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Production and Application of Galacto-oligosaccharides from Lactose by a Recombinant β-Galactosidase of *Bifidobacterium infantis* Overproduced by *Pichia pastoris*

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Abstract After overproduction of a recombinant β-galactosidase of *Bifidobacterium infantis* in *Pichia pastoris*, a synthesis of galacto-oligosaccharides (GOS) from 36% lactose using the enzyme (170.74 U/mg) was investigated. The transgalactosylation ratio reached up to 25.2% with 83.1% conversion of initial lactose and the maximum yield of GOS was 40.6%. The GOS syrup was composed of a 13.43% galacto-oligosaccharides, 5.06% lactose, and 8.76% monosaccharides. The prebiotic effect of GOS on the growth of bifidobacteria and lactobacilli strains was investigated *in vitro*. The maximum growth rate of *Bifidobacterium breve* and *Lactobacillus acidophillus* in GOS syrup (5%, v/v) media were 0.49 and 0.96/hr that are higher than those in 1%(w/v) galactose and 1%(w/v) lactose containing media. However, there was no significant difference between the specific growth rates of *L. acidophillus* in 1%(w/v) glucose and 5%(v/v) GOS syrup. Our data showed that GOS definitely promoted the growth of *B. breve* ATCC 15700^T and *L. acidophilus* ATCC 33323.

Key words: galacto-oligosaccharide, *Pichia pastoris*, recombinant β-galactosidase, non-digestible oligosaccharide, *Bifidobacterium, lactobacillus*

Introduction

Prebiotics are specific dietary components, usually carbohydrates, that are used to selectively stimulate certain bacterial group's resident in the colon such as bifidobacteria, and lactobacilli, considered beneficial for the human host (1). Such resistant short-chain carbohydrates (SCC) are also referred to as nondigestible oligosaccharides or low-digestible carbohydrates (2,3). Because of their high activity and low toxicity, more than 20 different types of the nondigestible oligosaccharides, such as lactulose, galacto-oligosaccharide (GOS), fructo-oligosaccharide (FOS), and soybean oligosaccharides are available in world markets (4). These compounds provide interesting possibilities for inclusion into conventional food products for their 'bifidogenic' effects. GOSs are one such group of food components that are known to promote the growth of bifidobacteria *in vivo*. Among several classes of oligosaccharides, GOSs have attracted particular attention because they are present in human breast milk which enhances the growth of bifidobacteria in the gastrointestinal (GI) tract of newly born, breast-fed infants (5). Human milk contains about 130 GOSs that provoke a reduction of microflora pathogens, an increase of bifidobacteria as well as minerals availability (6). In addition, it has been suggested that GOS can be used more readily and selectively by bifidobacteria *in vitro* than other oligosaccharides (7), but the mechanisms involved in selective utilization of GOSs by this group of bacteria are poorly known. The ingestion of GOS encourages the proliferation of bifidobacteria and lactobacilli in the intestine, which are considered to be beneficial health effects (8-12). Many studies *in vitro* have shown that GOSs are utilized by intestinal strains of bifidobacteria and lactobacilli (13-15), and many commercial carbohydrates and oligosaccharides have been reported to posses prebiotic effects. In the present study, a recombinant β-galactosidase of *Bifidobacterium infantis* overproduced by yeast, *Pichia pastoris* was used to synthesize GOSs from lactose and the prebiotic effects of the GOSs were studied *in vitro* before animal trials on the growth of 2 selected strains of probiotic bacteria, *Bifidobacterium breve* ATCC 15700^T and *Lactobacillus acidophilus* ATCC 33323.

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Materials and Methods

Strains and plasmid *Pichia pastiris* X-33 was used as a cloning host for the expression of a β-galactosidase gene from *Bifidobacterium infantis* HL96 (16). The pPICZB vector contains a zeocin select marker, an alcohol oxidase 1 (AOX1), Promoter-terminator cassette and a multiple cloning site. The bacterial strains used in this study were *Bifidobacterium breve* (ATCC 15700^T) and *Lactobacillus acidophilus* (ATCC 33323).

