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ARTICLE

Synthesis of Galactooligosaccharides in the Cheese Whey-based Medium by a Lactase from *Lactobacillus paracasei* YSM0308

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Abstract

An enzyme β-galactosidase or β-galactohydrolase [EC3.2.1.23], commonly called lactase, mediates galacto-oligosaccharide (GOS) synthesis under conditions of high substrate concentrations. Also, lactase hydrolyzes β(1→4) lactose into glucose and galactose, the latter is successively transferred to free lactose to make various oligosaccharides via transgalactosylation. GOS is non-digestible to human digestive enzymes and has been used as a functional prebiotics. Among the 24 lactic acid bacteria (LAB) strains used, *Lactobacillus paracasei* YSM0308 was selected based on its exhibition of the highest β-galactoside hydrolysis activity, and the crude lactase was prepared for examination of reaction conditions to affect the GOS synthesis. Lactase activity was measured with a spectrophotometer using ONPG (*o*-nitropheyl β-D-galactopyranoside) method. Lactase activity was not detected in the culture supernatant and was mostly present in the cell pellet after centrifugation. Activity of the crude lactase preparation ranges from102 to 1,053 units/mL, with the highest activity determined for *L. paracasei* YSM0308. Optimal conditions for GOS synthesis are as follows: concentration of whey powder, pH, temperature, and time were 30%, pH 6.5-7.0, 30°C, and 4 h, respectively. The final GOS concentration was 19.41% (w/v) by the crude YSM0308 lactase, which was obtained from strain YSM0308 grown in the 10% (w/v) reconstituted whey-based medium.

Key words: cheese whey-based medium, galacto-oligosaccharides (GOS), lactase, *Lactobacillus paracasei*, transgalactosylation activity

Introduction

Whey is opaque greenish yellow with total solids generally of 6.0 to 6.5%, commonly obtained as a by-product from the natural cheese making process, and amount of its production tends to increase as the cheese consumption is on the rise, allegedly due to westernization of eating habits in Korea. Fluid whey is rich nutritionally, retaining 52% of the nutrients of the whole milk used Cheddar cheese making. Yield of whey production is approximately 90% (Kosikowski, 1997). Liquid whey contains various useful substances including proteins, lactose, vitamins, minerals, essential amino acids, lactic acid, and various enzymes. It exerts several useful functionalities such as emulsification, foam formation, gas formation, gelatinization, and solubility (Park *et al.*, 1998). Despite the favorable nutrient balance of whey, it has been long neglected in industrial use.

As far as relative sweetness is concerned, oligosaccharides are low, compared to sucrose or fructose, which are commonly used sweetener in food industry. Therefore, oligosaccharides are frequently applied to manufacture the low-sweet foods by taking advantage of their low sweetness. Of the oligosaccharides commercially available, well known are malto-oligosaccharides (MOS), isomalto-oligosaccharides (ISO), galacto-oligosaccharides (GOS), fructo-oligosacchrides (FOS), and soybean oligosacccharides. They are utilized as a carbon source by few tooth-decaying germs, and able to inhibit the growth of harmful bacteria in the animal intestine by lowering the gut pH. In addition, oliosaccharides are recommended for hyper-glycemic patients, and used in the manufacture of infant formula, soft drinks, ice cream, bread, pharmaceuticals, and animal feeds as well (Boehm and Stahl, 2007).

GOS are the galactose-containing oligosaccharides of the form Glu α1–[β Gal 1–]n, where n = 2, meaning that 2 units of galactose are attached to glucose. They are usually produced from the concentrated lactose syrup using transgalactosylase activity of the enzyme β-galactosidase.

