Master's Thesis of Science in Agriculture

Enzymatic Production of Functional Indigestible Isomaltooligosaccharides using Glucansucrases from *Leuconostoc mesenteroides* B-512FMCM and *L. mesenteroides* B-1355CF10

류코노스톡 메센테로이즈 B-512FMCM과 NRRL B-1355CF10에서
유래한 글루칸수크라아제를 이용한 기능성 난소화성
이소말토올리고당의 효소적 생산

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Abstract

Enzymatic production of functional indigestible isomaltooligosaccharides using glucansucrases from *Leuconostoc mesenteroides* B-512FMCM and *L. mesenteroides* NRRL B-1355CF10

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The novel indigestible isomaltooligosaccharides (IDMOS) was synthesized by combination of two glucansucrases from *Leuconostoc mesenteroides* B-512FMCM ($DSase_{512}$) and *L. mesenteroides* NRRL B-1355CF10 ($GSase_{1355}$) from Kimchi, Korean traditional fermented food.

By acceptor reaction, glucose (donor) was covalently bonded to maltose (acceptor) with α-glycosidic linkages.

Conditions for synthesizing the IDMOS were tested by setting 4 variables. The synthesized the IDMOS was analyzed by TLC (thin layer chromatography) and HPLC (high-performance liquid chromatography). As a result, 85% of the IDMOS was remained after treatment with intestinal enzymes (α-amylase and amyloglucosidase).

Using yeast beads, simple sugars in the IDMOS were selectively removed.

One function of the IDMOS was prevention of insoluble glucan (mutan) formation as prebiotics. When 10 % (w/v) of the IDMOS was treated at 5 %

(w/v) sucrose concentration, the amount of mutan formation was reduced about 50%.

The IDMOS was also used as a carbon source for growth of *Bifidobacteria*. In IDMOS added media (MRS-IDMOS), *Bifidobacteria* were grown well and they used the IDMOS selectively. Especially, the *Bifidobacterium adolescentis* strain consumed the IDMOS the most by verifying TLC analysis. And as *Bifidobacteria* growing, growth of food pathogen like *Salmonella* was inhibited.

Synthesized the IDMOS by GRAS (generally recognized as safe) bacteria and having bifidogenic effect is expected matter for food industry.

Key words : glucansucrase, dextran, alternan, indigestible, isomaltooligosaccharides, acceptor reaction, prebiotics

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Introduction

Extracellular glucosyltransferases (GTFs) are mostly produced by lactic acid bacteria belonging to the genera *Leuconostoc*, *Streptococcus* and *Lactobacillus* [1, 2]. GTFs can be classified by their synthesized structure: dextransucrases (EC 2.4.1.5), which synthesize dextran mainly bonded α-(1,6) linkages into main chain; alternansucrases (EC 2.4.1.140), which produce an alternan composed of 50% α-(1,6) linkages and 50% α-(1,3) linkages in turns into main chain [3, 6]. GTFs are received attention because they synthesize unique branched glucooligosaccharides, which are fermented by beneficial bacteria species in human intestinal microflora and can be used as prebiotics [7, 8].

Koepsell *et al*. showed that when a sugar acceptor molecule exist, GTFs hydrolyze sucrose and then transfer the glucose from sucrose to the acceptor to produce oligosaccharides. This reaction named "acceptor reaction" [5]. Several authors categorized the acceptor molecules by their ability to make glucooligosaccharides and they demonstrated that maltose, isomaltose and α-methyl glucopyranoside are usually the most effective acceptor sugars [3, 4].

Leuconostoc species require sucrose in culture media to produce inducible glucansucrases. Kim and Robyt obtained *L. mesenteroides* mutants from strains B-512FM and B-1355 respectively that produce constitutive glucansucrases in glucose added media [9, 10]. A mutant, *L. mesenteroides* B-512FMCM, was obtained that made 13-times more activated enzyme than the parent mutant strain, B-512FMC, and over centuple more than the original B-512F, commercial strain [11, 12].

L. mesenteroides NRRL B-1355 produces two different glucosyltransferases: dextransucrase, capable of producing dextran which is

similar structure to that of the *L. mesenteroides* NRRL B-512F, and a second enzyme that has been named alternansucrase (EC 2.4.1.140) because of the alternating sequence of α-(1,6) and α-(1,3) glucosyl residues of the synthesized polymer, known as alternan [13].

Alternan and the low-molecular-weight oligoalternan have potential for food industry because of their peculiar structure and resistance to decomposition by hydrolytic enzymes such as amylases or dextranases. They can be especially useful in which characteristics of viscosity and bulk are needed without increasing calorific value [14].

Isomaltooligosaccharides (IMOs) are usually found as a mixture of oligosaccharides with predominantly α-(1,6) glycosidic bond . IMOs are produced using starch as raw material. IMOs are a product of an enzymatic transfer reaction, using combination of immobilized enzymes like α-amylase (EC 3.2.1.1) and pullulanase (EC 3.2.1.41), and in a second stage, the intermediary product is processed by both β-amylase (EC 3.2.1.2) and α-glucosidase (EC 3.2.1.20). Beta-amylase first hydrolyzes the liquenfied starch to maltose. The transglucosidase activity of α-glucosidase then produces IMOs mixtures [15].

There is evidence to suggest that IMOs induce a bifidogenic response [16]. These are widely used as food ingredients or additives [17] based on their nutritional and health benefits [18]. IMOs are interesting due to availability, high stability and low cost [19]. IMOs have been developed to prevent dental caries, as substitute sugars for diabetics [20], or to improve the intestinal flora [21].

A prebiotic can be defined as ‘a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth or activity of one, or limited number of, bacteria in the colon that can improve the host health’ [22]. Beneficial effects of prebiotics have been reported. Prebiotics acidifies colonic contents by increasing the concentration of short-chain

carboxylic acids by fermentation of lactic acid bacteria. They also help minerals absorption in colon, particularly Mg^{2+} and Ca^{2+} [23]. Above this, they effect alleviation of constipation and irritable bowel syndrome, protection against colon cancer, enhancement of immune system, anticarcinogenic effect and lowering cholesterol [24-27].

In a case of tooth decay, *Streptococcus mutans* is a main strain causing dental caries. Extracellular glucosyltransferase from S. mutans catalyze the synthesis of water-soluble and water-insoluble glucans, short α-(1,3) branches (almost certainly single residues) are attached to a linear α-(1,6) dextran, but insoluble glucans contain long sequences of α-(1,3) linked residues that can account for the majority of the glucosidic bonds in the polymer [28, 29].

Dental caries can be got when bacteria in mouth attached on tooth surface and form dental calculus. This calculus is made up of bacteria and non-cellular substances. First, *S. mutans* in mouth are attached on tooth surface and synthesize non-water soluble, sticky glucan (mutan) by producing glucosyltransferase (mutansucrase) using sucrose in taken foods. Mutan, one of the non-cellular substances, play a role in dealing with forming calculus. To prevent tooth decay, development of glucosyltransferase synthesis inhibitors or antimicrobial agent has studied until now.

Recently many prebiotics products are commercialized. However these products were composed with hydrolyzed starch by enzymes, and enzymes were expressed in *Escherichia coli*. Starch can be easily attacked by intestinal enzymes such as amylase or amyloglucosidase.

To pass over these limitation, in this study, first using enzymes from GRAS (generally recognized as safe) strains, *Leuconostoc mesenteroides*, food safety was about to secure. Second, through two enzymes treatment, nondigestibility was tried to retained. Third, with large-scale production, possibility of industrialization was tried to be opened. Finally, this new oligosaccharide was investigated as prebiotic functions.

Materials and Methods

1. Preparation of culture media for *Leuconostoc mesenteroides*

For cultivating *Leuconostoc mesenteroides*, LWG media were used. LWG media was composed of 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 2% (w/v) K_2HPO_4, 2% (w/v) glucose, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) NaCl, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, 0.0013% (w/v) $MnSO_4 \cdot H_2O$, and 0.0013% (w/v) $CaCl_2 \cdot H_2O$.

Yeast extract, peptone, K_2HPO_4, glucose and mineral solution were sterilized respectively at 121℃ for 10 min and the components were mixed at 60℃. LWS media was the same as LWG only substituted 2 % (w/v) glucose with 2 % (w/v) sucrose.

2. Enzyme preparation from the B-512FMCM strain ($DSase_{512}$)

Dextransucrase ($DSase_{512}$) was prepared by culturing the *Leuconostoc mesenteroides* B-512FMCM strain and in LWG media added 2% (w/v) glucose and purified as described previously [30].

3. Selecting high-activated colonies and subculture of the B-1355CF10 strain

100 colonies of the *L. mesenteroides* B-1355CF10 strain grown on LWS agar plates were picked and inoculated in 1 mL of LWG and LWS broth in eppendorf tube respectively. From LWG broths, glucose consumption of colonies were checked. And from LWS broths, enzyme activity was checked. To figure out the enzyme activity, 150 μL of each broth were taken and centrifuged under the condition of 13,600 g (12,000 rpm) for 5 min at room temperature. 100 μL of supernatant were taken and took them in water bath at 45℃ for 5 min to inactivate dextransucrase. The supernatant of heated samples were reacted with 100 mM sucrose added 20 mM sodium acetate buffer (pH 5.2) containing 0.02 % Tween 80 and 1 mM $CaCl_2$. The mixtures were incubated at 28℃ for 30 min. After the reaction, fructose released and alternan production were compared quantitatively respectively. From the top, to 10 of the highest activated colonies that produced high amount of fructose and alternan were picked and inoculated in 100 mL LWG broth. 2% (v/v) of seed culture was added. The cultivated 100 mL LWG broth were taken out to carry out glucose consumption and enzyme activity. The enzyme reaction went along for 30 min and then the broths were spotted on TLC plate. This plate was developed 4 times with acetonitrile:water (85:15 v/v) eluent and then coated with 0.3% (w/v) N-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol, followed by heating at 125 °C for 5 min. Following up this procedure, a colony was selected and inoculated in 1 L LWG broth. And this was chosen as a seed to 14 L fermentation using fermenter.

4. Fermentation of the B-1355CF10 strain using fermenter

14 L broth fermentation was carried out in 19 L volumetric fermenter (NLF 19L, Bioengineering, Switzerland). 5% (v/v) of seed were added and agitated at 150 rpm, 0.5 bar/min aeration (measured at 15°C) at 28°C. To verify

glucose consumption and enzyme activity, samples were collected every 2 h. In company with them, optical density (OD) was measured at 600 nm wave length to figure out the growth degree of microbes. Fermentation was suspended when glucose or sucrose consumption was completed. The samples were centrifuged with SUPRA 25K centrifugal separator (Hanil, Korea) at 9,118 g (6,000 rpm) and then the supernatant were collected and kept at 4°C.

5. Concentration of enzyme from the B-1355CF10 strain ($GSase_{1355}$)

The concentration of broth supernatant was flowed into polyethersulfone hollow fiber (Millipore, Bedford, USA) which has 100 kDa pole size at 4°C condition. The injection pump (LongerPump, BT300-2J, China) was used which set 167 mL/min (100 rpm) flow rate. Finally retentate was collected and lyophilized ($GSase_{1355}$).

6. Enzyme assay of the $GSase_{1355}$

Enzyme activity was evaluated by incubating the enzyme at 28°C with 200 mM sucrose as a substrate in 20 mM sodium acetate buffer (pH 5.2) containing 0.02 % Tween 80 and 1 mM $CaCl_2$. Each the 1 μL of enzyme-reacted sample was spotted on a silica gel $60F_{254}$ TLC plate (Merck) and then developed two times using eluent consists of nitromethane:n-propyl alcohol:water (2:5:1.5, by vol.). Next, sulfuric acid color reaction methods were carried out to analyze reacted sugar compounds. Products on a TLC plate was visualized by dipping into 0.3% (w/v) N-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol, followed by 5 min heating

at 125°C. The amount of fructose released from the reaction was analyzed using AlphaEaseFC 4.0 Image Program, with fructose standard solutions. One unit of dextransucrase or alternansucrase were defined as the amount of enzyme required to generate 1 μM fructose per min at 28°C. From these procedures, the $DSase_{512}$ and the $GSase_{1355}$ were calculated.

In order to find out the specific activity of the enzymes, protein concentration was measured by using a method known as Bradford assay. First, from 0.1 mg/mL to 1.0 mg/mL bovine serum albumin (BSA) solutions were prepared as standards. On the one hand, 5 times diluted Bio-Rad Protein Assay Dye Reagent Concentrate (catalog #500-0001) was prepared. 200 μL of this dye reagent was added in a 96-well plate. Then 5 μL of BSA standard solutions and samples were added in each wells. After 10 min, absorbance at 595 nm of wells was measured by SpectraMax M3 UV-Visible spectrophotometer (Molecular Devices, USA). Whole process was carried out at room temperature. Using a standard curve plotting protein concentration versus absorbance, protein concentration in products from fermentation was calculated and specific activity of the $GSase_{1355}$ was calculated.

7. Effects of factors on synthesis of the IDMOS

To maximize yield of indigestible isomaltooligosaccharides (IDMOS), the enzyme reaction was carried out at 45°C for 6 h under fixed 2 M of sucrose concentration. The variables were concentration of $DSase_{512}$, $GSase_{1355}$, maltose and reaction time. Reaction conditions were written in **Table 2–5**.