Media and culture conditions The expression clones were cultivated according to instructions in the EasySelect™ *Pichia* expression kit (Invitrogen, Burlington, ONT, Canada). Briefly, one colony of *P. pastoris* transformant was inoculated and grown in YPD medium supplemented with 100 μg/mL

Zeocin™ for 24 hr, and then inoculums (0.5%) were grown into 50 mL BMGY [1%(w/v) yeast extract, 2%(w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34%(w/v) YNB, 4×10^{-5} (w/v) biotin, and 1%(v/v) glycerol] at 30°C in a shaking incubator at 200 rpm. The cells grown in BMGY were harvested and resuspended in 100 mL buffered methanol complex medium (BMMY; the same as BMGY but with 0.5% methanol instead of glycerol), then incubated for 5 days. For the induction, 100% methanol was added to the culture to a final concentration of 0.5%(v/v) at 24 hr intervals.

Preparation of the recombinant β-galactosidase To prepare a native β-galactosidase from a recombinant *P. pastoris* X-33, cells were incubated in BMMY broth with shaking at 200 rpm for 4 days at 30°C. Cells were centrifuged (5,900×g, 10 min), washed twice with 50 mM sodium phosphate buffer (pH 7.0) and resuspended in the same buffer. The cells were disrupted by a sonicator (550 Sonic Dismembrator; Fisher Scientific, Mississauga, ONT, Canada) with the power level at 6 (10 min for 1 sec pulsing; 10 sec intervals) under constant cooling. The disrupted cells were centrifuged (2,300×g, 10 min, 4°C) and the supernatants (cell free extracts) were used to prepare the GOSs.

Enzyme activity and protein assay β-Galactosidase activity was assayed using 4 mg/mL of *O*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma-Aldrich, St. Louis, MO, USA) in 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 10 min and the reaction was stopped by adding 1.0 M sodium carbonate. The released *O*-nitrophenol was spectrophotometrically determined by measuring the absorbance at 420 nm of the reaction solution. One unit of the enzyme was defined as the amount of the enzyme that released 1 μmol of *O*-nitrophenol per min (17). The protein concentration was determined by the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. The specific activity was expressed in units of enzyme activity per mg of protein.

Analysis of GOS by high performance liquid chromatography (HPLC) Samples withdrawn at 2 hr internals were immediately heated at 100°C for 10 min to stop the reaction. The GOS syrup was passed through 0.45-μm filter. After dilution and filtration, samples were injected into HPLC for the GOS analysis. The Waters HPLC system (Milford, MA, USA) consists of a 600E system controller, a differential refractometer R401, a temperature control module, an autosampler (Waters 600 satellite WISP), a Millenium 2010 chromatography manager and a carbohydrate analysis column (polymeric column ION-300, 7.8 m×300 mm; Transgenomic Inc., CA, USA). The flow rate was adjusted to 0.25 mL/min with 0.02 N H_2SO_4 as the mobile phase and the column temperature was maintained at 55°C. The running time was set to be 40 min. Peaks were identified by comparing retention times with sugar standards. The sugars were eluted with the sequence of oligosaccharides, lactose, glucose, and galactose. The concentrations (w/v) of these sugars are proportional to their peak areas with the same proportionality constant. All the data from HPLC chromatogram was converted to the contents of sugar using a standard curve. Lactose conversion, transgalactosylation, and GOS yield were calculated according to the following formula (18,19);

$$\text{Conversion ratio (\%)} = [(\text{concen. of lactose}_{initial} - \text{concen. of lactose}_{final})/\text{concen. of lactose}_{initial}] \times 100$$

$$\text{Transgalactosylation ratio (\%)} = [(\text{concen. of glucose}_{initial} - \text{concen. of galactose}_{initial})/\text{concen. of glucose}_{final}] \times 100$$

$$\text{Yield (\%)} = (\text{concen. of oligosaccharide/concen. of lactose}_{initial}) \times 100$$

GOS synthesis Batch fermentations were conducted in a 2-L BIOSTAT M (B. Braun Biotech Inc., Midland, ONT, Canada) fermentor with working volume of 1.0 L. To maintain the induction in the BMMY medium, 100% methanol was added to the culture to a final concentration of 0.5%(v/v) at 24 hr intervals. Fermentations were maintained at pH 6.5, 30°C, 700 rpm with aeration (1.2 L/min). The cell-free extracts (20 U) were incubated with 36%(w/v) lactose solution in 50 mM sodium phosphate buffer (pH 7.0) for 24 hr at 45°C with constant stirring. The collected samples were boiled for 10 min to inactivate the enzyme and then analyzed for their carbohydrate content. After the solution was centrifuged (2,300×g, 10 min, 4°C), the supernatants were used as GOS syrup.