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Earlier studies on oligosaccharides have described about its desirable functions related to nutrient syntheses, promotion of metabolism, boost of immuno-modulation, and improved constipation (Kang *et al.,* 1996; Kim and chae, 1997). Despite the most oligosaccharides are distributed as a natural constituent in the plants, its efficient production process was established using a bacterial lactase from carbo- hydrates, representing sucrose, lactose, glucose, and starch. An enzyme responsible to GOS production is β-galactosidase, simply called lactase. This lactase hydrolyzes β(1→4) glycosides into monosaccharide form of glucose and gal- actose. Resulting galactose from lactose hydrolysis is in turn transferred to free lactose to make various oligosacchrides via transgalactosylation (Park and Oh, 2010). GOS is non-digestible to human digestive enzymes, discovered relatively abundant in human milk. Currently, it is mostly used as a prebiotic ingredient in the fermented food in- dustry. β-Galactosidase is widely distributed in microorganism including bacteria, yeast, and mold. Among bacterial sources, *Bacillus circulans* (Fujimoto *et al.*, 1998), *B. megaerium* (Li *et al.*, 2009), *Bifidobacterium adolescentis* (Hinz *et al.*, 2004), *Bif. infantis* (Hung and Lee, 2002), *Enterobacter agglomerans* (Lu *et al.*, 2007), *L. acidophilus* (Nguyen *et al.*, 2006), *L. reuteri* (Nguyen *et al.*, 2007), and *Streptococcus pneumonia* (Jeong *et al*., 2009) have been reported earlier. Yeast lactase was also studied from *Cryptococcus laurentii* (Ohtsuka *et al.*, 1990), *Kluy- veromyces fragilis* (Ladero *et al*., 2002), *K. lacits* (Kim *et al*., 2003), and *Sterigmatomyces elviae* (Onishi and Tanaka, 1998). As for mould lactase, *Aspergillus aculeatus* (van Casteren *et al*., 2000), *Asp. Oryzae* (Todorova-Balvay *et al*., 2006), *Bullera singularis* (Cho *et al*., 2003), *Sporobolomyces singularis* (Ishikawa *et al*., 2005) were published.

This experiment was conducted to examine the conditions for GOS synthesis in the reconstituted cheese whey-based medium, which is edible for human consumption, by a lactase from *Lactobacillus paracasei* YSM0308.

Materials and Methods

Chemicals and media

Dried whey (sweet whey) obtained from Cheddar cheese process was supplied from Samik Dairy Ltd. (Korea). For making growth medium for LAB, the whey powder was completely reconstituted at 10% (w/v) or proper concentrations in distilled water and partially digested by protease Alcalase (Novo Nordisk, Denmark) in the shaking water bath (Vision Scientific, Korea). The resulting hydrolysate was supplemented in common with 5% yeast extract, 0.1% sodium citrate, 1% Tween 80. Final pH was adjusted with 1 N-NaOH to 6.0 and then autoclaved at 90°C for 20 min. MRS medium (BD, USA) and other chemicals were purchased from Sigma (USA), unless specified otherwise.

Quantitative analysis of medium constituents

General compositions in whey and reconstituted cheese whey-based medium was determined with Milkoscan II FT120 (Foss, Denmark).

LAB strains

Primary screening experiment was carried out to isolate the LAB strains utilizing a lactose as a sole carbon source. Total 24 strains include *L. paracasei* YSM0308, *L. helveticus* FBT1, and 27 strains of LAB collections in this lab, (*L. acidophilus* KCCM32820, *Lactococcus* (*Lc.*) *lactis* ATCC11454, *L. rhamnosus* KCCM32405, *L. brevis* KCCM11904, *L. confusus* KCCM40015, *L. reuteri* ATCC 23272, *L. delbrueckii* KCCM35468, *L. plantarum* KCCM 12116, *Leuconostoc mesenteriodes* KCCM11324, *L. rhamnosus* KCCM32405 *L. bulgaricus* KCCM35463, *Weissella kimchii* KCCM 41287, and *Bif. infantis* KCTC 3249), and selected 12 strains based on their lactase activity. Each of 12 strains was cultured for inoculation before transferred in the MRS broth two times.