20 fold-diluted the reaction samples were treated 1% (v/v) α-amylase (from *Bacillus licheniformis*, Liquozyme Supra, Novozyme, Korea) at 90°C for 30 min. Then the samples were taken and kept in ice for 20 min. After that, 3% (v/v) amyloglucosidase (from *Aspergillus niger*, AMG300L, Novozyme,

Korea) were added in each samples at 60°C for 1 h. After treating two intestinal sugar-hydrolytic enzymes (digestive enzymes) to simulate human digestion system, 1 μL of each the enzyme reaction samples was spotted onto a TLC plate and then developed two times using eluent consists of nitromethane:n-propyl alcohol:water (2:5:1.5, by vol.). Products were visualized by the sulfuric acid color reaction methods. The densitometry of components was carried out using AlphaEaseFC 4.0 program.

With TLC densitometry results, statistical analysis was performed using IBM SPSS Statistics 23 program. To verify the homogeneity of variances between the different groups of treatments, Levene statistic was checked. If the Levene statistic of p-value was lower than the significance level (significance level (α) = 0.05), the homogeneity of variance between the different groups of treatments was able to reject. In this case, Welch test could be performed to know the homogeneity of means between the different groups of treatments. If the homogeneity of means between the different groups of treatments was rejected, Games-Howell test was performed as post hoc.

On the other hand, one-way ANOVA was performed if the Levene statistic of p-value was upper than the significance level. When the p-value of pooled between-group variances was lower than significance level, the Duncan post hoc test could be performed.

These final results are also shown in **Table 2-5** and **Figure 2–5**.

8. Analysis of the IDMOS after treating α-amylase and α-amyloglucosidase with HPLC and TLC

To figure out the amount of the IDMOS regarding treatment of α-amylase and α-amyloglucosidase, high performance liquid chromatography-refractive index detector (HPLC-RID) system were used. Prepared sample shown in

Table 1 were diluted 20-fold to adjust the concentration to after digestion samples (before treating digestion enzymes samples). After treating digestion sample were not diluted (after treating digestion enzymes samples). These samples were filtrated through 0.45 μm Minisart regenerated cellulose 15 mm filter (Sartorius, Germany) using syringe.

HPLC analytical conditions were composed as follows. Aminex HPX-42C Column (7.8 mm I.D. × 300 mm, BioRad, California, USA) which was incubated at 80°C was used. Injection volume of samples was 20 μL. The eluent was 100% distilled deionized water flowing 0.5 mL/min. The chromatograms using this HPLC system were shown in **Table 5**.

The sample of maximized condition was analyzed the same HPLC system using MCI GEL CK04SS (Mitsubishi Chemical Co., Japan) column (**Figure 9**).

The maximized IDMOS was compared with industrial oligosaccharide (Ottugi, Korea) by TLC analysis. Each of 1 μL samples were spotted on TLC plate and TLC plate was developed once with nitromethane:n-propyl alcohol:water (2:5:1.5, by vol.) and once with nitroethane:nitromethane:ethanol:water:n-propanol (1:2:3:4:5, by vol.). Through the sulfuric acid color reaction methods, components were detected (**Figure 15**).

9. Removal of monosaccharides using yeast beads and large-scale production of the IDMOS

To get large amount of the IDMOS, scale reaction volume was up to 500 mL. And to get higher purity of the IDMOS, yeast beads was treated in reaction sample. Methods was applied the described in Yoon *et al* [25]. 2 g of alginic acid sodium salt (from brown algae, low viscosity, Sigma Co.) was

dissolved in 80 mL hot water with stirring and kept in ice for 30 min. Meanwhile, 2 L of 4 % (w/v) calcium chloride dihydrate (Duksan, Korea) solution was prepared. 4 g of yeast powder (*Saccharomyces cerevisiae* 98.5%, Saf Instant Yeast Red, Societe Industrielle Lesaffre, France) was mixed in 20 mL water. This yeast dispersion was added in alginate and degassed for 20 min. Then with injection pump (LongerPump, BT300-2J), alginate beads trapped the yeast powder was dropped in calcium chloride solution with stirring in ice. After washing with pure water, the wet beads were kept in 4°C for 2 h.

These beads were added to 500 mL the maximized the IDMOS reaction sample. After incubation at 37°C, 150 rpm for a week, consumption of monosaccharides was verified by TLC analysis. The TLC plate was twice developed with nitromethane:n-propanol:water (2:5:1.5, by vol.) solvent. Finally this sample was lyophilized in order to do further studies.

10. Prevention of insoluble glucan (mutan) formation by the IDMOS

Mutansucrase was prepared by growing *S. mutans* in Brain Heart Infusion (BHI) medium containing 0.5% (w/v) glucose and purified as described previously [23]. One unit of mutansucrase activity (U) was definied as the amount of enzyme that liberates 1 μM of fructose per min at 37°C and pH 6.8. The mutansucrase and the IDMOS added solution were filtered using a 0.2 μm minisart syringe filter (Sartorius Stedim, Germany) before using.

Reaction conditions were set at different condition of sucrose concentration [1 – 5% (w/v)] with different concentration of the IDMOS [0 – 10% (w/v)] and 10 % (v/v) of mutansucrase (1.1 U/mL) in 20 mM sodium phosphate buffer (pH 6.8) at 37°C for 6 h. Final volume of all reaction samples were 200

μL. The reaction condition is shown **Table 6**. Water-insoluble glucan produced the reaction was harvested by centrifugation at 13,572 g (12,000 rpm) for 10 min. After washing with distilled water and centrifugation two times, each pellet of samples was dissolved in 200 μL of 1 M NaOH.

For quantification of insoluble glucan, phenol-sulfuric acid total carbohydrate method was applied. 25 μL of samples were taken and added to 25 μL of 5% (v/v) phenol solution. Meanwhile glucose standard solutions [0.025 – 0.2 (mg/mL)] were prepared. After slight mixing, 125 μL of concentrated sulfuric acid was added to each sample. After slight mixing again, samples were kept in 80°C water bath for 30 min. Cooled samples at room temperature were taken into 96-well plate and measured the absorbance at 492 nm using SpectraMax M3 spectrophotometer (Molecular Devices LLC, Sunnyvale, CA, USA).

11. Effect of the IDMOS for the growth of microorganisms

Bifidobacterium bifidum MR57 (*B. bifidum*), *Bifidobacterium infantis* KCCM 11207 (*B. infantis*), *Bifidobacterium longum* KCCM 11953 (*B. longum*), *Bifidobacterium adolescentis* KCCM 11206 (*B. adolescentis*), and *Salmonella typhimurium* ATCC 14028 (S. *typhimurium*) were obtained from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea).

For growth of *Bifidobacterium* strains, deMan-Rogosa Sharpe broth (MRS) broth (Difco Lab., USA) (pH 6.3) added 0.5 g/L of cysteine-HCl anhydrate (Sigma) (MRS-Cys) to lower redox potential was used. 20 mL of MRS-Cys broth was poured in 30 mL-volume test tubes and added 2 mL of paraffin oil onto broth to make anaerobic condition and autoclaved for 5 min themselves. The carbon sources were used with 2% (w/v) glucose or the IDMOS. Carbohydrates-free MRS (cfMRS) broth was used as negative control.

Components of media are described in **Table S1**.

Table S1. Composition of MRS broth (in 1 L)

Components (Company)	MRS-Glucose (g)	Carbohydrates-free MRS (cfMRS) (g)	MRS-IDMOS (g)
Peptone (BD Difco)	10	10	10
Beef extract (BD Difco)	10	10	10
Yeast extract (BD Difco)	5	5	5
Carbohydrates	20, Glucose	0	20, IDMOS
Tween 80 (Duksan)	1	1	1
Ammonium citrate dibasic (Duksan)	2	2	2
Sodium acetate trihydrate (Duksan)	5	5	5
Magnesium sulfate heptahydrate (Duksan)	0.1	0.1	0.1
Manganese sulfate monohydrate (Duksan)	0.05	0.05	0.05
Potassium phosphate dibasic (Duksan)	2	2	2

12. Antimicrobial effect of *Bifidobacteria* against pathogen bacteria through mixed cultivation

To make seed cultures, *Bifidobacteria* and *Salmonella typhimurium* was grown in MRS broth for 24 h respectively. Autoclaved MRS-IDMOS and paraffin oil on the broth were used for culture media. The temperature of all media set 37℃. The seed broths were diluted to log(CFU, cell forming unit) around 3. 2% (v/v) of the seed broths were inoculated to MRS-IDMOS media. At each scheduled time, small volume of vortexed broths were taken out to measure viable cell count and pH. In case of viable cell count, MRS agar plate for *Bifidobacteria* and BHI agar plate for *Salmonella* were used. Through serial dilution of each media from 10^{-1} to 10^{-9}, 100 μL of diluted samples with vortexed were spreaded on agar plates. After 24 h, cell counts were measured. pH was checked by using pH meter (Suntex SP-2100, Taiwan).

Results

1. Enzyme assay

Through unit assay methods, the $DSase_{512}$ was 2.0 U/mg and of the $GSase_{1355}$ was 4.0 U/mg. And after incubation of the $GSase_{1355}$ at 45°C for 5 min, 2.9 U/mg alternansucrase and 1.1 U/mL dextransucrase were determined respectively from 4.0 U/mg of the $GSase_{1355}$.

2. Fermentation using fermenter and concentration of the B-1355CF10 strain ($GSase_{1355}$)

First, glucose consumption of each colonies was checked (**Figure 1**) and supernatant was treated with sucrose to verify enzyme activity (**Figure 2**). The supernatant treated incubation at 45°C for 5 min to figure out alternansucrase activity (**Figure 3**). By densitometry using AlphaEaseFC 4.0 program, number 91 colony was selected (**Figure 4**) for 1 L and 14 L fermentation.

In case of 14 L fermentation, glucose consumption was over after 20 h. In this time, OD was 5.3 (**Figure 5**). With this culture media, concentration and lyophilization were carried out and total units of $GSase_{1355}$ finally increased 1,282 fold. These results were described in **Table 1**.

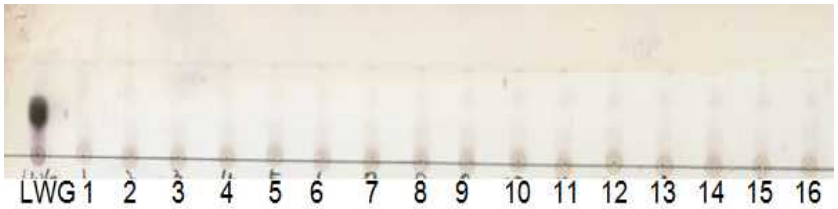


Figure 1. Glucose consumption of the B-1355CF10 strain by TLC analysis. Lane LWG: LWG broth not cultivated, lane 1-16: colony numbers. Other colonies were omitted.

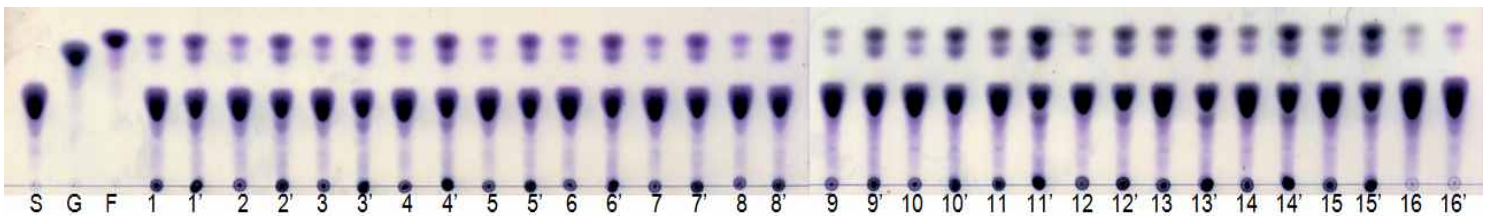


Figure 2. The $GSase_{1355}$ (dextransucrase and alternansucrase) activity by TLC analysis. Lane S: sucrose, lane G: glucose, lane F: fructose, lane 1: colony number, lane 1': colony number after enzyme reaction for 30 min, lane 1-16: colony numbers. Other colonies were omitted.

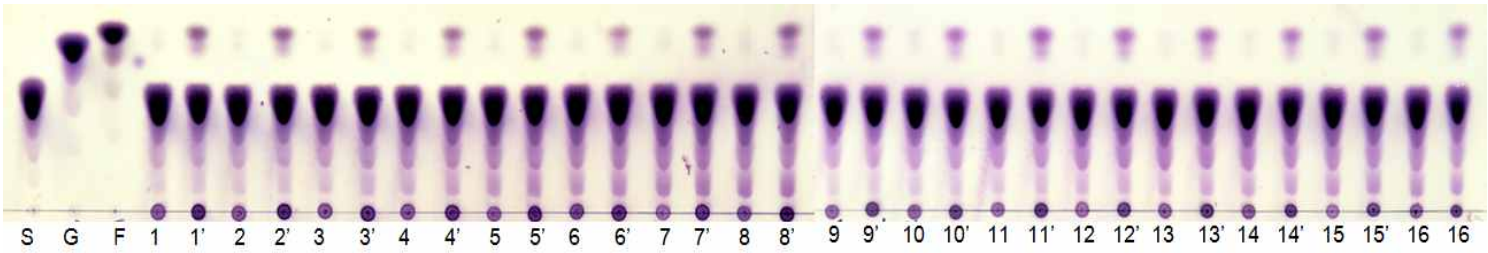


Figure 3. Alternansucrase activity of the $GSase_{1355}$ by TLC analysis. Lane S: sucrose, lane G: glucose, lane F: fructose, lane 1: colony number, lane 1': colony number after enzyme reaction for 30 min and heating at 45°C for 5 min, lane 1-16: colony numbers. Other colonies were omitted.

