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Effective Enzymatic Synthesis of Lactosucrose and Its Analogues by β-D-Galactosidase from *Bacillus circulans*

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In the present study, β-D-galactosidase from *Bacillus circulans* was proved to be a suitable biocatalyst for the production of lactosucrose (β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→2)-β-D-Fru*f*, **I**) and its analogues from lactose and sucrose. During the hydrolysis of lactose, the formation of four transfer products was followed by high performance liquid chromatography with refraction index detector. In addition, the transfer products were isolated from the reaction mixture and identified to be **I**, β-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→2)-β-D-Fru*f* (**II**), β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α,β-D-Glc*p* (**III**), and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→2)-β-D-Fru*f* (**IV**) by mass spectrometry with an electrospray ionization source and nuclear magnetic resonance spectroscopy. The order for the production of the transfer products was **III** > **I** > **IV** > **II** in the initial stage of the reaction, and the same relationship was also observed for the hydrolytic rates of transfer products. Furthermore, the effects of synthetic conditions including reaction temperature, reaction time, concentration of substrate, molar ratio of donor/acceptor, and enzyme concentration on the formation of transfer products were examined. We found that the optimal synthetic conditions were different for the production of **I** and **II**. Under the optimal conditions, the amount of total transfer products kept increasing during the early 4 h incubation, and a maximum yield of 146 g/L for total transfer products was obtained at 4 h of reaction.

KEYWORDS: Analogues; enzymatic synthesis; β-D-galactosidase; lactosucrose; transgalactosylation

INTRODUCTION

It is well-known that the intestinal tracts of humans are colonized by complex and vast arrays of bacterial cells and that a right balance of intestinal bacterial flora is important for human health. It has also been reported that the growth of intestinal bifidobacteria and lactobacilli, the mainly health-promoting organisms in the colon, are facilitated by prebiotics (*1*). Prebiotics are nondigestible food ingredients that have a beneficial effect on the health of the host by selectively stimulating the growth and/or the activity of a limited number of bacteria in the colon (*2*). Lactosucrose (**I**), β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→2)-β-D-Fru*f*, is one kind of nondigestible oligosaccharide which is recognized as a prebiotic (*3*). Other health benefits such as the prevention of allergic disease, reduction of cancer risk, and enhancement of calcium absorption have also been described (*4*, *5*). Because of the health benefits and favorable characteristics of **I**, its use as a food ingredient has grown rapidly, particularly in Europe and Japan (*3*, *6*). Generally, **I** can be synthesized from sucrose and lactose as substrates through transfructosylation catalyzed by β-fructofuranosidase from *Arthrobacter* sp. *K-1* (*7*) and levansucrase or the cellular biosynthetic system derived from *Bacillus natto*, *B. subtilis*, *Paenibacillus polymyxa*, or *Rahnella aquatilis* (*8*, *9*).

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During the investigation of the prebiotic properties of carbohydrates, it has been observed that chemical structures of oligosaccharides (the number or type of saccharide moieties and the position and conformation of links between the saccharides) are important in determining the prebiotic properties of oligosaccharides, especially the selectivity of fermentation and digestibility in the colon (*10*, *11*). Therefore, there is a high interest in obtaining new prebiotic oligosaccharides with improved prebiotic potential.

β-D-Galactosidases (EC 3.2.1.23) are members of glycosyl hydrolases. They are widely present in plants, animals, and microorganisms (*12*). Generally, β-D-galactosidases are used for the hydrolysis of milk lactose and the synthesis of galactooligosaccharides (GOS) from lactose (*13*−*17*). However, there is little information on the formation of **I** and its analogues using β-D-galactosidases with lactose and sucrose as substrates (*18*−*20*). Since **I** plays a major role in the refinement of sugars in the food industry, the use of β-D-galactosidase-mediated transgalactosylation may provide a new synthetic strategy for the production of **I** and an opportunity for the discovery of new prebiotics. Therefore, it is essential to gain more insight into the formation of **I** and its analogues during the transgalactosylation reaction catalyzed by β-D-galactosidases.

Here, we report in detail the synthesis, purification, and characterization of transfer products with lactose and sucrose as substrates catalyzed by β-D-galactosidase from *B. circulans*.

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The effects of synthetic conditions such as reaction temperature, reaction time, concentration of substrate, molar ratio of donor/acceptor, and enzyme concentration on the formation of transfer products were also reported. Furthermore, the hydrolytic reaction by β-D-galactosidase on transfer products was investigated.

MATERIALS AND METHODS

Chemicals and Enzyme. Commercially, β-D-galactosidase from *B. circulans* was obtained from Daiwa Kasei Co., Ltd. (Osaka, Japan) and used without further purification. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG), lactose, sucrose, glucose, galactose, and activated charcoal (Darco G-60, 100 mesh) were purchased from Sigma−Aldrich Chemical Co. (St. Louis, MO, USA). The standard of **I** was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Celite 535 was purchased from Fluka Co. (Buchs, Swiltzerland). Acetonitrile (HPLC grade) was purchased from Hanbon Science and Technology Co., Ltd. (Jiangsu, China). Millipore membrane filters (0.45 μm) were obtained from Millipore Co. (Bedford, MA, USA). All other chemicals were of analytical grade.