Growth and viable cell counts

Bacterial cell count was done on both 10% (w/v) reconstituted whey-based medium and the MRS agar medium, if necessary, enumerated the colonies grown on the surface of the medium at 48 h incubation. Growth curve was drawn by based on viable cell counts in the samples taken at different time points up to 60 h of incubation at 37°C.

Assay for the crude lactase activity

Test strains were transferred for activation in 10 mL of MRS broth and grown at 30°C overnight and then inoculated into 100 mL of 10% (w/v) reconstituted whey medium at same temperature for 18 h. Cultured broth was centrifuged at 2,000 *g* for 20 min. The cell pellet was completely suspended in the original volume for washing. After draining off the supernatant, the pellet was completely dissolved in 20 mL distilled water, which was mixed with a lysozyme stock solution (1 mg/mL) in order to induce cell lysis, followed by incubation at 37°C for about 60 min. Crude lactase solution was prepared by sonicating (VCX130, Sonics, USA) the lysozyme-treated cul-

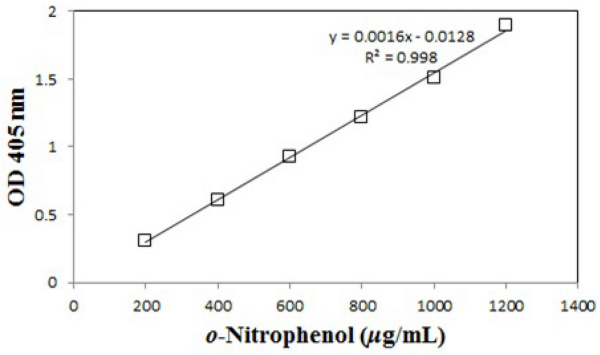

Fig. 1. Standard curve of *o*-nitrophenol in 100 mM Na-phosphate buffer (pH 7.0).

ture 30 times at every 10 s on ice-bath. Lactase activity was assayed by a slight modification previously described by Nguyen *et al*. (2006), determined amount of *o*-nitrophenol liberated from ONPG (Sigma) per min. Compositions of reaction mixture are as follows; 1.4 mL of 100 mM phosphate buffer (pH 7.0), 500 μL crude lactase solution, 100 μL ONPG stock solution (20 mM). After incubation at 30°C for 10 min, 200 μL Na_2CO_3 (0.2 M) was added to stop the reaction, yellow color was measured at 405 nm using a spectrophotometer (Shimadzu, model UV-1601PC). Lactase unit is defined as amount of μmole *o*-nitrophenol (ONP) liberated from ONPG per min. Enzyme unit was calculated from the standard curve of *o*-nitrophenol (Fig. 1).

GOS assay on thin-layer chromagtography (TLC)

TLC method by Itoh *et al*. (1982) was slightly modified in order to determine GOS production by lactase under defined conditions. 15 mL of 30% (w/v) whey medium prepared as described above was mixed with 5 mL of crude lactase solution (206 U/mg protein), and incubated at 30°C for 4 h. Samples were properly diluted and spotted on TLC (Merck, Germany) for GOS production. Developing solution was a mixture of isopropanol and water (4:1) and color was developed by treating the solution mixed equal amount (5 mL) of aniline (dissolved in 1% acetone) and 5 mL diphenylamine (1% dissolved in acetone) with 1 mL of 85% phosphoric acid.

Analysis of the GOS

For HPLC determination, 15 mL sample was mixed with active carbon powder (Active Carbon, Ilsan) for brief boiling, and then the cooled samples were filtered with a filter paper (Whatman No.2). Samples filtered again by syringe filter (0.45 μm, Advantec, Japan) was subjected to inject to the High Performance Liquid Chromatography equipment (HPLC, e2695, Waters, USA) with RI detector and YMC-Pack Polyamine II column.