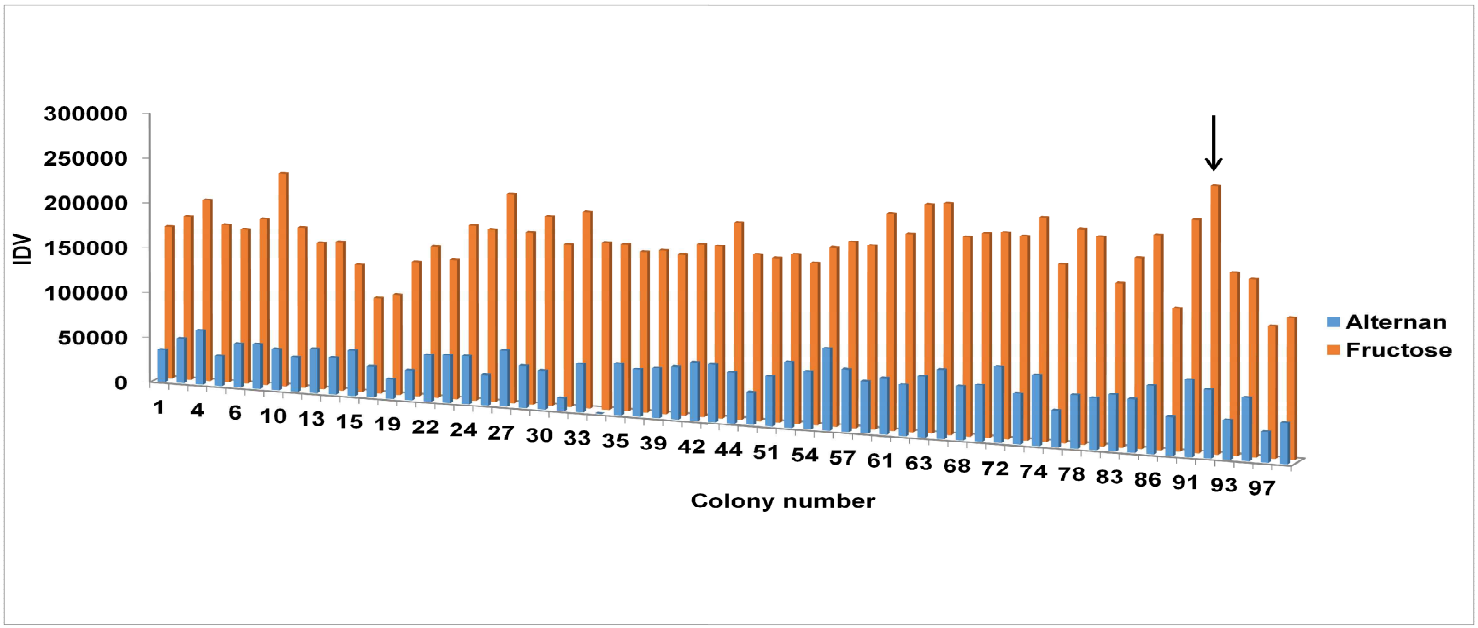


Figure 4. Confirmation of alternan production and fructose release from the $GSase_{1355}$ by AlphaEaseFC 4.0 program from TLC analysis. IDV: Integrated Density Value. The arrow directs the colony showing the highest alternan and fructose release.

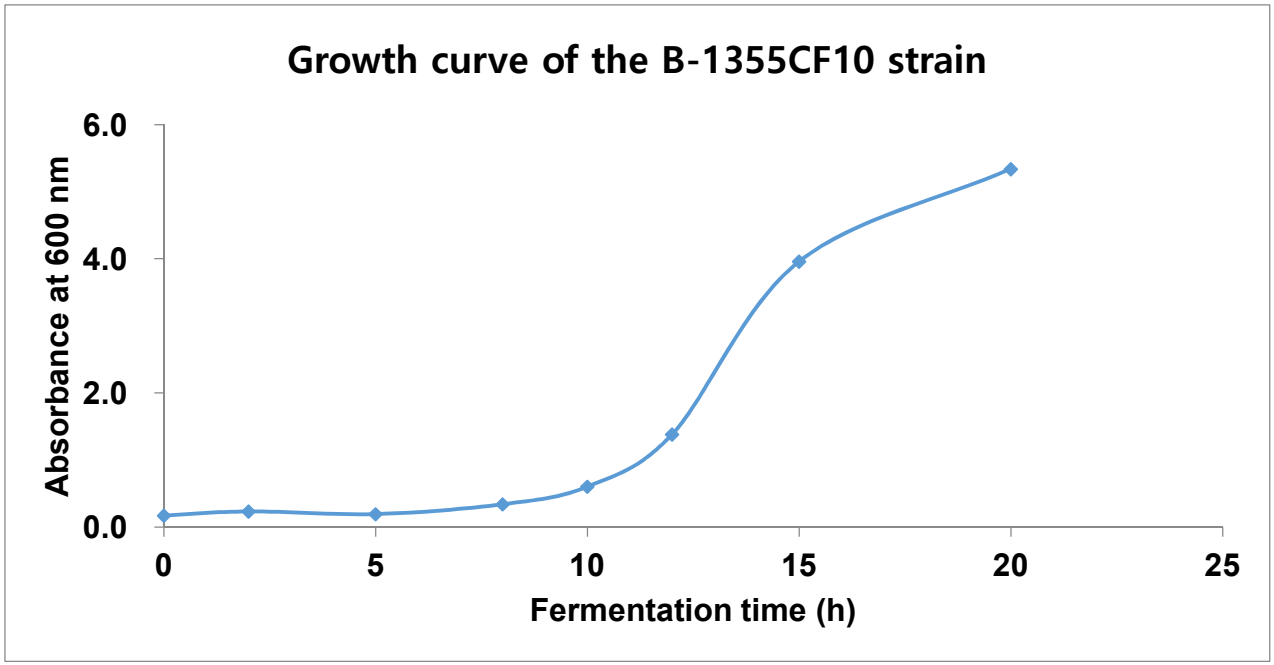


Figure 5. Growth curve of the B-1355CF10 strain during 14 L fermentation using fermenter.

Table 1. Change of activity of the $GSase_{1355}$ by concentration

Procedure	Fraction (L)	Total units (U/mL)	Specific activity (U/mg protein)
Culture supernatant	26.0	3.1	8.7
Concentration	0.56	61.2	183.3
	(g)	(U/g)	(U/mg protein)
Lyophilization	5.52	3975.0	183.3

3. Effects of factors on synthesis of the IDMOS

In statistic analysis, after digestion of oligosaccharides part (DP≥3) was focused on. In case of reaction time maximization, sucrose contents were focused.

By statistical analysis, maximized concentration of the $GSase_{1355}$ was 1 U/mL. As the $GSase_{1355}$ concentration increased, the IDMOS also continuously increased. Even though optimal condition from statistical analysis was 0.75 U/mL, 1 U/mL was set the maximized condition because of conversion yield (**Figure 6** and **Table 2**). Continuously optimal condition of the $DSase_{512}$ was 1 U/mL and maltose concentration was 0.25 M (**Figure 7**, **8** and **Table 3**, **4**). And optimal reaction time was 4 h (**Figure 9** and **Table 5**). All statistical analysis was carried out at α=0.05. The same superscript letters mean they are significantly not different (p-value<0.05).

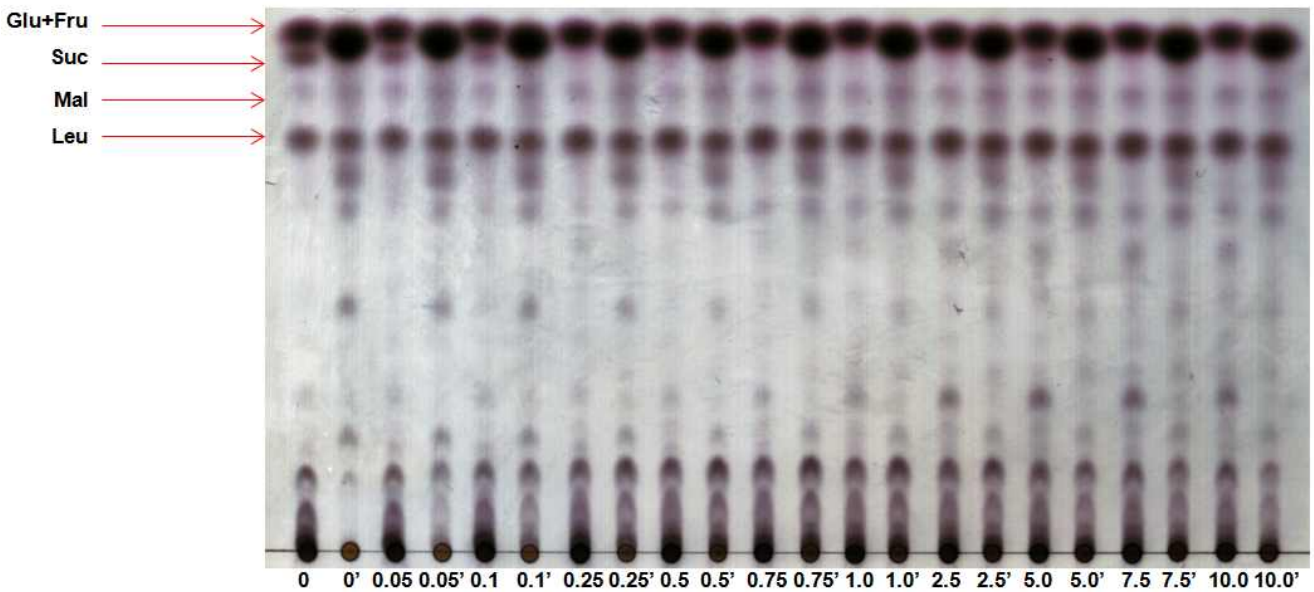


Figure 6. TLC analysis for maximization of the $GSase_{1355}$ concentration synthesizing the IDMOS. Conditions were described in Table 2. Lane 0–10.0 means reacted samples under different the $GSase_{1355}$ concentration (U/mL). Lane 0'–10.0' means samples treated digestion enzymes of 0–10.0 samples respectively.

Table 2. Conditions for maximization of the $GSase_{1355}$ concentration synthesizing the IDMOS and results of TLC analysis

Conditions				Before digestion (relative ratio, %)				After digestion (relative ratio, %)				DP≥3 conversion ratio (%)
$DSase_{512}$	$GSase_{1355}$	Suc	Mal	Mono	Di	Leu	DP≥3	Mono	Di	Leu	DP≥3	
10.0	0	2.0	0.2	28.3±5.2	22.4±3.2	8.8±1.7	40.5±7.5	51.1±7.7	15.0±2.7	8.8±1.7	25.1^{a}±6.9	61.9
	0.05			25.9±4.3	20.1±2.9	9.1±1.6	44.8±6.8	46.4±5.9	14.1±2.2	8.8±1.7	$30.8^{a,b,c,d,e,f}$±6.0	68.7
	0.1			25.6±4.4	18.0±3.2	9.4±1.5	47.0±7.4	43.2±6.6	13.5±1.9	8.8±1.5	$34.5^{a,b,c,d,e,f,g}$±7.0	73.3
	0.25			25.9±4.8	15.4±3.1	9.9±1.7	48.9±8.0	40.5±5.8	13.0±1.9	8.9±1.7	$37.7^{b,c,d,e,f,g,h,i}$±6.5	77.2
	0.5			27.0±5.4	15.4±3.7	10.2±1.6	47.4±9.4	39.8±6.5	12.9±2.1	9.0±1.6	$38.3^{b,c,d,e,f,g,h,i}$±7.1	80.8
	0.75			26.1±5.2	16.0±3.4	10.3±1.9	47.6±8.9	37.8±5.2	12.7±2.0	8.8±1.4	$40.7^{b,c,d,e,f,g,h,I,j,k}$±5.9	85.4
	1.0			25.0±5.1	15.3±3.7	10.0±1.6	49.7±8.6	36.2±4.7	12.4±2.0	8.6±1.4	$42.9^{c,d,e,f,g,h,I,j,k}$±5.3	86.2
	2.5			22.6±4.3	14.9±3.2	9.8±1.5	52.7±7.5	33.8±3.8	12.3±1.9	8.9±1.3	$45.0^{d,e,f,g,h,I,j,k}$±4.4	85.5
	5.0			21.0±3.6	14.7±2.8	9.6±1.5	54.7±6.5	32.3±3.2	12.2±1.9	8.9±1.2	$46.6^{d,e,f,g,h,I,j,k}$±3.6	85.2
	7.5			19.5±2.9	14.4±2.6	9.4±1.4	56.7±5.6	30.1±2.5	12.4±1.9	8.8±1.1	$48.7^{f,g,h,I,j,k}$±3.1	85.8
	10.0			18.2±2.5	14.6±2.7	9.1±1.5	58.1±5.0	29.6±2.1	12.6±2.1	8.8±1.1	$49.0^{f,g,h,I,j,k}$±2.7	84.4

($DSase_{512}$: concentration of the $DSase_{512}$ (U/mL), $GSase_{1355}$: concentration of the $GSase_{1355}$ (U/mL), Suc: sucrose concentration (M), Mal: maltose concentration (M), Mono: monosaccharides, Di: disaccharides, Leu: leucrose, DP: degree of polymerization, conversion ratio: (after digestion / before digestion) × 100 (%). Groups not sharing a common letter are significantly different when analyzed by ANOVA followed by the Games-Howell post hoc test (α=0.05, p-value<0.05).)