Assay for β-D-Galactosidase Activity. The activity of β-D-galactosidase was determined using ONPG as substrate according to our previously reported method (*21*). The reaction was carried out in 5.0 mL of sodium phosphate buffer (50 mM, pH 6.0) containing 2.0 mM ONPG and an appropriate amount of enzyme at 37 °C for 10 min. The reaction was stopped by adding 2.0 mL of 1.0 M Na_2CO_3 solution. The liberated *o*-nitrophenol was determined spectrophotometrically at 420 nm. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of ONPG per minute. As a result, the commercial enzyme preparation expressed an activity of 860 U per gram of enzyme preparation.

Enzymatic Synthesis of I and Its Analogues. The synthesis of **I** and its analogues was carried out with lactose and sucrose as substrates and with β-galactosidase as catalyst in an eppendorf tube. In order to investigate the influences of synthetic conditions on the formation of transfer products, the reactions were done at four different temperatures (20, 30, 40, and 50 °C), four different concentrations of substrates (30%, 40%, 50%, and 60%, w/v), five different molar ratios of donor/acceptor (4:1, 3:1, 2:1, 1:1, and 1:2), and four different enzyme concentrations (0.33, 0.66, 1.0, and 1.3 U/mL). In general, to a solution (1.0 mL) containing substrates dissolved in sodium phosphate buffer (50 mM, pH 6.0) was added β-galactosidase, and the reaction mixture was incubated at the required temperature. During the incubation, samples (10 μL) were withdrawn at appropriate time intervals and terminated by heating at 100 °C for 5 min to inactivate the enzyme. All samples were analyzed by high performance liquid chromatography (HPLC). All assays were run in triplicate.

Chromatographic Determination of Carbohydrates. The carbohydrates in the reaction mixture were determined by HPLC with refraction index detector (RID) on an Agilent 1100 series HPLC system, which consisted of a G1311A pump, a G1362A RID, a G1379A degasser, and a G1316A column oven. The separation of sugars was completed on a Sugar-D column (4.6 × 250 mm, Nacalai Tesque Inc., Japan) using acetonitrile−water (80:20, v/v) as the mobile phase at a flow rate of 1.0 mL/min (*22*), or a Shodex Sugar KS-801 column (8.0 × 300 mm, Showa Denko Co., Tokyo, Japan) eluted with ultrapure water at a flow rate of 0.8 mL/min (*23*). Injection volume was 20 μL. Sugar compounds in the reaction mixture were identified by comparing the retention times with those of standard sugars. Quantification of each sugar was performed by an external calibration curve using its corresponding standard solutions.

Purification and Structural Characterization of Transfer Products. To a solution of lactose (30%, w/v) and sucrose (30%, w/v) dissolved in sodium phosphate buffer (50 mM, pH 6.0, 6.0 mL) was added β-D-galactosidase (6.0 U). After 4 h of incubation at 40 °C, the reaction was terminated by heating at 100 °C for 5 min.

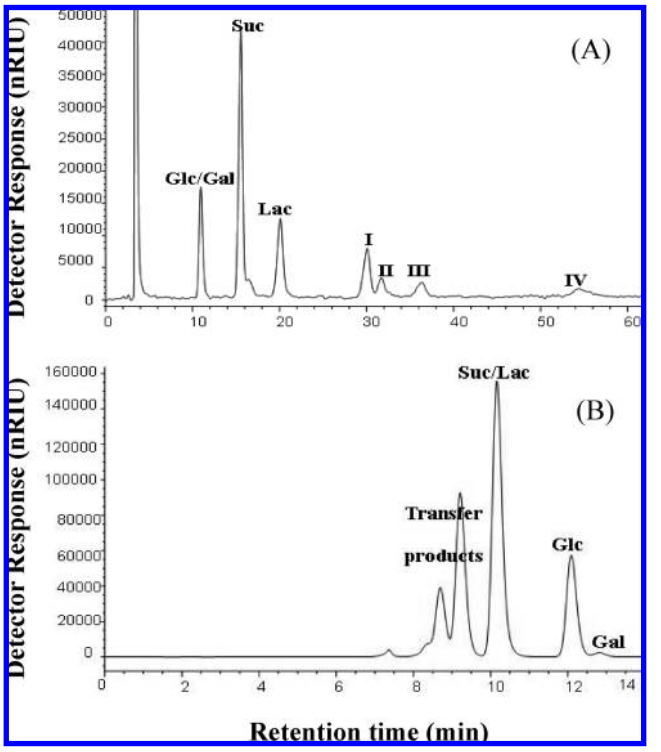

Figure 1. HPLC−RID profiles of a carbohydrate mixture obtained by the transglycosylation of β-D-galactosidase after 4 h of reaction. The reaction was performed at 40 °C and using 60% (w/v) of lactose and sucrose at a molar ratio of 1:1 and 1.0 U/mL of enzyme. The reaction mixture was analyzed by a column of Sugar-D (**A**) or a column of Shodex Sugar KS-801 (**B**). Glc, glucose; Gal, galactose; Suc, sucrose; Lac, lactose; **I**, β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→2)-β-D-Fru*f*; **II**, β-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→2)-β-D-Fru*f*; **III**, β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α,β-D-Glc*p*; **IV**, β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→2)-β-D-Fru*f*.