Growth experiments The selected *B. breve* ATCC 15700^T and *L. acidophilus* ATCC 33323 strains were grown in MRS broth with 0.05%(w/v) L-cysteine hydrochloride, except the carbon source was replaced by different GOS syrup concentrations. Carbon sources were supplied at a final concentration of 1%(w/v). All carbon sources were filter sterilized (0.45-μm). The cells were grown anaerobically in 100 mL MRS containing carbohydrate and GOS syrup with different concentrations for 48 hr at 37°C without shaking. After the cells were grown in an anaerobic jars using BD BBL™ GasPak™ Plus (GasPak Jar Systems, Fisher, Pittsburgh, PA, USA), growth was determined by measuring the absorbance at 600 nm (20). The specific growth rate (μ) for each culture was calculated using the following equation

$$\mu = (\ln X_2 - \ln X_1)/(t_2-t_1)$$

where, X_2 and X_1 are the cell densities at times t_2 and t_1 (21).

The results are the mean of triplicate experiments and triplicate sample analyses ± the standard error.

Results and Discussion

Growth and enzyme production Figure 1 shows the time course of growth and enzyme production. When the recombinant *P. pastoris* was grown at 30°C, the growth was exponential, but the maximum cell density [2.54 OD (optical density)] and β-galactosidase activity (170.74 U/mg) were obtained at day 4. The growth patterns of *P. pastoris* containing other recombinant enzymes in this laboratory were similar (22-24).

HPLC analysis Concentrations of all sugars found in sample solutions (oligosaccharides, lactose, glucose,

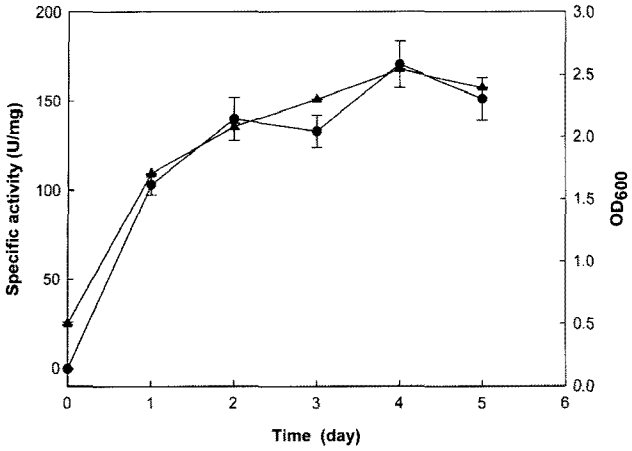

Fig. 1. Growth and enzyme production from *P. pastoris* X-33. ▲, Optical density; ●, specific activity.

galactose) were determined by HPLC. Figure 2 shows a typical chromatogram from the lactose hydrolysis and transgalactosylation by a recombinant β-galactosidase. As the reaction progressed, the hydrolysis of lactose resulted in monosaccharides glucose and galactose, disaccharide lactose, trisaccharide, and small amount tetrasaccharide. Calculated retention times of oligosaccharide, lactose, glucose, and galactose were 14.7-16.2, 18.4, 22.9, and 24.5 min, respectively. At least 2 types of oligosaccharide (14.7, 16.2 min) were produced in most of the reaction. Quantitatively, trisaccharides were the most abundant galactooligosaccharides.

GOS synthesis When a recombinant β-galactosidase was reacted with 36% lactose, transgalactosylation ratio reached up to 25.2% at the 83.1% conversion of initial lactose and the maximum yield of GOS was 40.6%. The carbohydrate composition of final product is shown in Table 1. The GOS syrup was composed of a 13.43% galactooligosaccharide, 5.06% lactose, and 8.76% monosaccharides. It is known that GOSs are formed when lactose is subject to an enzymatic hydrolysis by β-galactosidase. β-Galactosidase is known to catalyze both hydrolysis and synthesis (transgalactosylation reaction). Hydrolytic activity has been applied in the food industry for decades for reducing lactose content in milk. However, transgalactosylation activity, which yields GOSs has been studied less than the hydrolytic reaction. Figure 3 shows the time course of GOS production from 36% lactose by β-galactosidase at 45°C in 50 mM sodium phosphate buffer (pH 7.0). Initially, a high rate of GOS formation was achieved by a rapid decrease in lactose concentration. The maximum amount of GOS obtained after 11 hr incubation was 14.61 g/100 mL. The concentration of monosaccharides and GOSs increased with time, but after 12 hr reaction, the concentration of GOSs started to decrease, probably due to the enhanced inhibitory effect of the monosaccharides. The by-products such as glucose and galactose were found to restrain the formation of oligosaccharides when the concentration of the monosaccharides reaches a certain level (25,26).