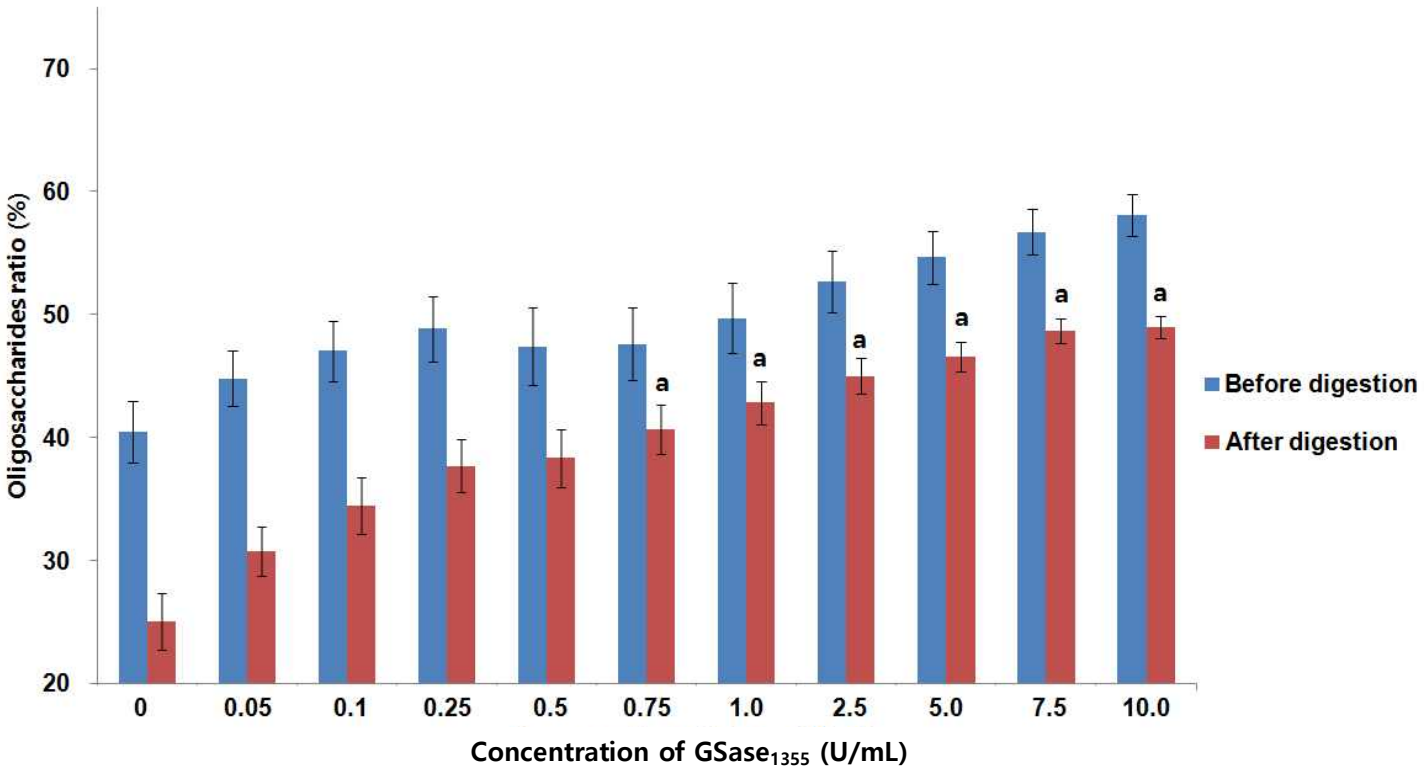


Figure 7. Change of DP≥3 (oligo) part of maximization for the $GSase_{1355}$ concentration. Error bar means standard error. [a]: significantly not different group.

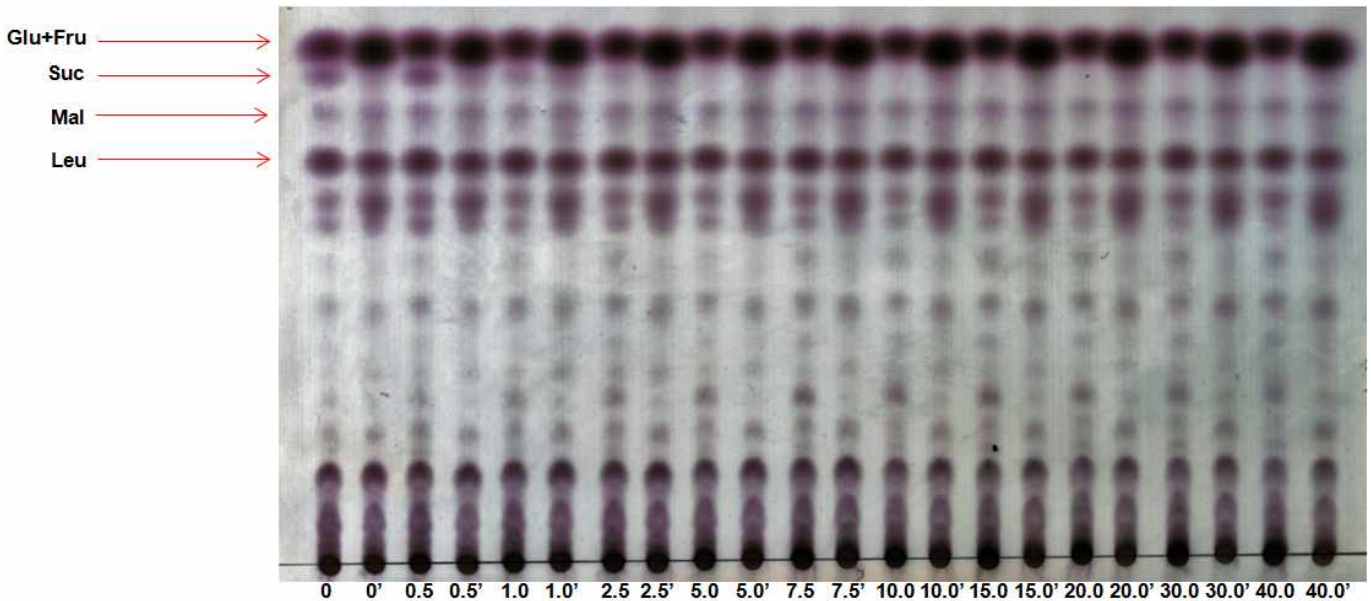


Figure 8. TLC analysis for maximization of the $DSase_{512}$ concentration synthesizing the IDMOS. Conditions were described in Table 3. Lane 0–40.0 means reacted samples under different the $DSase_{512}$ concentration (U/mL). Lane 0'–40.0' means samples treated digestion enzymes of 0–40.0 samples respectively.

Table 3. Conditions for maximization of the $DSase_{512}$ concentration synthesizing the IDMOS and results of TLC analysis

Conditions				Before digestion (relative ratio, %)				After digestion (relative ratio, %)				DP≥3 conversion ratio (%)
$DSase_{512}$	$GSase_{1355}$	Suc	Mal	Mono	Di	Leu	DP≥3	Mono	Di	Leu	DP≥3	
0				24.0±3.8	11.6±4.7	11.9±1.2	52.5±2.2	28.6±4.0	12.5±5.5	10.0±0.4	48.9±2.0[a]	93.1
0.5				22.2±2.9	11.6±4.5	11.3±0.9	54.8±2.2	27.0±3.6	12.5±5.4	9.7±0.6	50.8±1.9[a,b]	92.7
1.0				21.0±2.3	10.5±4.2	10.8±0.9	57.7±2.7	25.2±3.1	12.3±5.6	9.3±0.5	53.2±3.1[b,c]	92.2
2.5				20.3±1.9	9.9±3.7	10.7±0.8	59.1±2.5	24.9±3.0	12.3±5.4	9.3±0.5	53.6±2.5[b,c]	90.6
5.0				20.4±1.8	9.9±3.5	10.7±0.9	59.0±2.5	25.3±3.2	12.4±5.3	9.2±0.7	53.0±2.5[b,c]	90.0
7.5	1.0	2.0	0.2	20.4±2.0	10.1±3.7	10.4±0.8	59.1±3.0	24.9±2.9	12.6±5.5	9.1±0.6	53.4±3.0[b,c]	90.3
10.0				20.4±1.8	10.0±3.3	10.3±0.8	59.3±2.7	24.4±2.8	12.4±5.3	8.9±0.7	54.3±2.7[c]	91.5
15.0				19.7±1.7	10.0±3.5	10.2±0.9	60.1±3.0	24.3±3.2	12.4±5.1	8.8±0.7	54.5±2.7[c]	90.7
20.0				19.7±2.0	10.0±3.5	10.0±0.8	60.2±3.2	24.1±2.7	12.4±5.2	8.7±0.7	54.8±3.0[c]	91.0
30.0				19.1±2.0	10.1±3.5	9.8±0.8	61.0±3.7	23.4±3.2	12.8±5.7	8.5±0.7	55.2±3.8[c]	90.6
40.0				18.7±2.5	10.4±3.7	9.6±0.9	61.3±4.2	23.6±3.2	12.9±5.4	8.6±0.6	54.9±4.1[c]	89.6

($DSase_{512}$: concentration of the $DSase_{512}$ (U/mL), $GSase_{1355}$: concentration of the $GSase_{1355}$ (U/mL), Suc: sucrose concentration (M), Mal: maltose concentration (M), Mono: monosaccharides, Di: disaccharides, Leu: leucrose, DP: degree of polymerization, conversion ratio: (after digestion / before digestion) × 100 (%). Groups not sharing a common letter are significantly different when analyzed by ANOVA followed by the Duncan post hoc test (α=0.05, p-value<0.05).)

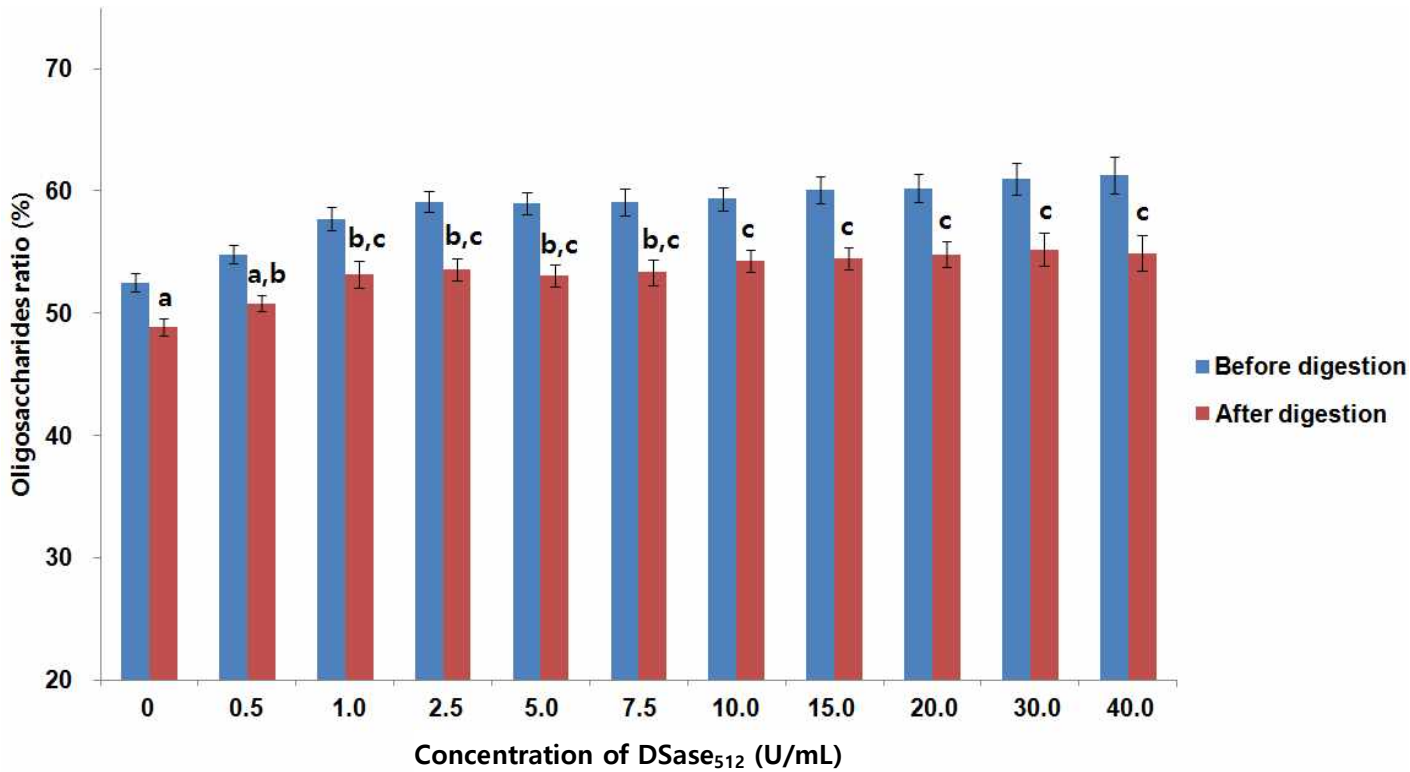


Figure 9. Change of DP≥3 (oligo) part of maximization for the $DSase_{512}$ concentration. Error bar means standard error. $^{a\text{-}c}$: significantly not different group respectively.