The reaction mixture was filtrated, and the filtrate was loaded directly onto an activated charcoal and Celite column (3.0 × 50 cm). The column was first eluted with 1000 mL of water and then eluted with a linear gradient of 0−30% ethanol in water (2000 mL) at a flow rate of 4 mL/min. The eluate was collected and monitored by measuring the absorbance at 495 nm (carbohydrate content, determined by the phenol−sulfuric acid method) (*21*). Those with sugars were further analyzed by HPLC with a Sugar-D column. As results, four fractions (F_1, F_2, F_3, and F_4) containing transfer products were obtained. Among the four fractions, F_1, F_3, and F_4 were collected, evaporated by a rotary evaporator (Heidolph Laborota 4000 efficient, Schwabach, Germany), and lyophilized, affording transfer products **I**, **III**, and **IV**, respectively. However, F_2 was found to contain transfer product **II** and sucrose. Therefore, F_2 was collected, concentrated, and further loaded onto a column of Biogel P 2 (1.5 × 90 cm). The column was eluted with ultrapure water at a flow rate of 0.4 mL/min, and the eluate was treated as mentioned above. As a result, the fractions containing **II** were collected, concentrated, and lyophilized to afford transfer product **II**.

The structures of transfer products (**I**−**IV**) were identified by mass spectrometry with an electrospray ionization source (ESI−MS) and nuclear magnetic resonance spectroscopy (NMR). ^1H, ^{13}C NMR, and two-dimensional HMBC spectra were recorded in D_2O as solvent at 300 K with a Bruker Avance DRX-500 spectrometer (Bruker, Karlsruhe, Germany) using the residual solvent signal as internal standard. The chemical shifts (δ) are given in ppm, and coupling constants (*J*) are given in Hz. MS were obtained by direct injection using the Mariner system 5304 mass spectrometer (Applied Biosystems, Foster City, CA, USA).

Hydrolytic Reaction by β-D-Galactosidase from *B. circulans* on Transfer Products. To a solution containing transfer product (0.1 mM) in 50 mM sodium phosphate buffer (pH 6.0,

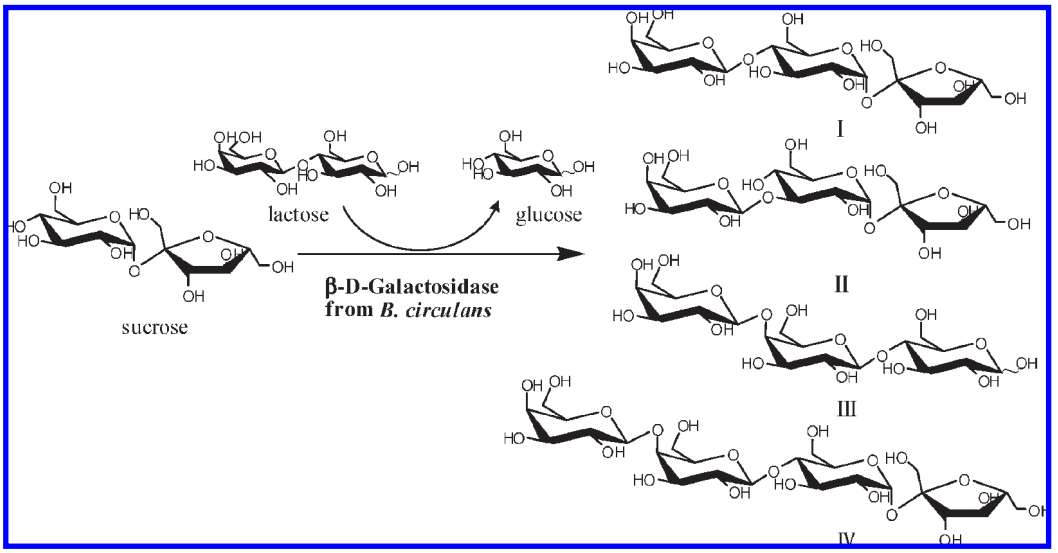

Figure 2. Synthetic scheme for compounds **I**−**IV** by use of β-D-galactosidase from *B. circulans* with lactose and sucrose as substrates.

1.0 mL) was added β-D-galactosidase (0.5 U). The solution was incubated at 40 °C, and samples (100 μL) were taken out at appropriate time intervals (0, 5, 10, 15, and 20 min) during the incubation and immediately immersed in boiled water for 5 min to inactivate the enzyme. All samples were analyzed by HPLC as mentioned above.