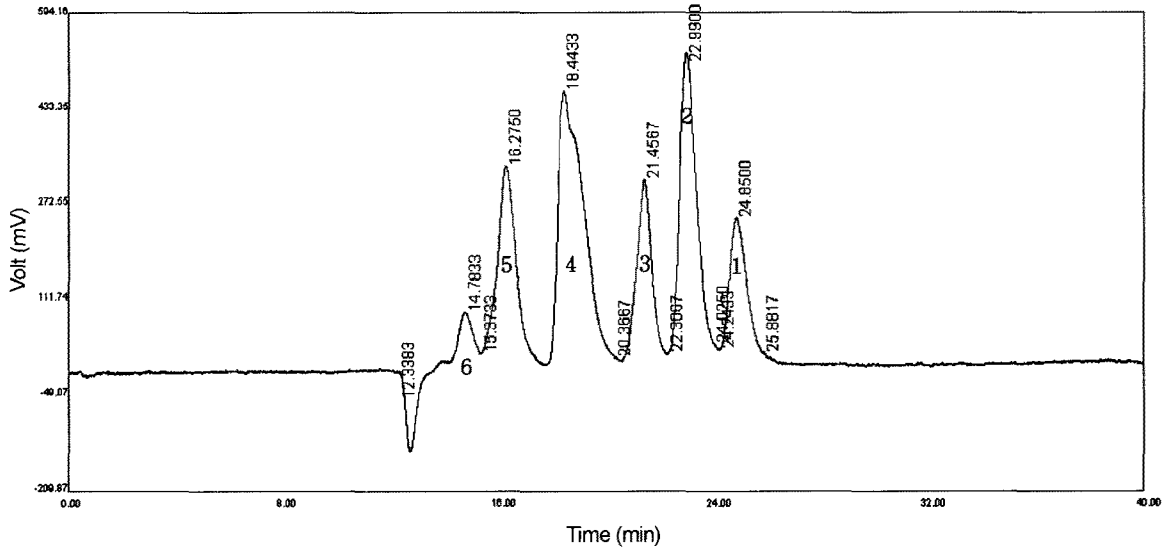

Fig. 2. The HPLC chromatogram from the lactose hydrolysis by the recombinant β-galactosidase. 1, Galactose; 2, glucose; 3, buffer; 4, lactose; 5, trisaccharide, 6, tetrasaccharide.

Table 1. The carbohydrate compositions of GOS synthesis reaction (g/100 mL)

	Lactose	Galacto-oligosaccharide	Glucose	Galactose
GOS syrup	5.06	13.43	4.96	3.80
GOS syrup (2%)[1)]	0.1	0.27	0.1	0.08
GOS syrup (5%)[2)]	0.25	0.67	0.25	0.19

[1,2)]GOS syrup (v/v): GOS syrup/broth.

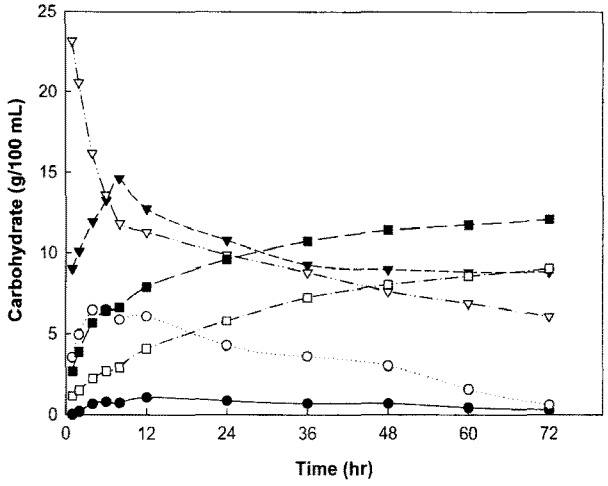

Fig. 3. Changes in sugar concentrations during hydrolysis of lactose by the β-galactosidase. ●, Tetrasaccharide; ○, trisaccharide; ▼, total GOS; ▽, lactose; ■, glucose; □, galactose.