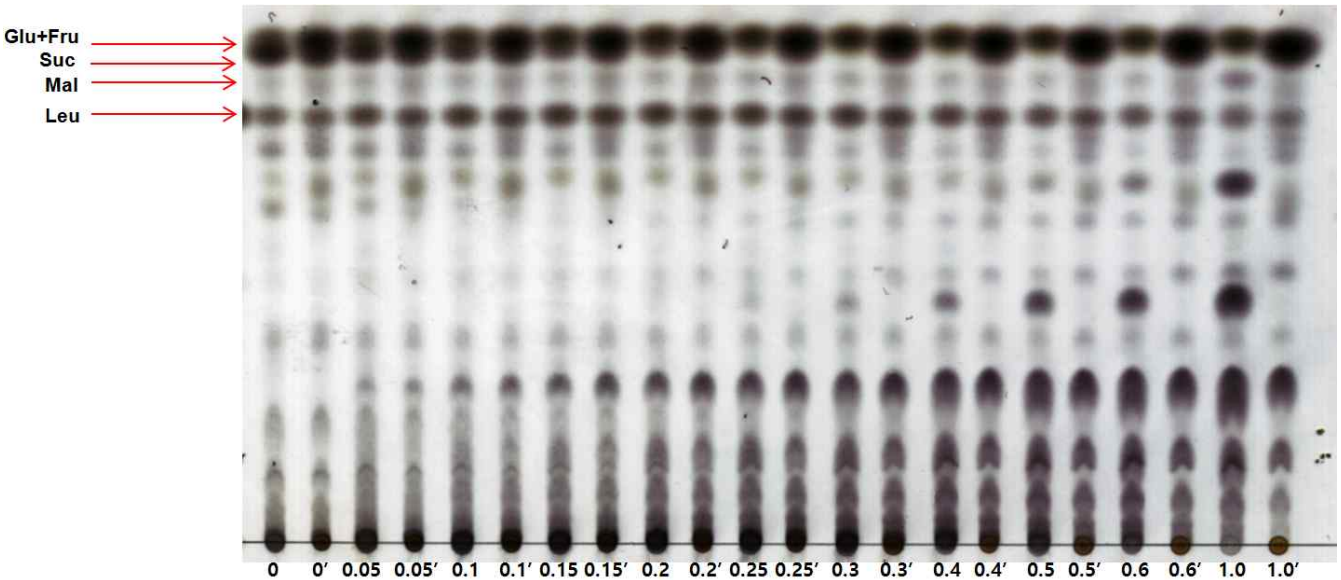


Figure 10. TLC analysis for maximization of maltose concentration synthesizing the IDMOS. Conditions were described in Table 4. Lane 0–1.0 means reacted samples under different maltose concentration (M). Lane 0'–1.0' means samples treated digestion enzymes of 0–1.0 samples respectively.

Table 4. Conditions for maximization of maltose concentration synthesizing the IDMOS and results of TLC analysis

Conditions				Before digestion (relative ratio, %)				After digestion (relative ratio, %)				DP≥3 conversion ratio (%)
$DSase_{512}$	$GSase_{1355}$	Suc	Mal	Mono	Di	Leu	DP≥3	Mono	Di	Leu	DP≥3	
1.0	1.0	2.0	0	26.1±13.2	35.9±15.1	12.8±2.1	25.2±9.1	42.8±14.5	22.3±11.4	10.5±1.6	24.4±8.4^{a}	96.7
			0.05	23.6±8.1	24.6±9.6	12.5±1.9	39.29±7.7	37.5±10.4	17.8±9.4	11.0±1.2	33.8±5.4^{b}	86.0
			0.1	21.4±5.5	20.2±7.6	12.2±1.9	46.3±8.0	32.8±6.2	15.6±8.2	10.4±0.7	41.2±4.3^{c}	89.0
			0.15	20.8±4.7	15.8±5.6	11.8±1.5	51.6±7.2	31.0±5.1	14.2±7.3	10.2±0.5	44.5±4.7^{c,d}	86.2
			0.2	20.8±4.4	12.5±4.2	11.5±1.7	55.2±6.9	29.9±3.8	12.8±6.5	9.8±0.6	47.5±5.0^{d,e}	86.0
			0.25	19.8±3.9	10.5±3.4	10.3±1.7	59.4±6.7	28.8±3.2	12.3±6.4	8.9±0.6	50.0±5.1^{d,e,f}	84.1
			0.3	18.3±3.4	9.8±3.3	8.9±1.4	63.1±6.7	27.0±2.3	11.9±6.3	8.0±0.7	53.1±5.5^{e,f}	84.2
			0.4	16.3±3.2	8.7±3.0	7.3±1.3	67.7±6.4	27.0±2.6	11.8±6.2	7.1±0.5	54.1±5.5^{f}	79.9
			0.5	15.9±3.6	8.1±2.5	6.3±1.1	69.7±5.7	28.2±2.6	11.4±5.9	6.6±0.5	53.7±5.5^{f}	77.0
			0.6	14.7±3.0	8.2±2.5	5.6±1.0	71.4±5.2	28.9±3.2	12.0±6.2	6.4±0.5	52.7±5.4^{e,f}	73.8
			1.0	12.7±2.6	10.9±3.2	4.8±0.7	71.6±5.2	32.6±4.5	12.3±6.2	6.3±0.6	48.8±4.7^{d,e,f}	68.1

($DSase_{512}$: concentration of the $DSase_{512}$ (U/mL), $GSase_{1355}$: concentration of the $GSase_{1355}$ (U/mL), Suc: sucrose concentration (M), Mal: maltose concentration (M), Mono: monosaccharides, Di: disaccharides, Leu: leucrose, DP: degree of polymerization, conversion ratio: (after digestion / before digestion) × 100 (%). Groups not sharing a common letter are significantly different when analyzed by ANOVA followed by the Duncan post hoc test (α=0.05, p-value<0.05).)

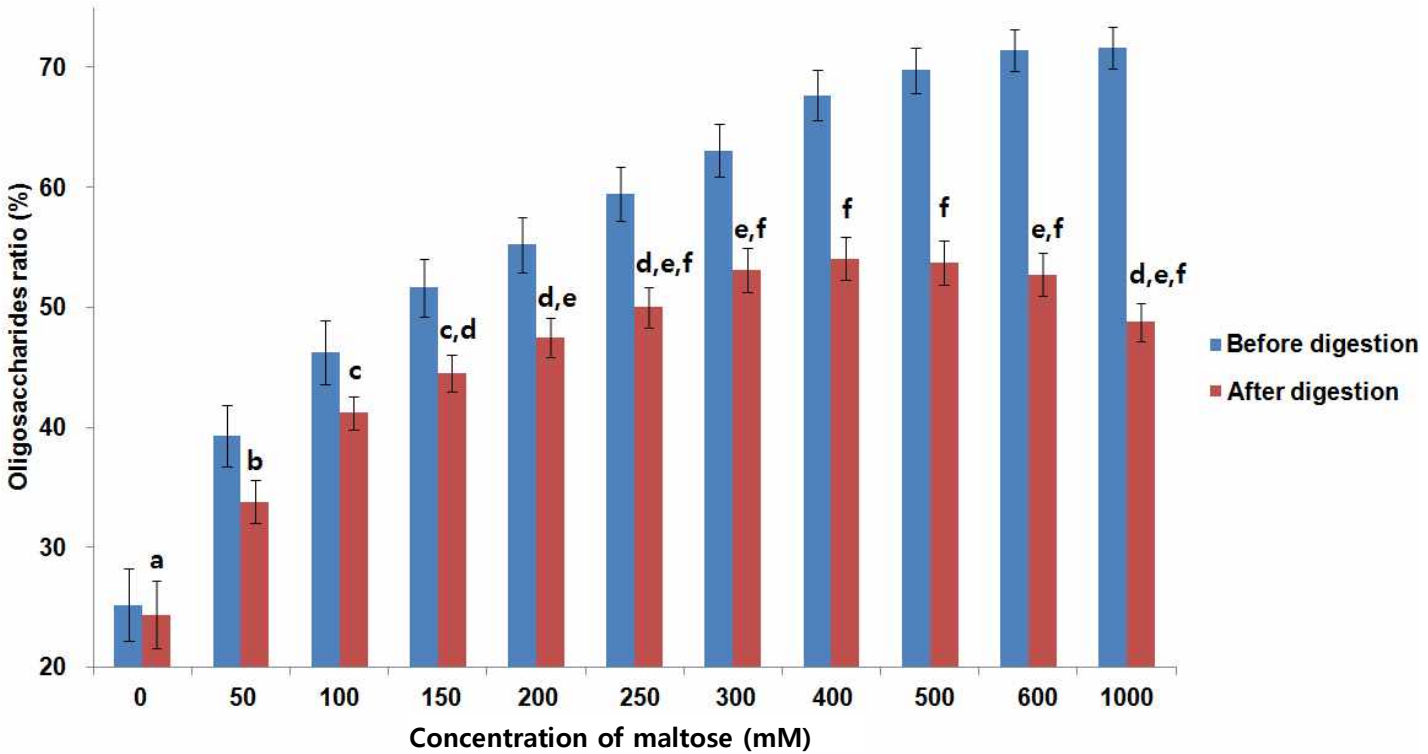


Figure 11. Change of DP≥3 (oligo) part of maximization for maltose concentration. Error bar means standard error. [a-f]: significantly not different group respectively.

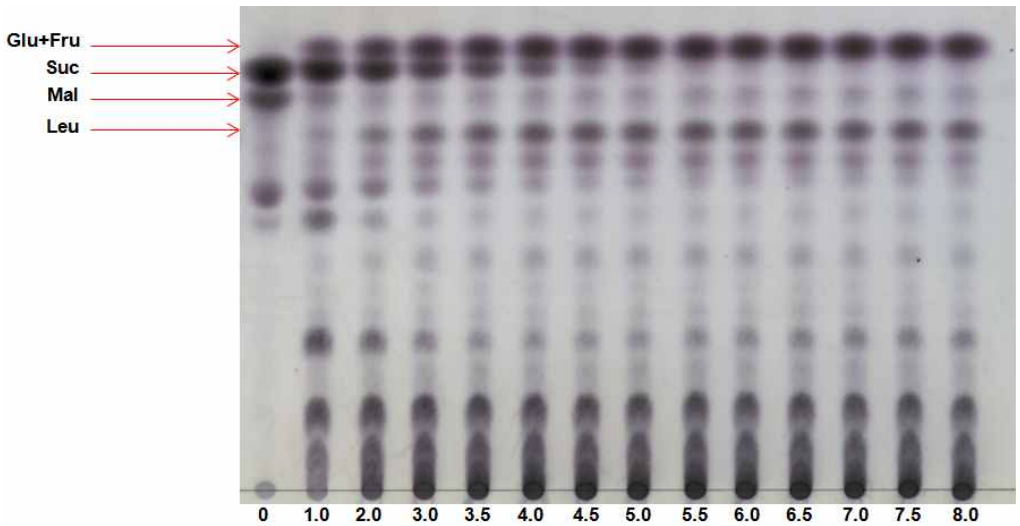


Figure 12. TLC analysis for maximization of reaction time synthesizing the IDMOS. Conditions were described in Table 5. Lane 0–8.0 means reacted samples under different reaction time (h).