RESULTS AND DISCUSSION

Synthesis of Transfer Products by Using β-D-Galactosidase from *B. Circulans*. **Figure 1** shows the chromatographic profiles of transfer products formed in a reaction with lactose and sucrose as substrates catalyzed by β-D-galactosidase. In the present study, two kinds of HPLC columns were used for the separation of the reaction mixture, one for the separation of the whole transfer products (**Figure 1A**) and another one for the separation of galactose and glucose (**Figure 1B**). As observed, transfer products (**I**−**IV**) were formed during the hydrolysis of lactose into galactose and glucose, resulting from the β-D-galactosidase-mediated transgalactosylation as shown in **Figure 2**. The transfer products were isolated from the reaction mixture by using an activated charcoal−Celite column and Biogel P 2 column to afford compounds **I**−**IV**. The structures of transfer products (**I**−**IV**) were identified by MS, NMR, and HPLC.

Mass spectrometry analysis of compounds **I**−**III** gave a peak at m/z 527.2 for $[M + Na]^+$ corresponding to trisaccharides, whereas **IV** gave an ion at m/z 689.3 for $[M + Na]^+$, implying that **IV** was a tetrasaccharide. Assignment of the NMR data as shown in Supporting Information to each sugar including glucose and galactose was done through a comparison of the chemical shifts with those reported previously (*18*, *19*, *24*). The ^{1}H NMR and ^{13}C NMR data showed that the chemical shifts of **I** and **III** were identical to those reported for β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→2)-β-D-Fru*f* (*18*) and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α,β-D-Glc*p* (*25*), respectively. In the ^{13}C NMR spectrum of **II**, 18 carbon signals containing the 6 carbon signals of the terminal galactosyl moiety appeared, and the chemical shift of C′-3 in the glucose unit of sucrose shifted down-field from 72.95 to 79.56 ppm. In addition, the inter-residual HMBC correlation between H-1″ and C-3′ determined that the transferred galactosyl moiety (H-1″) was connected to position 3′ of the glucose (C-3′) unit in sucrose. Furthermore, the characteristic signal at δ 4.51 ppm (J = 7.7 Hz) due to the H-1 of galactose in the ^{1}H NMR spectrum indicated the connection of the galactosyl residue to sucrose through a β-linkage. Therefore, transfer product **II** was identified as the isomer of **I**, β-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→2)-β-D-Fru*f* (*19*). Compound **IV** showed 24 carbon signals containing those of a terminal galactose in the spectrum of ^{13}C NMR, and the chemical shifts of C-4″ in the galactose unit of **I** shifted greatly from 69.0 to 77.2 ppm, indicating that the transferred galactosyl group was connected to C-4″ in the galactose unit of **I**. In addition, ^{1}H NMR data revealed that the galactosyl residue of lactose was transferred to C-4″ in the galactose unit of **I** by β-anomeric configuration based on the coupling constant (J = 7.7 Hz) of the galactose anomeric proton signal observed at 4.59 ppm. Accordingly, the structure of **IV** was identified to be a novel nonreducing oligosaccharide, β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→2)-β-D-Fru*f*. Apparently, **IV** was produced by further regioselective galactosylation of **I** formed initially.

As mentioned above, the transfer products **I**−**III** (trisaccharides) were the main transfer products formed as a result of transgalactosylation catalyzed by β-D-galactosidase. Compounds **I** and **II** were formed by transferring the galactosyl moiety from lactose to sucrose, while **III** was formed by transferring the galactosyl moiety from lactose to lactose. The result is similar to those obtained by Onishi et al. (*24*) and Boon et al. (*25*). In those reports, trisaccharides are the main GOS synthesized with lactose as the substrate by β-D-galactosidases from *Sterigmatomyces elviae* CBS8119, *B. circulans*, *Aspergillus oryzae*, *Kluyveromyces lactis*, and *K. fragilis*. However, Splechtna et al. reported that nonlactose disaccharides were the main transfer products in the case of lactose as substrate catalyzed by β-D-galactosidases from *Lactobacillus reuteri* (*15*). Kim et al. also observed that lactulose (disaccharide) was the main transfer product with lactose and fructose as substrates by a thermostable β-galactosidase from *Sulfolobus solfataricus* (*26*). Therefore, the nature of substrate and enzyme source may affect the polymerization degree of transfer products. In addition, it should be noted that transgalactosylation is a complex, kinetically controlled process, involving intermolecular as well as intramolecular reactions (*15*). Through transgalactosylation, di-, tri-, and tetra-saccharides and eventually higher oligosaccharides are formed. Any sugar molecule in the reaction mixture, even the hydrolytic products of galactose