Results and Discussion

Screening of lactase activity in LAB strains

For total 12 LAB strains grown in MRS broth, lactase activity was measured by ONPG disc method for both cell pellet and culture supernatant (Table 1). Lactase acti-

Table 1. Detection of lactase activity of LAB strains grown on MRS agar

	List of LAB stocks	Activity	
		Culture Supernatant	Cell Pellet
A	*Lactobacillus rhamnosus* KCCM32405	–	+
B	*Lactobacillus brevis* KCCM11904	–	+
C	*Lactobacillus confusus* KCCM40015	–	+
D	*Lactobacillus reuteri* ATCC23272	–	+
E	*Lactobacillus delbrueckii* KCCM35468	–	+
F	*Lactobacillus acidophilus* KCCM32820	–	+
G	*Lactobacillus plantarum* KCCM12116	–	+
H	*Lactococcus lactis* ATCC11454	–	+
I	*Leuconostoc mesenteriodes* KCCM11324	–	–
J	*Weissella kimchii* KCCM41287	–	–
K	*Lactobacillus helveticus* FBT1	–	+
L	*Lactobacillus paracasei* YSM0308	–	+

+, positive; –, negative

Table 2. Comparisons of lactase activity of the commercial LAB starters

LAB strains	*L. acidophilus*	*Lc. lactis*	*L. helveticus*	*L. paracasei*
Enzyme activity (U)[1)]	228	102	520	1,053

[1)]Enzyme activity is defined as the transformation of 1 mol of the *o*-nitrophenol per minute under standard conditions.

vity was only found in the cell pellet but no lactase activity in the culture supernatant, indicating this lactase is intracelluar which is synthesized inside the cell without excretion out of the cells. Lactase activity was found in all LAB strains except two strains of *Leuconostoc mesenteriodes* KCCM11324 and *Lactobacillus* GG, both were confirmed lactose-negative (data not shown). Nguyen *et al*. (2007) disrupted the bacterial cell wall with French Press (Amico, USA) for extracting a lactase from *L. acidophilus.*

Selection of LAB strain for GOS synthesis

The 14% (w/v) cheese whey powder medium contains 1.70% protein, 9.84% lactose, and 1.02% ash. In order to induce lactase, all LAB strains were cultivated in the 14% whey medium (pH 6.0), in which yeast extract is indispensable for growth of LAB as suggested by Rech *et al*. (1999). By using ONPG method, lactase activity was determined for the primary isolates along with four LAB strains currently used as commercial yogurt starter strains As shown in Table 2, their lactase activities ranged from 102 units to max 1,053 units, the latter was highest which was produced by *L. paracasei* YSM0308.

Simple assay of GOS synthesis on TLC

As shown in Fig. 2, TLC chromatogram has shown that few spots responding to GOS were clearly positioned on the sample lanes (5-8) just below the monosaccharide spots of glucose, galactose, and lactose as a control (lane 7 to 8). These smeared spots explains because the GOS in general consists inherently of 3-mer to 5-mers (Lee *et al.,* 2011). Protein concentration of the *L. paracasei* cell-free lysate was measured at 5.1 mg/mL in the crude lactase solution, with specific activity of 206 U/mg protein. According to Nguyen *et al*. (2006, 2007), protein concentration and specific activity of *L. acidophilus* and *L. reuteri* were 230 mg/mL and 180 units/mg, respectively.

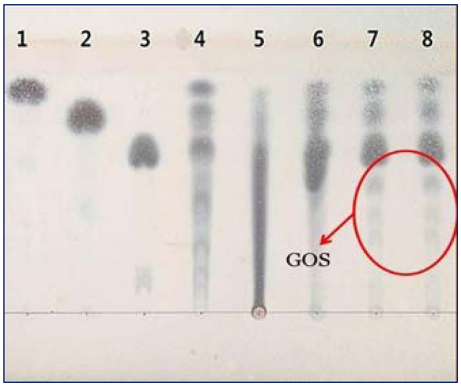

Fig. 2. Detection of GOS synthesis by a thin layer chromatography (TLC) method. Lane 1: glucose, Lane 2: galactose, Lane 3: lactose.