Table 5. Conditions for maximization of reaction time synthesizing the IDMOS and results of TLC analysis

Conditions					Before digestion (relative ratio, %)				
$DSase_{512}$	$GSase_{1355}$	Suc	Mal	Time	Mono	Suc	Mal	Leu	DP≥3
1.0	1.0	2.0	0.25	0	2.5±0.9	$39.6^{a,b,c}$±4.2	24.5±2.7	5.4±0.8	28.0±4.9
				1.0	8.8±0.5	$23.2^{a,b,c,d}$±1.8	7.3±0.3	3.9±0.3	56.8±1.2
				2.0	11.0±0.2	$19.8^{a,b,c,d,e}$±1.8	4.5±0.4	5.3±0.2	59.3±1.5
				3.0	13.4±0.5	$15.9^{b,c,d,e,f}$±1.4	3.9±0.4	6.7±0.1	60.1±0.9
				3.5	13.9±0.5	$13.4^{c,d,e,f,g,h}$±1.5	3.8±0.4	7.3±0.2	61.6±1.2
				4.0	14.6±0.3	$10.9^{d,e,f,g,h,i}$±1.0	3.9±0.1	7.9±0.1	62.7±1.3
				4.5	15.1±1.3	$8.7^{e,f,g,h,i}$±1.1	3.9±0.4	8.3±0.1	64.0±0.8
				5.0	15.4±0.6	$7.2^{e,f,g,h,i}$±0.5	4.0±0.5	8.2±0.3	65.3±0.7
				5.5	15.3±0.2	$6.9^{e,f,g,h,i}$±0.4	4.1±0.5	8.4±0.2	65.3±0.3
				6.0	15.3±0.8	$6.6^{e,f,g,h,i}$±0.7	4.1±0.6	8.5±0.2	65.5±0.4
				6.5	15.8±0.7	$6.4^{e,f,g,h,i}$±0.5	4.2±0.5	8.6±0.2	65.0±0.4
				7.0	15.9±0.8	$6.3^{f,g,h,i}$±0.9	4.2±0.4	8.5±0.2	65.1±0.4
				7.5	15.9±1.0	$6.1^{e,f,g,h,i}$±0.6	4.2±0.4	8.5±0.2	65.3±0.5
				8.0	16.6±1.1	$6.2^{f,g,h,i}$±0.6	4.3±0.4	8.5±0.2	64.5±0.6

($DSase_{512}$: concentration of the $DSase_{512}$ (U/mL), $GSase_{1355}$: concentration of the $GSase_{1355}$ (U/mL), Suc: sucrose concentration (M), Mal: maltose concentration (M), Mono: monosaccharides, Leu: leucrose, DP: degree of polymerization, Groups not sharing a common letter are significantly different when analyzed by ANOVA followed by the Games-Howell post hoc test (α=0.05, p-value<0.05).)

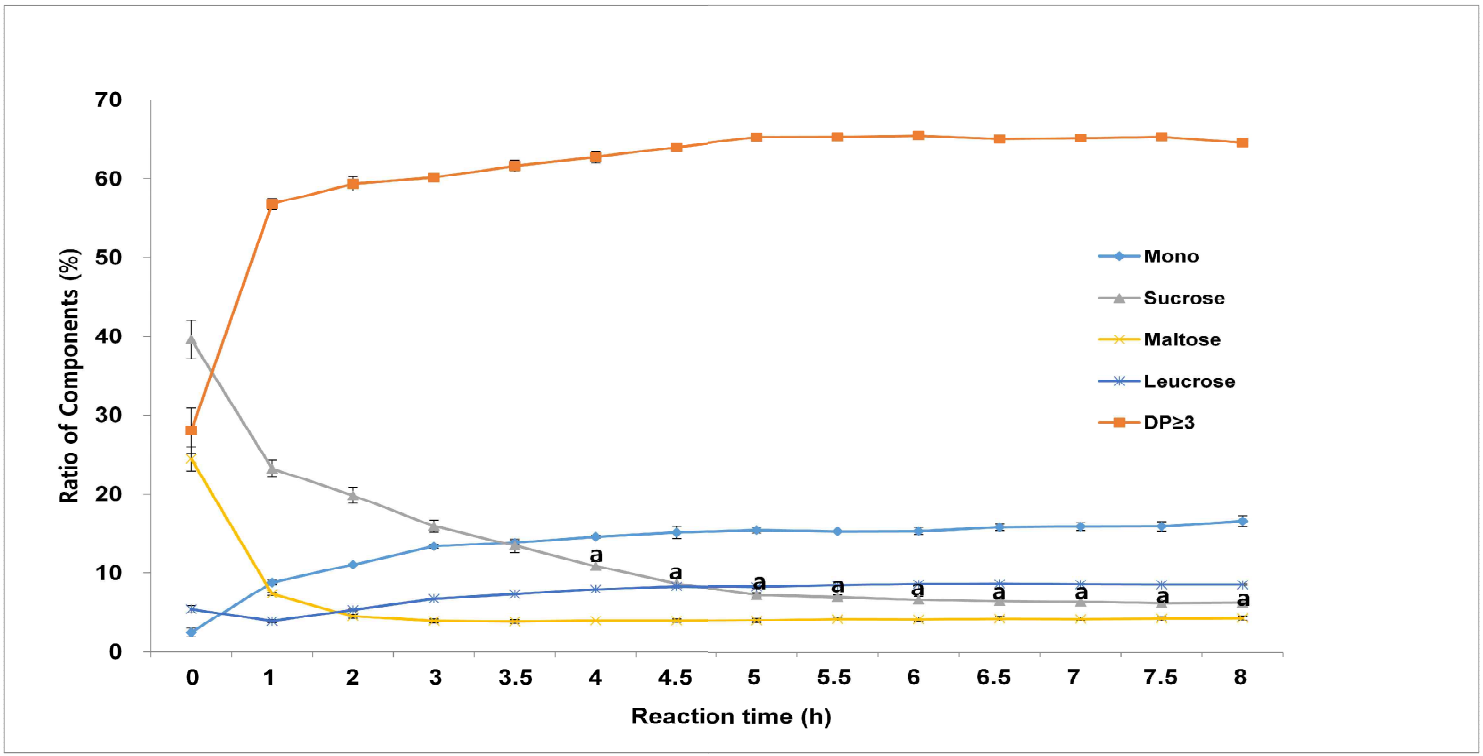


Figure 13. Change of components of maximization for reaction time. Mono: monosaccharides including glucose and fructose, DP: degree of polymerization. [a]: significantly not different group.

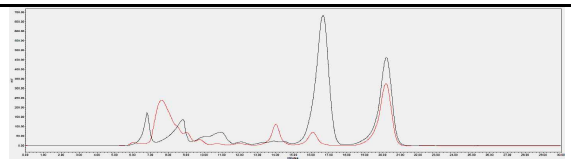
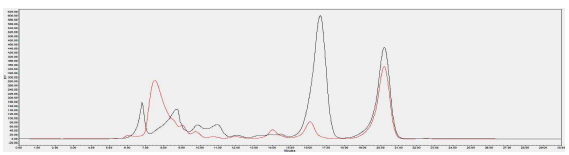
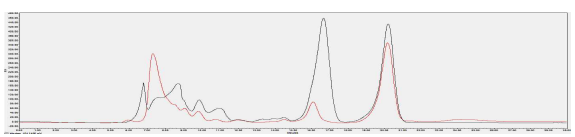
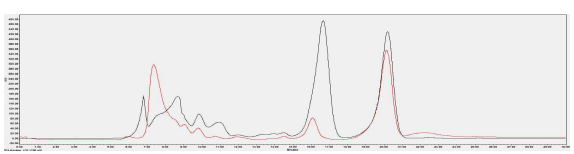
4. Analysis of the IDMOS after treating α-amylase and α-amyloglucosidase using HPLC and TLC

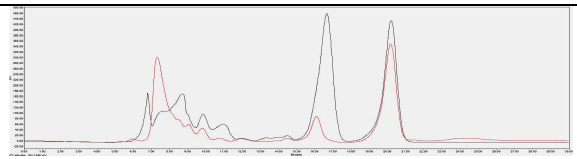
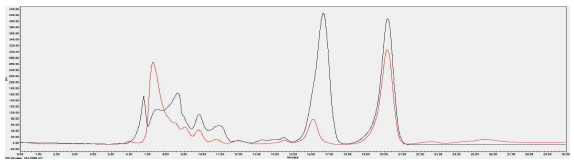
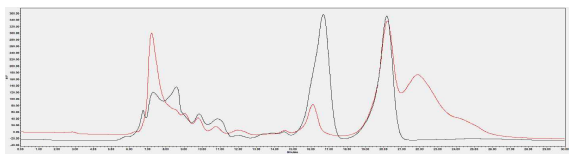
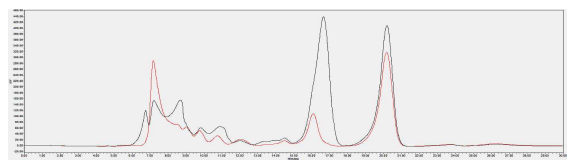
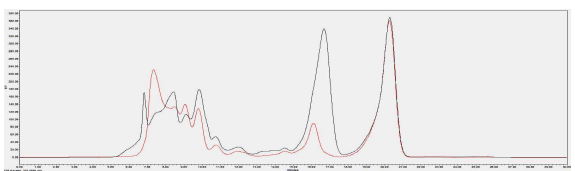
To supplement for TLC analysis, HPLC analysis was carried out. Peak areas of DP≥3 part except enzymes peak areas were calculated and compared samples between before and after treatment of sugar-digestive enzymes. Likewise the results of TLC analysis, HPLC analysis also showed optimal concentration was 1.0 U/mL of $GSase_{1355}$ from conversion yield (%) (**Table 6**).

Optimal condition sample was injected to analyze using the MCI GEL CK04SS column to see peaks of DP≥3 products. Result from comparison between before and after digestion, conversion yield (%) was also the same value as 102% (**Figure 14**).

By TLC analysis with the IDMOS and industrial oligosaccharides, indigestibility of oligosaccharides was confirmed (**Figure 15**).

Table 6. HPLC analysis for maximization of $GSase_{1355}$ synthesizing the IDMOS

$GSase_{1355}$ concentration (U/mL)	Chromatogram	Sum of DP≥3 peak areas		Conversion ratio (%)
		Before digestion	After digestion	
0		21904776±447713	15207880±253745	69.4
0.1		23347450±203849	17510312±288202	75.0
0.25		22658714±166785	20560969±480897	90.7
0.5		23040889±38333	23789165±357717	103.3

0.75		23184630±71894	25439936±249968	109.7
1.0		20274077±150215	24574175±760952	121.2
2.5		23657371±61090	26092115± 389119	110.3
7.5		21663332±306784	24657618±334279	113.9
Maximized		28823672±128989	29491139±153530	102.3

(Conditions were written in Table 2. Red line: before treating digestion enzymes, black line: after treating digestion enzymes.)

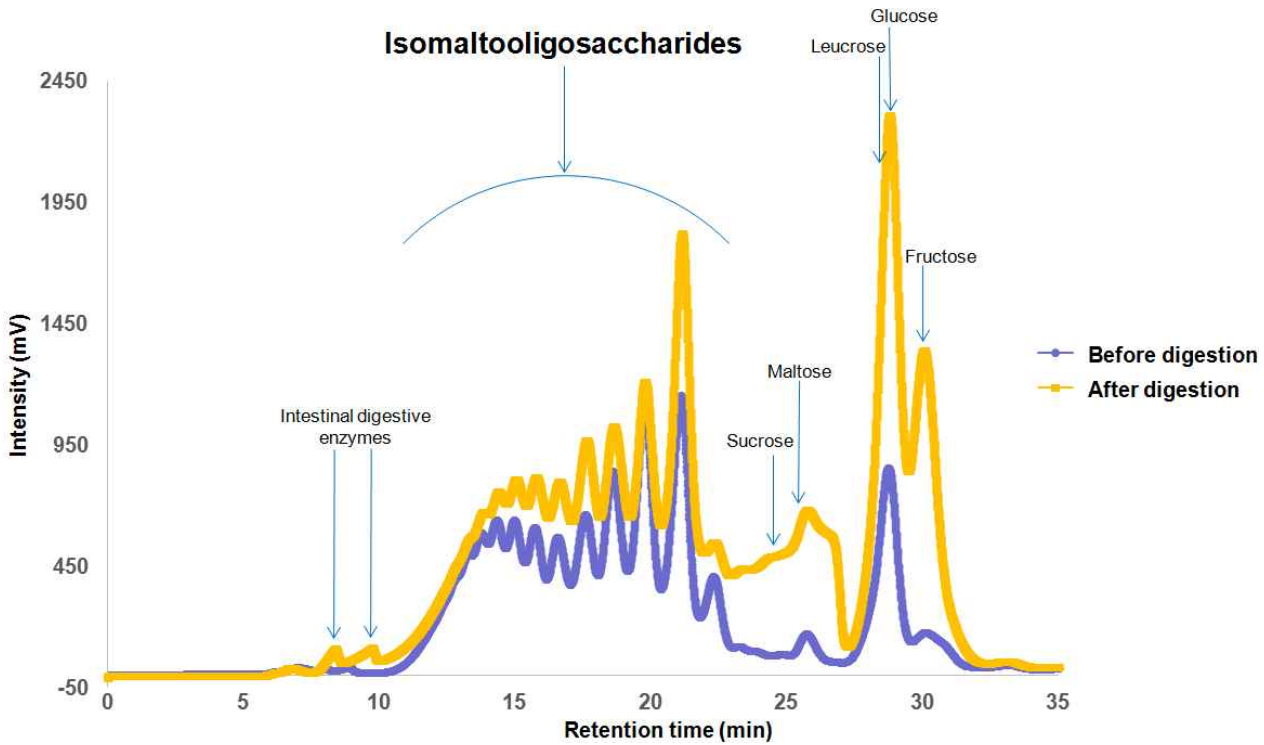


Figure 14. Chromatogram of maximized condition of the IDMOS. Blue line: before treating digestion enzymes, yellow line: after treating digestion enzymes treated.