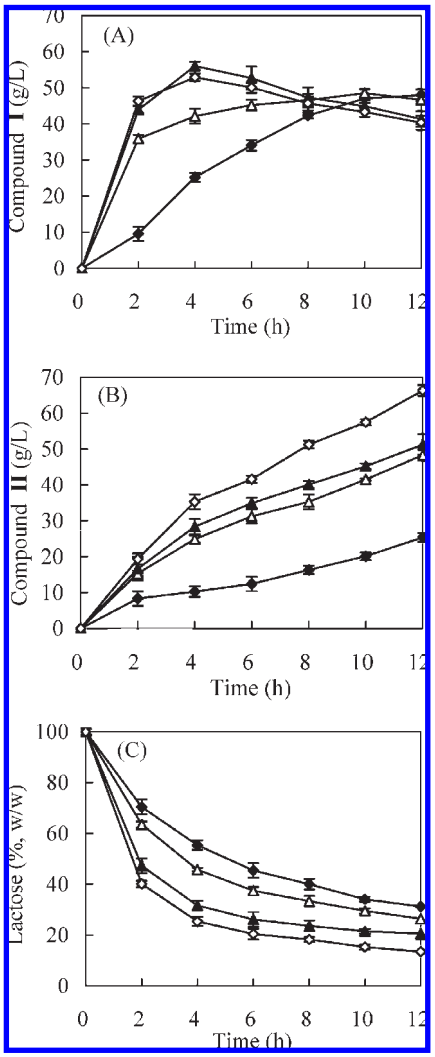

Figure 3. Effect of temperature on the production of **I** (**A**) and **II** (**B**) and the remaining lactose content (**C**) by β-D-galactosidase during 12 h of incubation. The reactions were performed at 20 °C (♦), 30 °C (△), 40 °C (▲), and 50 °C (◇) using 60% (w/v) of lactose and sucrose at a molar ratio of 1:1 and 1.0 U/mL of enzyme. Error bars indicate standard derivations.

and glucose, can be the nucleophile to accept the galactosyl moiety, whereas transfer products are not the end products. They are only transiently formed as they are also subject to hydrolysis, which becomes more and more pronounced toward the end of the reaction when the donor lactose becomes depleted. Therefore, the amount and composition of transfer products change dramatically as the reaction proceeded further.

Effects of Synthetic Conditions on the Formation of Transfer Products I and II. Since transfer products of **I** and **II** were the major trisaccharides in all reactions, the effects of synthetic conditions on their formation were investigated.

Effect of Temperature. To determine the influence of temperature on the transfer products of **I** and **II**, experiments were performed at 20, 30, 40, and 50 °C using 60% (w/v) of lactose and sucrose at a molar ratio of 1:1 and 1.0 U/mL of enzyme, following a time course of reaction of up to 12 h. As shown in **Figure 3**, the rates of lactose hydrolysis increased with temperature (**Figure 3C**), as well as the formation of transfer products (**Figure 3A** and **B**). The yields of **I** and **II** varied significantly with the reaction temperature. The yield of **I** was found to be 47.0 g/L at 20 °C, and increased with the increase of reaction temperature (**Figure 3A**), reaching a maximum of 56.0 g/L at 40 °C. These results are in agreement with that of Hsu et al. (*14*) who reported that the GOS yield increased along with the increase of temperature from 25 to 45 °C. We also found that further increase of the reaction temperature from 40 to 50 °C resulted in a decrease in the yield of **I**, which indicated that **I** was susceptible to transgalactosylation by the same enzyme under high temperature, resulting in the formation of **II** or **IV** (data not shown). The result is similar to that reported for the production of trisaccharide using β-galactosidase from Pectinex Ultra SP-L (*27*). In contrast, the formation of **II** increased constantly during the whole reaction at the four reaction temperatures investigated (**Figure 3B**), and the maximum yield of **II** increased progressively up to 66.3 g/L at 50 °C from 25.4 g/L at 20 °C.

Effect of Substrate Concentration. It is well known that substrate concentration is the most significant factor for the transglycosylation reaction (*12*, *17*). Thus, assays were performed at 40 °C and 1.0 U/mL of enzyme using different initial substrate concentrations of 30, 40, 50, and 60% (w/v) of lactose and sucrose at a molar ratio of 1:1.

As shown in **Figure 4A** and **B**, both of the maximum yields of **I** and **II** increased sharply with the increase of initial substrate concentration in the range of 30 to 60% (w/v), and reached the maximum yields of 56.0 g/L and 51.3 g/L, respectively. These results are in agreement with those reported for the production of **I** by levansucrase (*9*) and the production of GOS by β-D-galactosidase (*14*). Although both the maximum yields were obtained at the highest substrate concentration, the reaction time required for the maximum yield of **I** was greatly shorter than that for **II**.

Under low substrate concentration (30%, w/v), the yield of **I** was low (31.4 g/L), and **I** formed at the initial stage was hydrolyzed quickly during subsequent reaction (**Figure 4A**). These observations are consistent with results shown in **Figure 4C**, where lactose was converted more rapidly with lower substrate concentration. The result demonstrates that the hydrolysis reaction dominates in lower substrate concentration for galactosidase since the amount of hydroxyl groups of carbohydrate is lower as compared with that of water, which can act as an acceptor of galactose. Therefore, high concentration of substrate is usually required in order to enhance transgalactosylation.