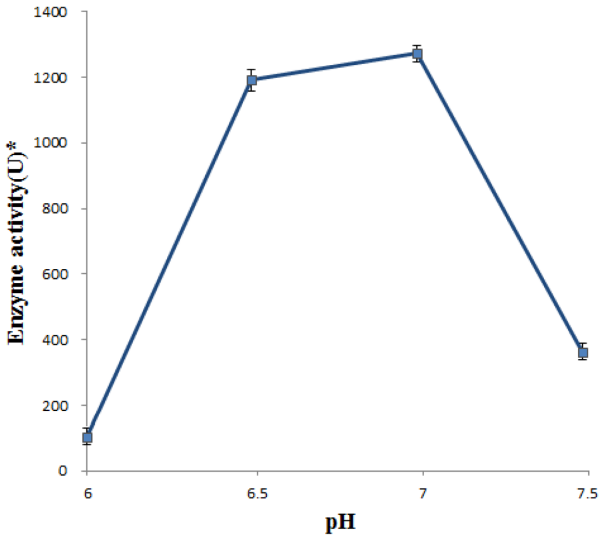

Fig. 3. Effect of pH on *L. paracasei* YSM0308 lactase activity. *Enzyme activity is defined as the transformation of 1 μmol of the *o*-nitrophenol per minute under standard conditions.

Effect of pH on *L. paracasei* lactase activity

As shown in Fig. 3, YSM0308 lactase activity based on the ONPG method was optimal at pH 6.5-7.0. Previous works have described that optimal pH for *Bif. infantis* lactase was 6.5 (Hung *et al.,* 2002), pH 6.0 for *Bif. adolescentis* lactase (Hinz *et al*., 2004), pH 6.5-8.0 for *L. acidophilus* lactase (Nguyen *et al*., 2007).

Effect of lactose concentrations on GOS synthesis

Initial lactose concentration is by far the most significant factor affecting GOS formation (Boon *et al*., 2000) as the GOS was synthesized using lactose by an enzyme lactase. Fig. 4 has shown that GOS increased with the whey concentration up to 40%, followed by being slow down in the production rate at the higher than 30% of whey concentrations. GOS production increased with increasing lactose conversion until a maximum was reached at approximately 50% conversion rate. Before the lactose conversion reached 40%, there was a constant increase in GOS concentration as well as increased in glucose and galactose concentrations. However, at higher conversion rates, there was a shift in the reactions to favor the hydrolysis, which resulted in increased formation of monosaccharides (glucose and galactose) and decreased amounts of GOS. Carlos *et al*. (2012) reported that synthesis of GOS with *Aspergillus oryzae* galactosidase were maxi-

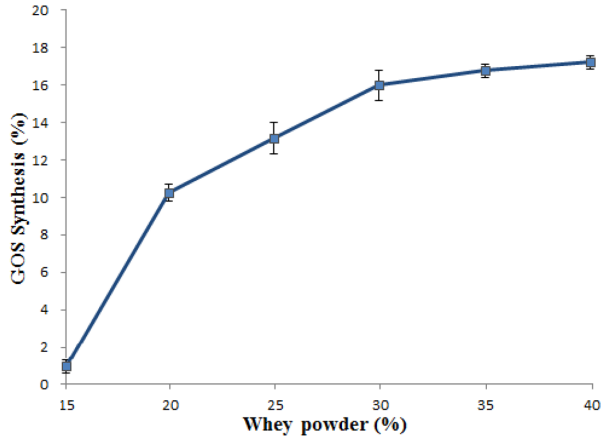

Fig. 4. Effect of powdered whey concentrations on GOS production by *L. paracasei* YSM0308 lactase at 30°C for 4 h.

mally obtained at 50% (w/w) lactose monohydrate of initial concentration at 47.5°C.