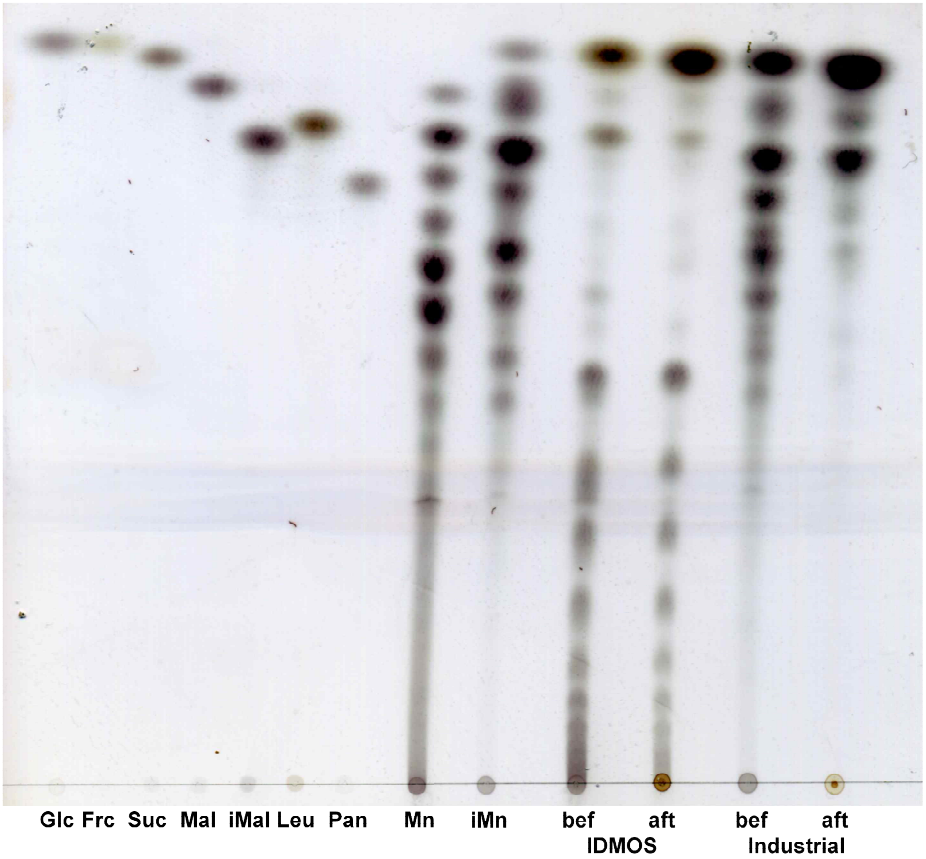


Figure 15. Comparison of indigestibility between the IDMOS and an industrial oligosaccharides product (Ottugi). Lane Glc: glucose, lane Frc: fructose, lane Suc: sucrose, lane Mal: maltose, lane iMal: isomaltose, lane Leu: leucrose, lane Pan: panose, lane Mn: maltooligosaccharides standard, lane iMn: isomaltooligosaccharides standard, lane bef: before digestion enzymes treated, lane aft: after digestion enzymes treated.

Table 7. Components of saccharides in the IDMOS and an industrial oligosaccharide product by TLC analysis

	IDMOS (relative ratio, %)		Industrial oligosaccharides (relative ratio, %)	
	Before digestion	After digestion	Before digestion	After digestion
Monosaccharides	15.4	27.3	16.1	34.1
Maltose	2.7	4.6	10.3	15.6
Isomaltose	-	-	13.6	20.2
Leucrose	8.0	6.8	-	-
Oligosaccharides	73.9	61.3	60.0	30.1

5. Removal of monosaccharides using yeast beads

After yeast bead treatment, fructose of the IDMOS in the sample was almost removed and other compounds were not affected by yeast beads. So by treating yeast beads, monosaccharides were removed selectively (**Figure 16**).

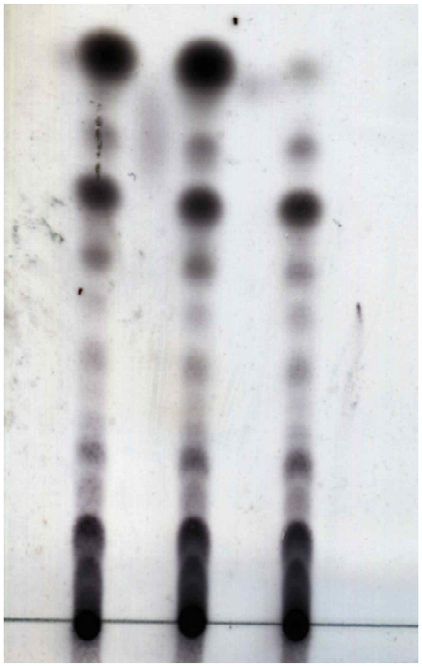


Figure 16. Comparison of the IDMOS after scale-up and treating yeast beads. Lane 200 µL and 500 mL: total volume of maximized reaction of the IDMOS, lane After yeast: after treating yeast beads.

6. Prevention of insoluble glucan (mutan) formation by the IDMOS

Results showed that under fixed sucrose concentration, the IDMOS concentration increased, amount of produced insoluble glucan (mutan) reduced. Especially, at each sucrose concentration, when 10 % (w/v) of the IDMOS was treated, formation of insoluble glucan was reduced below 50% (**Table 8** and **Figure 17**).

Table 8. Formation of insoluble glucan by the IDMOS concentration

Sucrose concentration (%)	Soluble sugar concentration from mutan (mg/mL) (formation yield (%))									
	IDMOS concentration (%)									
	0	0.1	0.5	1.0	1.5	2.0	3.0	5.0	7.5	10.0
1.0	1.12±0.09 (100.0)	0.91±0.03 (81.4)	0.84±0.11 (75.5)	0.76±0.14 (67.5)	0.76±0.12 (67.5)	0.69±0.05 (61.7)	0.58±0.07 (52.2)	0.49±0.02 (43.8)	0.64±0.07 (57.1)	0.56±0.06 (50.0)
3.0	2.98±0.21 (100.0)	2.86±0.27 (95.7)	2.77±0.05 (92.9)	2.40±0.07 (80.5)	2.43±0.09 (81.4)	2.13±0.01 (71.4)	1.81±0.14 (60.5)	1.41±0.06 (47.3)	1.45±0.28 (48.5)	1.25±0.17 (41.8)
5.0	4.07±0.25 (100.0)	4.09±0.19 (100.5)	3.73±0.07 (91.8)	3.41±0.12 (83.8)	3.62±0.31 (89.1)	3.01±0.20 (73.9)	2.56±0.19 (63.0)	2.29±0.26 (56.2)	2.11±0.52 (51.9)	1.84±0.19 (45.3)

(Formation yield: relative amount of components when 0 % of the IDMOS concentration is 100 % under same sucrose concentration.)

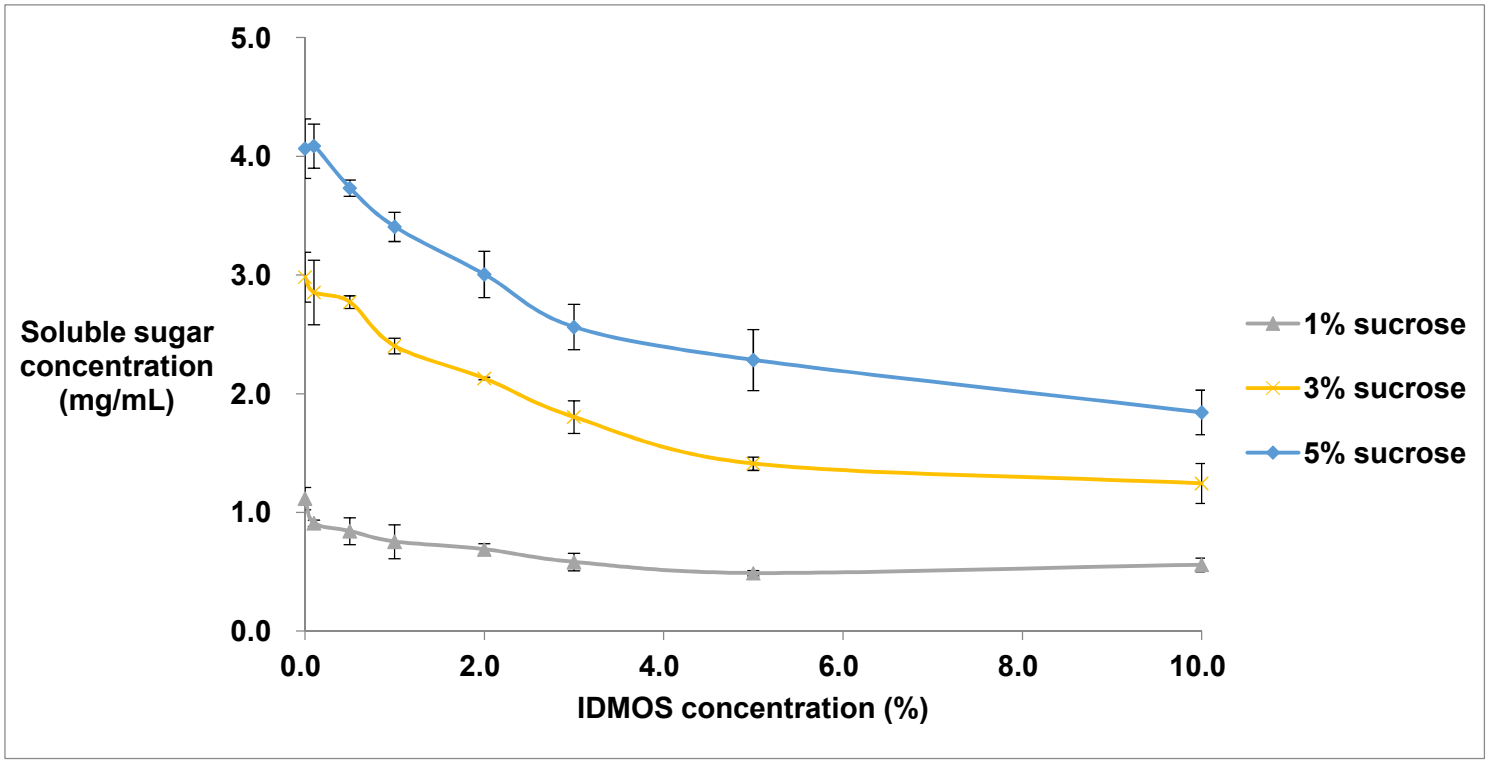


Figure 17. Prevention of mutan formation by the IDMOS concentration. Error bar means standard error.

7. Effect of the IDMOS for growth of *Bifidobacteria*

4 *Bifidobacteria* strains were cultivated in media which have different carbon source. pH was measured to figure out specific carbon source consumption. All strains were grown well in MRS-glucose and MRS-IDMOS media with the comparison of cfMRS media as negative control. And the 4 strains, *B. bifidum*, *B. infantis*, *B. longum* and *B. adolescentis* were grown well in glucose added media rather than the IDMOS added media. Among the 4 strains, *B. adolescentis* showed the least pH of the IDMOS added media (**Table 9**).

Table 9. Change of pH by cultivation of *Bifidobacteria* strains

Strain	Initial (0 h)			After cultivation (24 h)		
	cfMRS	MRS-Glucose	MRS-IDMOS	cfMRS	MRS-Glucose	MRS-IDMOS
B. bifidum	6.4	6.0	6.2	6.1±0.0	4.3±0.0	5.4^{a}±0.0
B. infantis				6.1±0.0	4.3±0.0	5.5^{a}±0.0
B. longum				6.0±0.1	4.3±0.0	5.4^{a}±0.0
B. adolescentis				6.3±0.0	4.2±0.0	4.8^{b}±0.0

(Tests were carried out 3 times repeatedly. average±standard deviation. Duncan post hoc statistical analysis was carried out (α=0.05, p-value<0.05). $^{a-b}$: significantly not different group respectively.)

8. Antimicrobial effect of *Bifidobacteria* against pathogen bacteria through mixed cultivation

To figure out inhibition effect of pathogen growth by growing *Bifidobacteria* in the IDMOS added media, mixed cultivation between *Bifidobacteria* and *Salmonella typhimurium* as pathogen was carried out.

First, cultivation of *B. bifidum* and *S. typhimurium* was done. In case of single culture of *B. bifidum* and *S. typhimurium*, two strains was grown up to around log(CFU) 9 (**Table 10** and **Figure 18**). However, when mixed cultivation between two strains, *B. bifidum* was grown similar to single cultivation. *S. typhimurium* was showed log(CFU) 5 when mixed cultivation and after 72 h the bacteria was died out (**Table 10** and **Figure 19**).

Next, cultivation between *B. adolescentis* and *S. typhimurium* was carried out. In single cultivation, *B. adolescentis* was grown like *B. bifidum*. However, in mixed culture *B. adolescentis* was grown like single culture (**Table 11** and **Figure 20**) meanwhile *S. typhimurium* was rarely grown and died out after 48 h earlier time than the case of *B. bifidum* (**Table 11** and **Figure 21**).