Effect of the Molar Ratio of the Donor/Acceptor. To determine the optimal molar ratio of the donor/acceptor for the production of **I** and **II**, the effect of different molar ratios of lactose/sucrose at 40 °C and 1.0 U/mL of enzyme was investigated. The substrate concentration was fixed at 60% (w/v) while varying the molar ratio of lactose/sucrose at 4:1, 3:1, 2:1, 1:1, and 1:2.

To a large extent, the formation of **I** and **II** was greatly dependent on the molar ratio of the donor/acceptor as shown in **Figure 5A** and **B**. The maximum production (56.0 g/L) of **I** was obtained at the molar ratio of 1:1, which was 1.51-, 1.47-, 1.36-, and 1.38-fold of that obtained at the molar ratios of 4:1, 3:1, 2:1, and 1:2, respectively (**Figure 5A**). These results could be explained by a lower degree of secondary hydrolysis and a higher transgalactosylation rate for galactosidase at equimolar concentration of donor/acceptor (*26*). In addition, the lower yield of **I** at unequal ratio compared with an equal one might be attributed to relative insufficient supply of donor or acceptor. As for **II**, the maximum yield increased dramatically from 17.6 to 51.3 g/L with the increase of sucrose concentration (**Figure 5B**), and the reaction time

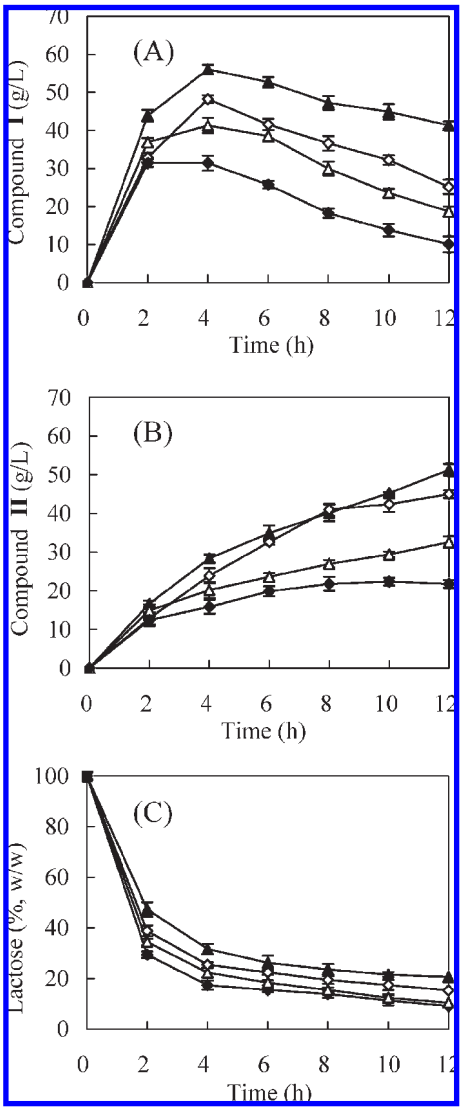

Figure 4. Effect of substrate concentration on the production of **I** (**A**) and **II** (**B**) and the remaining lactose content (**C**) by β-D-galactosidase during 12 h of incubation. The reactions were performed at 40 °C, with the initial substrate concentrations of 30% (♦), 40% (△), 50% (◇), and 60% (▲) of lactose and sucrose at a molar ratio of 1:1 and 1.0 U/mL of enzyme. Error bars indicate standard derivations.

required for the maximum production of **II** was shortened greatly at a molar ratio of 1:2.

It should be noted that the formation of both **I** and **II** was lower under a higher molar ratio of the donor/acceptor. One possible explanation for this is that a higher rate of byproduct formation (galactose and glucose) would be expected with a relative abundant donor (lactose), although the higher molar ratio of donor/acceptor led to a lower lactose hydrolytic rate (**Figure 5C**). Since both glucose and galactose are strong competitive inhibitors of β-D-galactosidase (*14*, *25*, *27*), it can be concluded that a favorable production of transfer product is possible when equal or low donor/acceptor ratio is used.

Effect of Enzyme Concentration. Using different enzyme concentrations of 0.33, 0.66, 1.0, and 1.3 U/mL, an investigation on the influence of enzyme concentrations on transfer product formation was carried out at 40 °C and 60% (w/v) lactose and sucrose at a molar ratio of 1:1.