Effect of temperature on GOS synthesis

Incubation temperature is known as one of critical factors affecting the enzyme-mediated transgalactosylation reactions. For GOS synthesis by a YSM0308 lactase, temperature was adjusted from 20 to 60°C by 10°C increment, and the amount of GOS was determined in the medium at the predetermined temperatures. As the results shown in Fig. 5, maximum synthesis was obtained at 30°C and gradually decrease at the temperature above. This result indicated that optimal temperature of GOS synthesis is a little lower than that of growth for strain YSM0308 in the 30% cheese whey medium. According to a previous work, optimal temperature for GOS synthesis with *L. reuteri* lactase was 30°C (Splechtna *et al*., 2006). They also reported that the temperatures higher than 37°C was not possible due to the lack of enzyme thermostability. As for transgalactosylation temperature using a lactase from *Geobacillus stearothermophilus* KVE39 (Placier *et al*., 2009), the reaction were optimal at 37°C in 0.1 M potassium phosphate buffer, pH 6.5. The result was very similar to the transgalactosylation of *L. paracasei* YSM0308 lactase in this study.

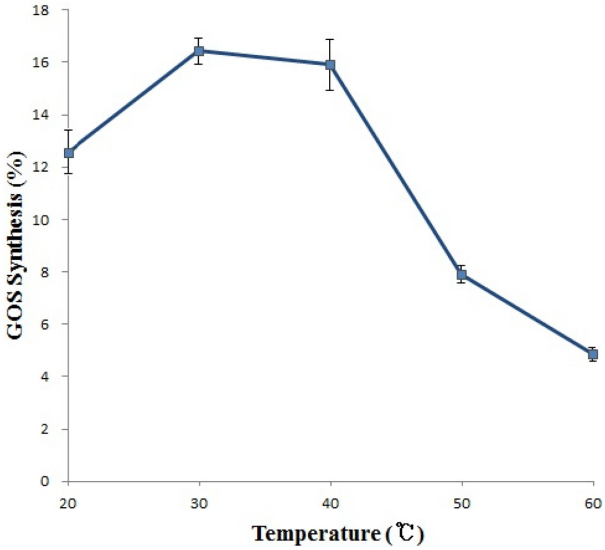

Fig. 5. Effect of temperature on GOS synthesis by *L. paracasei* YSM0308 lactase in 30% whey-based medium for 4 h.

Effect of reaction time on GOS synthesis

Incubation time affect significantly the yields of GOS synthesis, which was determined during incubation up to 8 h at 30°C. *L. paracasei* YSM0308 lactase has steadily produced galacto-oligosaccharides when its production was monitored at every two hours up to 8 h, confirmed a steady increase for the first 4 h and then slightly increased

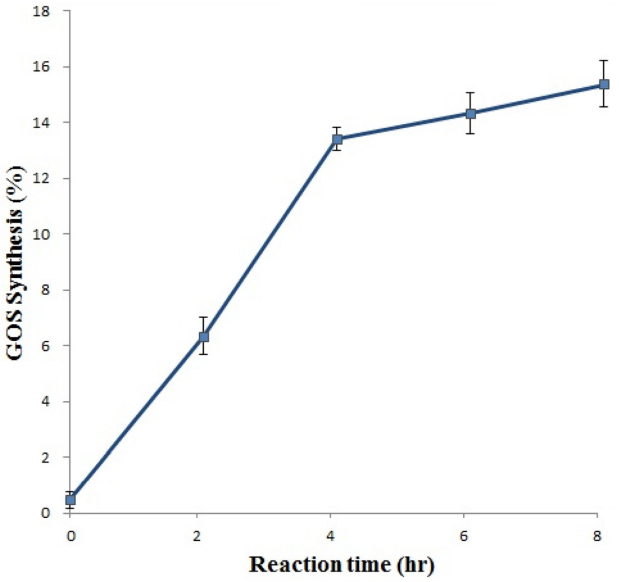

Fig. 6. Effect of reaction time on GOS synthesis by *L. paracasei* YSM0308 lactase in 30% whey-based medium at 30°C.

