1*, *26*, *27*). It follows that, at a low lactose concentration, transgalactosylation is inferior to hydrolysis, since the amount of hydroxyl groups of carbohydrates is low, and this results in a higher amount of glucose and galactose in the reaction solution. Therefore, to increase transgalactosylation, high concentrations of lactose are usually required (*21*, *25*, *27*, *28*).

Effects of pH and Temperature on GOS Production. pH was found to affect the production of GOS in the reaction mixture catalyzed by β-galactosidase of *B. longum* BCRC 15708 (**Figure 5**). A maximum production of GOSs, ca. 33.3−33.7% of the GOS yield, was noted in the reaction mixture with pH between 5.8 and 6.8, while a lower or higher pH value (4.8, 7.8, and 8.8) resulted in a significant reduction in the production of GOSs in the reaction solution ($p < 0.05$). The optimal pH for GOS production found is rather close to the pH (7.0) optimal for this enzyme to exhibit the highest hydrolytic activity, which was determined using ONPG as the substrate at 37 °C (*14*). The significant effect of pH on the GOS production by β-galactosidase of *B. longum* BCRC 15708 observed in the present study differed from the reports of Shin and Yang (*5*) and Iwasaki et al. (*9*), who reported that the pH value showed minimal or no effects on GOS production by β-galactosidase from *B. singularis* and *A. oryzae*. However, our observations are consistent with the reports of Huber et al. (*28*) and Ji et al. (*29*). The former found GOS production, catalyzed by β-ga-

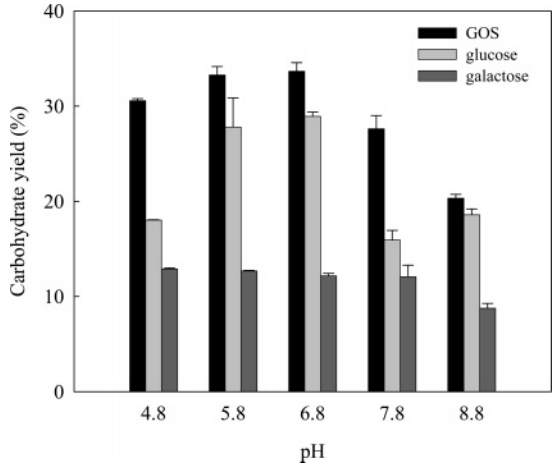

Figure 5. Effect of pH on the GOS production catalyzed by β-galactosidase from *B. longum* BCRC 15708. The reaction was performed at 45 °C with an initial concentration of 40% lactose for 10 h. Bars indicate standard deviations.

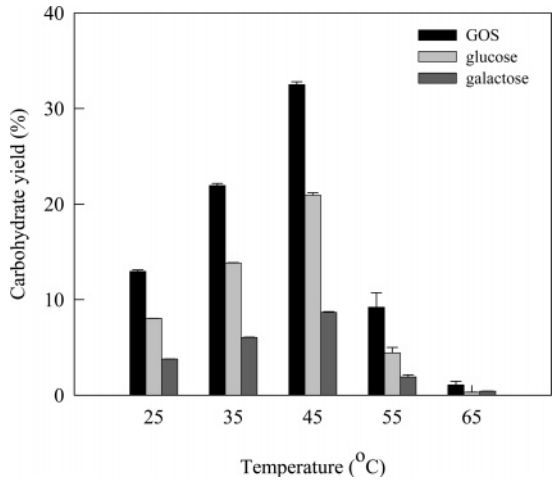

Figure 6. Effect of temperature on the GOS production catalyzed by β-galactosidase from *B. longum* BCRC 15708 at pH 6.8 with an initial concentration of 40% lactose for 10 h. Bars indicate standard deviations.

lactosidase from *Thermotoga maritima*, was affected by the pH value. The latter also observed that pH values dramatically affect the ratio of transgalactosylation to hydrolysis of β-galactosidase from *Escherichia coli*. One possible explanation for these discrepancies is that the effect of pH on the production of GOSs may vary with the source of β-galactosidase.

As shown in **Figure 6**, production of GOSs, glucose, and galactose all varied significantly with the reaction temperature. The GOS yield was found to be ca. 13.0% at 25 °C, increasing as the temperature of the reaction increased, reaching a maximum of ca. 32.5% at 45 °C. This observation was consistent with that observed for *B. circulans* by Boon et al. (*7*), who demonstrated that the GOS yield increased along with temperature (20−50 °C). Roy et al. (*30*) also showed that an increase of temperature from 40 to 65 °C resulted in a higher GOS production catalyzed by β-galactosidase from *Bacillus infantis* RW-8120. On the other hand, in the present study, we also found that increasing the temperature from 25 to 45 °C would increase the lactose conversion from 24.1% to 59.4%. This might be the main reason for the increase of carbohydrate yields. Hung and Lee (*31*) observed that increasing the temperature from 30 to 60 °C would accelerate lactose hydrolysis, as well as GOS synthesis. They indicated that high temperatures