As expected, an increase of hydrolytic rate for lactose with the increase of enzyme concentration was observed

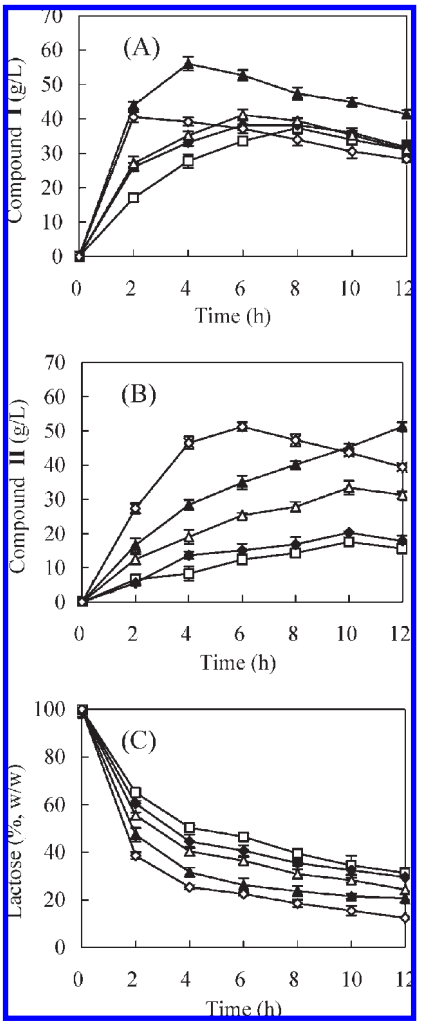

Figure 5. Effect of molar ratio of donor/acceptor on the production of **I** (**A**) and **II** (**B**) and the remaining lactose content (**C**) by β-D-galactosidase during 12 h of incubation. The reactions were performed at 40 °C using 60% (w/v) substrate concentration at molar ratios of lactose/sucrose 4:1 (□), 3:1 (♦), 2:1 (△), 1:1 (▲), 1:2(◇), and 1.0 U/mL of enzyme. Error bars indicate standard derivations.

(**Figure 6C**). The initial formation rate of **I** increased with the increase of enzyme concentration (**Figure 6A**). At an enzyme concentration of 0.33 U/mL, a maximum production of 50.4 g/L for **I** was obtained. As enzyme concentration was over 0.66 U/mL, the reaction time required for the maximum production of **I** decreased markedly. At an enzyme concentration of 1.3 U/mL, the maximum amount of **I** was reached at 2 h of incubation. Among the tested enzyme concentrations, the optimal enzyme concentration was 1.0 U/mL, and the maximum amount of **I** was obtained at a reaction of 4 h. In contrast, a higher enzyme concentration was favorable for the production of **II** as shown in **Figure 6B**. The production of **II** increased with the increase of enzyme concentration, reaching a maximum yield of 62.7 g/L at a concentration of 1.3 U/mL.

Time Course of the Formation of Transfer Products under Optimal Reaction Conditions. Figure 7 shows a time course of transgalactosylation under optimal reaction conditions by using β-D-galactosidase. The reaction was carried out as follows: substrate concentration (lactose/sucrose, 1:1), 60%; enzyme amount, 1.0 U/mL; 50 mM sodium phosphate buffer, pH 6.0; reaction temperature, 40 °C. At the initial stage of reaction, both sucrose and lactose were

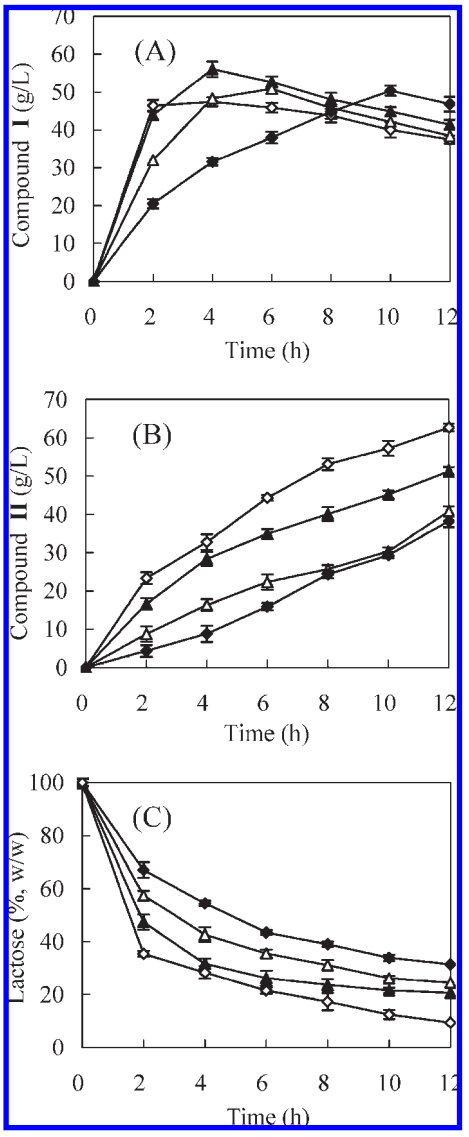

Figure 6. Effect of enzyme concentration on the production of **I** (**A**) and **II** (**B**) and the remaining lactose content (**C**) by β-D-galactosidase during 12 h of incubation. The reactions were performed at 40 °C and 60% (w/v) of substrates (lactose/sucrose, 1:1) using 0.33 (♦), 0.66 (△), 1.0 (▲), or 1.3 U/mL (◇) of enzyme. Error bars indicate standard derivations.