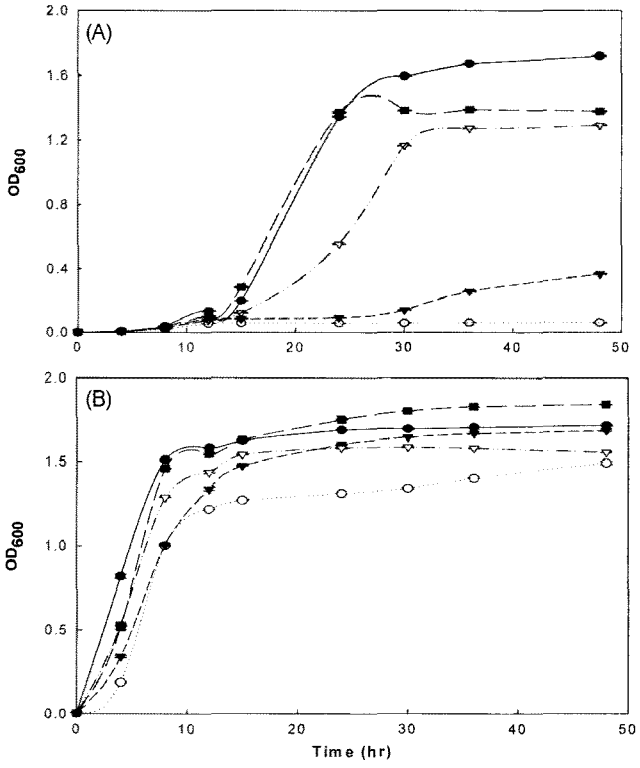

Fig. 4. Growth of *B. breve* (A) and *L. acidophillus* (B) in MRS broth with different carbohydrates at 1%(w/v). ●, Glucose; ○, galactose; ▼, lactose; ▽, GOS syrup 2%; ■, GOS syrup 5%.

Effect of GOS on *B. breve* B 20S and *L. acidophilus* L-5 Figure 4 and Table 2 show the growth of bifidobacteria and lactobacilli in carbohydrate-free MRS broth supplemented with different concentration of GOS and carbohydrate at 37°C for 48 hr. From the optical density, it can be observed that two strains exhibited the higher capacity to grow on the GOS syrup. The specific growth rates of *L. acidophillus* in the GOS syrup supplemented media were higher than those of *B. breve*, indicating a better efficiency in GOS utilization. The maximum growth rates of *B. breve* and *L. acidophillus* in GOS syrup (5%, v/v) media were 0.49 and 0.96/hr, respectively that higher than those in 1%(w/v) galactose and 1%(w/v) lactose containing media. However, no significant difference in the specific growth rate of *L. acidophilus* was found between 1%(w/v) glucose and 5%(v/v) GOS. From the known metabolic diversity of the lactobacilli, considerable variation in prebiotic activity appears to be found in using different prebiotics utilized by a single probiotic strain. *L. plantarum* and *L. acidophilus* had significantly higher scores for GOS compared with the other commercial prebiotics (27). Some suggest that two genera, *Bifidobacterium* and *Lactobacillus*, are indigenous bacterial species that may protect the host from disease (28-32). From our initial studies (data not shown), GOSs were found to stimulate the growth of both bifidobacteria and lactobacilli tested *in vitro*, but not of *E. coli* and other pathogens, suggesting a potential prebiotic effect (33-36).

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Table 2. Specific growth rates of *B. breve* and *L. acidophillus* in individual carbohydrate sources (1%, w/v) and GOS syrup

Strains	Specific growth rate[1] (1/hr)				
	Glucose	Galactose	Lactose	GOS syrup (2%)[2]	GOS syrup (5%)[3]
B. breve	0.58±0.0003	NG[4]	NG	0.29±0.001	0.49±0.002
L. acidophillus	0.98±0.061	0.71±0.062	0.80±0.059	0.88±0.059	0.96±0.054

[1] Results are mean values of 3 determinations±SD.
[2,3] GOS syrup (v/v): GOS syrup/broth.
[4] No growth.

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