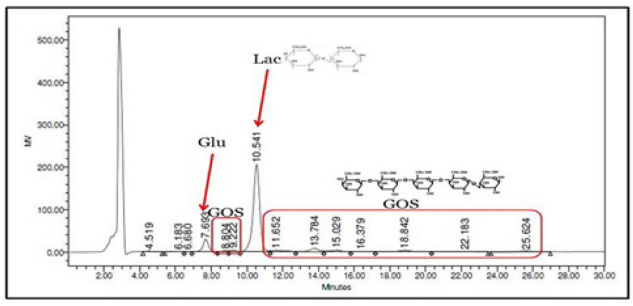

Fig. 7. HPLC chromatogram of GOS synthesis.

Table 3. Carbohydrate compositions of GOS synthesis by the crude lactase of YSM0308

	Fru	Glu	GOS-DP2	Lac	GOS-DP3	4'-galactosylactose	6'-galactosylactose	GOS-DP4<	Total GOS(%)
1	0.76	5.13	1.66	80.73	1.70	3.59	1.24	4.65	12.84
2	0.47	2.41	1.42	86.7	0	4.13	0.47	4.01	10.03
3	0.51	6.21	1.74	81.58	0.23	4.10	1.37	3.94	11.38
4	0.56	6.60	2.07	80.07	0	3.95	1.84	4.61	12.47
5	0	5.38	1.56	82.33	1.80	3.65	1.10	4.18	12.29
6	0	4.06	5.04	79.81	2.20	4.05	1.14	3.70	16.13
7	0	5.25	6.44	75.34	0	5.02	1.91	6.04	19.41
8	0	2.34	5.50	83.69	0	4.39	0.58	3.50	16.31
9	0.53	9.06	2.09	73.80	2.65	4.26	2.48	4.82	16.3
10	0.5	7.60	1.03	78.69	2.5	4.12	0.76	4.62	13.03
11	0.32	7.81	1.19	79.61	1.78	3.51	0.75	4.83	12.06
12	2.12	8.95	0.73	76.95	1.8	3.39	0.74	5.14	11.8

Total GOS = GOS-DP2 + GOS-DP3 + 4'-galactosylactose + 6'-galactosylactose + GOS-DP4<
Each sample is reacted at 30% cheese whey medium, 30°C for 4 h.

(Fig. 6). Therefore, 8 hr incubation is thought to be optimal in terms of cost-effective production.

HPLC Determination of GOS by *L. paracasei* lactase

For determination of galacto-oligosaccharides, HPLC analysis was performed (Fig. 7). As average amount of lactose in whey is about 30%, GOS is synthesized through a mechanism that free lactose molecule binds to free galactose molecule via transgalactosylation reaction. In this experiment using 30% whey medium, lactose level decreased, glucose and galactose increase, and peaks corresponding to GOS were confirmed on the chromatogram during the passage of time. As shown in Table 3, maximum production was obtained from 19.41% (w/v). The total GOS yield from 500 g/L of lactose under the optimal conditions was about 32%, which corresponded to 160.4 g/L of GOS (Lee *et al*., 2011). Lee *et al*. (2011) reported that GOS synthesized using active *E. coli* β-galactosidase was mostly galactosyl lactose, indicating that a galactose moiety was most likely transferred to a lactose molecule during GOS synthesis.

To conclude, GOS production from lactose by *L. paracasei* YSM0308 β-galactosidase was mainly affected by the lactose concentrations in the reconstituted whey medium. More GOS could be produced in the presence of higher lactose concentrations. Temperature and pH significantly affected the overall reaction rate. Therefore, further study needs to increase GOS synthesis in the reconstituted whey-based medium.

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