quickly utilized. Afterward, the sucrose concentration in the reaction mixture was kept at approximately 160 g/L, whereas lactose was further hydrolyzed to glucose and galactose, resulting in the increase of the concentrations of glucose and galactose. When the samples were analyzed by HPLC with a Shodex Sugar KS-801 column, it was found that the amount of galactose in the reaction mixture was lower than that of glucose in all samples (data not shown), which suggested that galactose from the hydrolysis of lactose was used for transgalactosylation, whereas glucose was not. For the formation of transfer products, the transgalactosylation reaction led to preferential synthesis of **I** and **III** at the initial stage of reaction. However, once the formation of **I** and **III** reached their maximum, the amounts decreased markedly during the subsequent reaction. However, the amount of **II** kept increasing during the whole reaction. It indicates that **I** and **III** produced can also act as the galactosyl donor, resulting in the increase of yield for **II**. The formation trend of **IV** was similar to that of **I**, and the ratio of **I** to **IV** at the time of maximum production

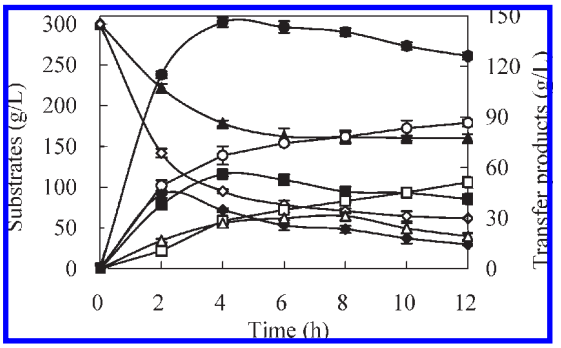

Figure 7. Time course of transfer product production by β-D-galactosidase during 12 h of incubation. Refer to **Figure 1** for reaction conditions. Lactose (◇), sucrose (▲), **I** (■), **II** (□), **III** (♦), **IV** (△), glucose/galactose (○), total transfer products (●). Error bars indicate standard derivations.

Table 1. Hydrolytic Rates of β-D-Galactosidase Acting on Compounds **I**–**IV**

compounds	relative rate (%)
β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→2)-β-D-Fru*f* (**I**)	63
β-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→2)-β-D-Fru*f* (**II**)	13
β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α,β-D-Glc*p* (**III**)	100[a]
β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→2)-β-D-Fru*f* (**IV**)	51

[a] The hydrolytic rate on **III** was arbitrarily set at 100.

of **I** was 2:1. Notably, the formation of oligosaccharides with β-(1→4) linkage was in the order of trisaccharides (**I** and **III**) > tetrasaccharide (**IV**) and **III** > **I** in the initial stage of reaction. In addition, the formation of transfer products with β-(1→4) linkage (**I**, **III** and **IV**) was favorable compared to that with β-(1→3) linkage (**II**). Under optimal conditions, the amount of total transfer products kept increasing during the early 4 h incubation, and a maximum yield of 146 g/L for total transfer products was obtained at 4 h of reaction.

Hydrolytic Rates by *B. circulans* β-D-Galactosidase on Transfer Products. In many cases, it has been noticed that the hydrolytic specificity of isomeric oligosaccharides by galactosidase can be useful for predicting the regioselectivity of transgalactosylation. Several attempts have therefore been made to establish a relationship between hydrolytic specificity and regioselectivity during transgalactosylation (*28*, *29*). In a separate experiment, hydrolytic reactions of transfer products were carried out with β-D-galactosidase. As a result, the hydrolytic rates of **I**, **II**, and **IV** compared with that of **III** (set at 100) were 63, 13, and 51, respectively (**Table 1**). A 5-fold difference between **I** and **II** was observed. Compounds **I**, **III**, and **IV** should be much better substrates than **II**. Thus, the order of the hydrolytic rates of **I**–**IV** closely corresponds to that of their transgalactosylation, which is useful for predicting the regioselectivity of transgalactosylation.

In conclusion, our results suggested that *B. circulans* β-D-galactosidase was a suitable catalyst for the production of **I** and its analogues with lactose and sucrose as substrates because it is readily available, and these compounds would be of great interest because of their potential application in food. In addition, the results indicated that the synthetic conditions such as reaction temperature, reaction time, concentration of substrate, molar ratio of donor/acceptor, and enzyme amount were important factors for the production of transfer products. Further work on the potential function of transfer products is in progress.

Supporting Information Available: ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for compounds **I**−**IV**, ^{1}H NMR spectrum of **I**, ^{13}C NMR spectrum of **I**, ^{1}H NMR spectrum of **II**, ^{13}C NMR spectrum of **II**, ^{1}H NMR spectrum of **III**, ^{13}C NMR spectrum of **III**, ^{1}H NMR spectrum of **IV**, ^{13}C NMR spectrum of **IV**, and two dimensional HMBC spectrum of **II**. This material is available free of charge via the Internet at http://pubs.acs.org.

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