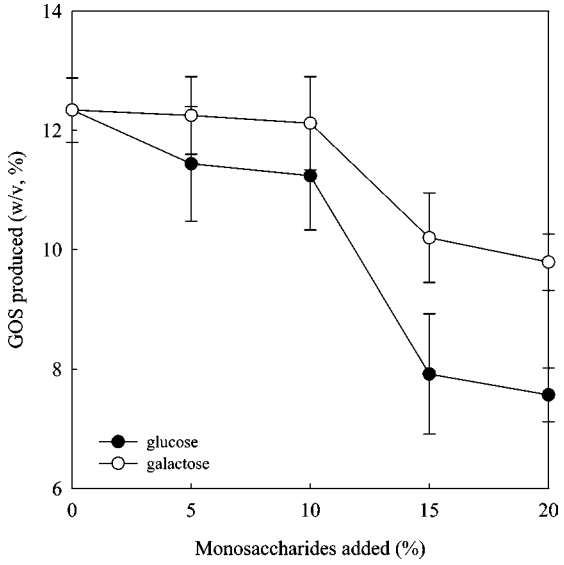

Figure 7. Effect of the monosaccharide concentration on the GOS production catalyzed by β-galactosidase from *B. longum* BCRC 15708. The reaction was performed at 45 °C and pH 6.8 with an initial concentration of 40% lactose and the indicated concentration for 10 h. Bars indicate standard deviations.

were preferable for GOS production by *B. infantis* HL96 β-galactosidase. However, in the present study we noted that further increasing the reaction temperature from 55 to 65 °C resulted in a sharp reduction in the GOS yield as catalyzed by β-galactosidase of *B. longum* BCRC 15708 (**Figure 6**). This may be attributed to the instability of β-galactosidase from *B. longum* BCRC 15708 at a temperature higher than 50 °C, although this enzyme exhibited the highest hydrolytic activity at 50 °C (*14*). Comparing the results obtained in the present study and reports of other investigators (*7*, *9*, *14*, *28*−*31*), the effect of pH and temperature on the catalytic production of GOSs by β-galactosidase may vary with the source of the enzyme. Furthermore, the maximum GOS yield as shown in **Figure 6** corresponded to a productivity of ca. 13.0 g/(L·h), which was higher than those reported for *B. singularis* (4.8 g/(L·h)) (*5*), *B. bifidum* (0.6 g/(L·h)) (*24*), *B. infantis* HL96 (12.7 g/(L·h)) (*31*), *L. reuteri* (7.8 g/(L·h)) (*19*), *Sterigmatomyces elviae* CBS8119 (6.8 g/(L·h)) (*32*), and *Talaromyces thermophilus* CBS 236.58 (1.9 g/(L·h)) (*33*). However, it was lower than that for *A. oryzae* (24.3 g/(L·h)) (*9*) and *T. maritima* (18.2 g/(L·h)) (*29*).

Effects of Galactose and Glucose on GOS Production. Competing for the enzyme active site with lactose, galactose is known as a strong competitive inhibitor to β-galactosidase (*1*, *34*−*36*). Moreover, glucose is also a strong inhibitor for β-galactosidase from some species, such as *B. singularis*, *S. elviae* CBS8119, or *K. lactis* (*5*, *32*, *37*). The effects of glucose and galactose on GOS production by β-galactosidase from the test organism were also examined. As shown in **Figure 7**, no significant difference ($p > 0.05$) in the production of GOSs was observed in the reaction mixture added with 5% or 10% glucose or galactose. However, as the amount of glucose or galactose added increased to 15% or 20%, there was a significant reduction ($p < 0.05$) in GOS production. Furthermore, it was noted that the inhibitory effect of glucose to transgalactosylation was greater than that of galactose. This suggests that the greater part of the galactosyl moiety was transferred to lactose to form GOSs. Thus, glucose inhibited transgalactosylation more seriously than galactose (*5*).

Despite the inhibitory effect, the transgalactosylation activity of β-galactosidase from *B. longum* BCRC 15708 seems to be less sensitive to glucose and galactose than that of β-galactosidase from other organisms (*5*, *8*, *32*). Onishi et al. (*32*) reported that adding 4% glucose strongly inhibited the transgalactosylation activity of β-galactosidase from *S. elviae* CBS8119 by more than 50%. Shin and Yang (*5*) reported that the amount of GOSs formed by β-galactosidase from *B. singularis* decreased markedly by more than 50% when 2−6% glucose or galactose was added to the reaction mixture. Albayrak and Yang (*8*) also showed that 10% glucose or galactose strongly inhibited the transgalactosylation catalyzed by β-galactosidase from *A. oryzae* immobilized on cotton cloth by as much as 15%. On the other hand, GOS production by *B. longum* BCRC 15708 β-galactosidase as examined in the present study was inhibited less by 17.3−35.8% as 15−20% glucose or galactose was added to the reaction mixture. With this nature and the high productivity of GOSs, β-galactosidase from *B. longum* BCRC 15708 may be a potentially useful food industrial enzyme for the production of GOSs.

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