To figure out consumption of the IDMOS, cultivated media were analyzed by TLC (**Figure 22**). *B. bifidum* consumed monosaccharides selectively, but *B. adolescentis* used monosaccharides for growth and oligo part, around DP 7, of the IDMOS.

Table 10. Mixed cultivation of *B. bifidum* and *S. typhimurium*

Cultivation time (h)	Measurement	Single culture		Mixed culture	
		S. typhimurium	*B. bifidum*	*S. typhimurium*	*B. bifidum*
0	log(CFU)/mL	3.1±0.1	4.4±0.1	3.1±0.1	4.4±0.1
	pH	6.2	6.2	6.2	
6	log(CFU)/mL	3.3±0.2	5.9±0.1	3.4±0.1	5.5±0.2
	pH	6.1	6.1	6.1	
12	log(CFU)/mL	4.6±0.2	8.5±0.1	4.5±0.1	8.6±0.1
	pH	6.1	5.9	5.9	
24	log(CFU)/mL	7.8±0.0	9.1±0.1	4.6±0.1	8.9±0.3
	pH	6.2	5.5	5.6	
36	log(CFU)/mL	8.3±0.1	9.1±0.1	4.8±0.2	9.1±0.2
	pH	6.0	5.5	5.6	
48	log(CFU)/mL	8.4±0.1	9.0±0.1	4.4±0.1	9.1±0.0
	pH	6.2	5.5	5.6	
72	log(CFU)/mL	6.6±0.0	9.2±0.2	0	9.1±0.0
	pH	6.4	5.5	5.6	

(average±standard deviation)

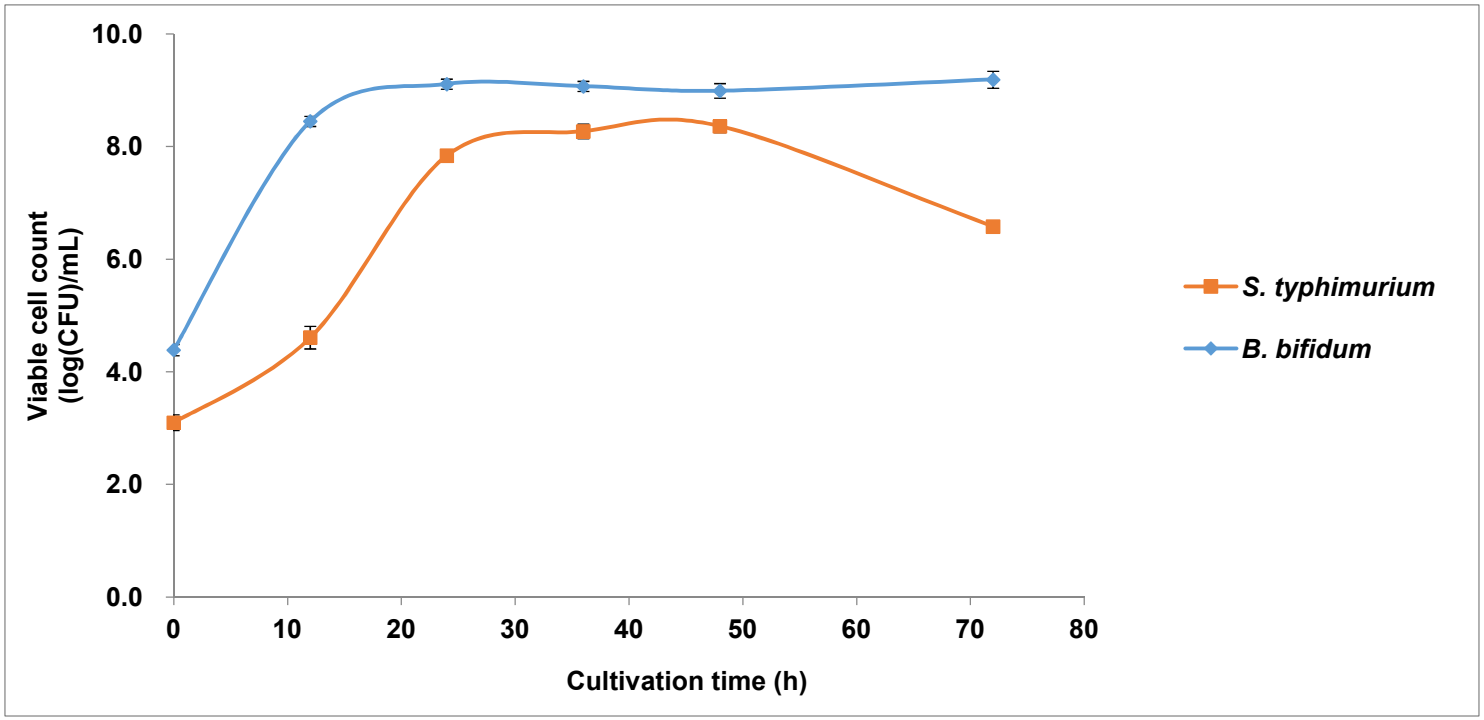


Figure 18. Single cultivation of *B. bifidum* and *S. typhimurium*.

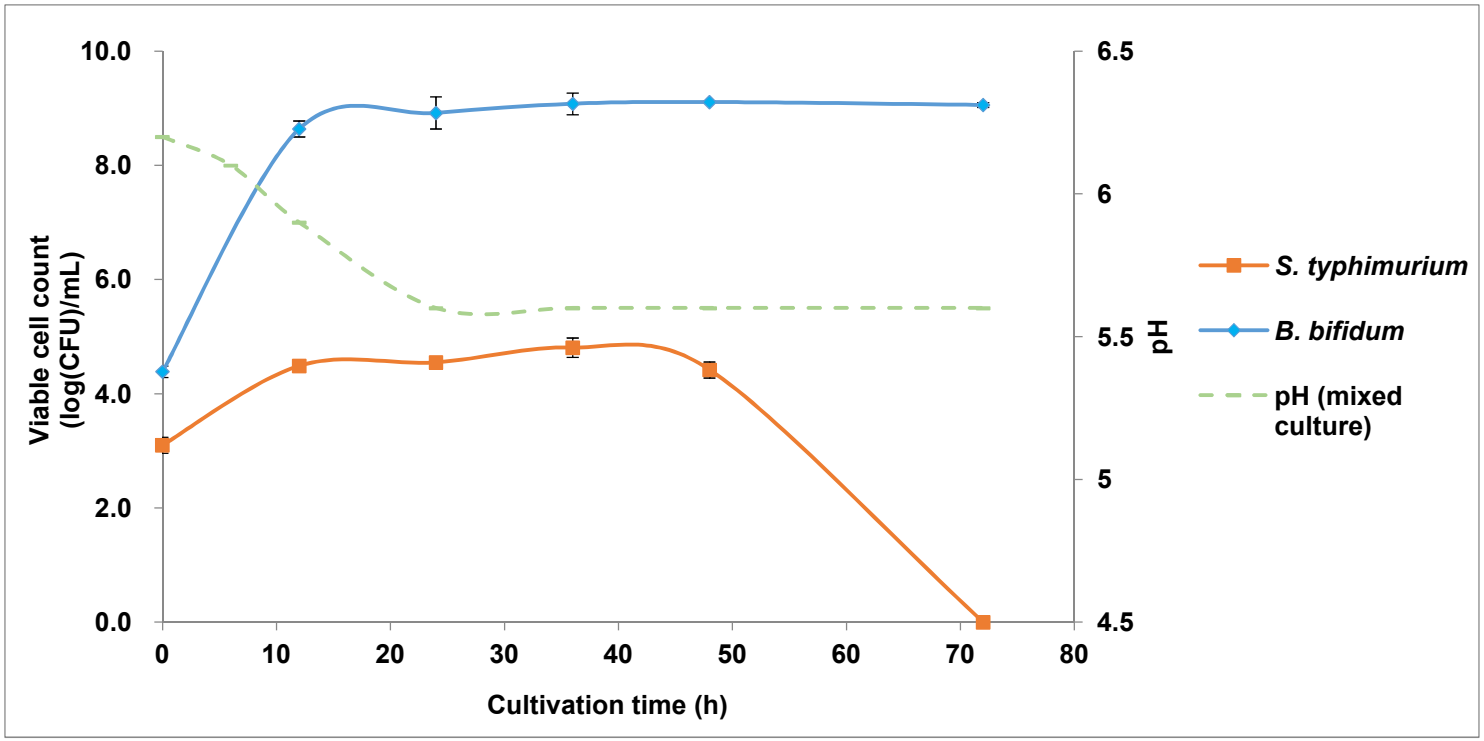


Figure 19. Mixed cultivation of *B. bifidum* and *S. typhimurium*.

Table 11. Mixed cultivation of *B. adolescentis* and *S. typhimurium*

Cultivation time (h)	Measurement	Single culture		Mixed culture	
		S. typhimurium	*B. adolescentis*	*S. typhimurium*	*B. adolescentis*
0	log(CFU)/mL	3.3±0.0	4.0±0.7	3.3±0.0	4.0±0.7
	pH	6.2	6.2	6.2	
12	log(CFU)/mL	3.3±0.1	7.0±0.1	3.4±0.2	6.8±0.0
	pH	6.2	6.1	6.2	
24	log(CFU)/mL	5.2±0.1	8.0±0.3	3.8±0.2	8.9±0.3
	pH	6.1	5.3	5.3	
36	log(CFU)/mL	7.7±0.1	8.5±0.4	2.9±0.2	8.0±0.8
	pH	6.2	4.9	4.9	
48	log(CFU)/mL	8.7±0.2	7.9±0.2	0	8.1±0.2
	pH	6.0	4.9	4.9	

(average±standard deviation)

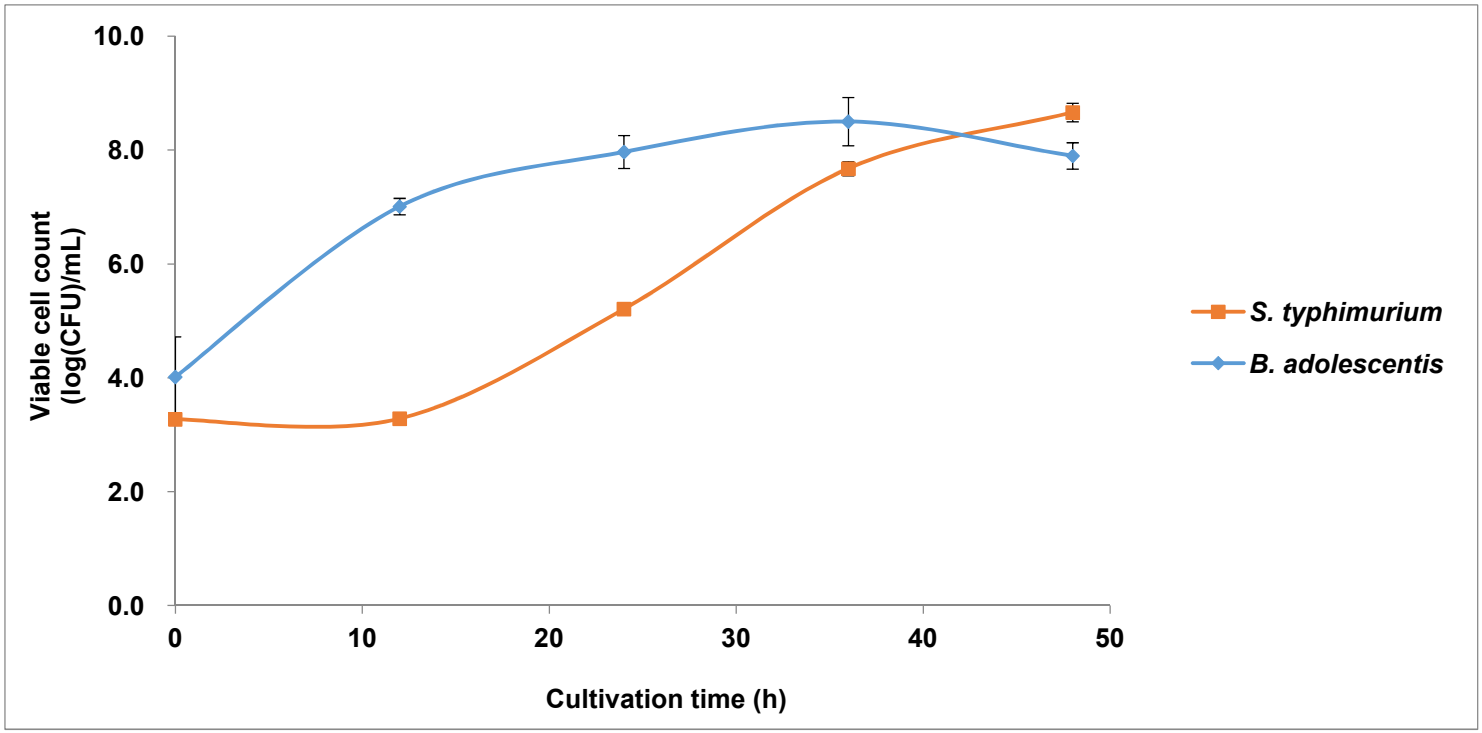


Figure 20. Single cultivation of *B. adolescentis* and *S. typhimurium*.

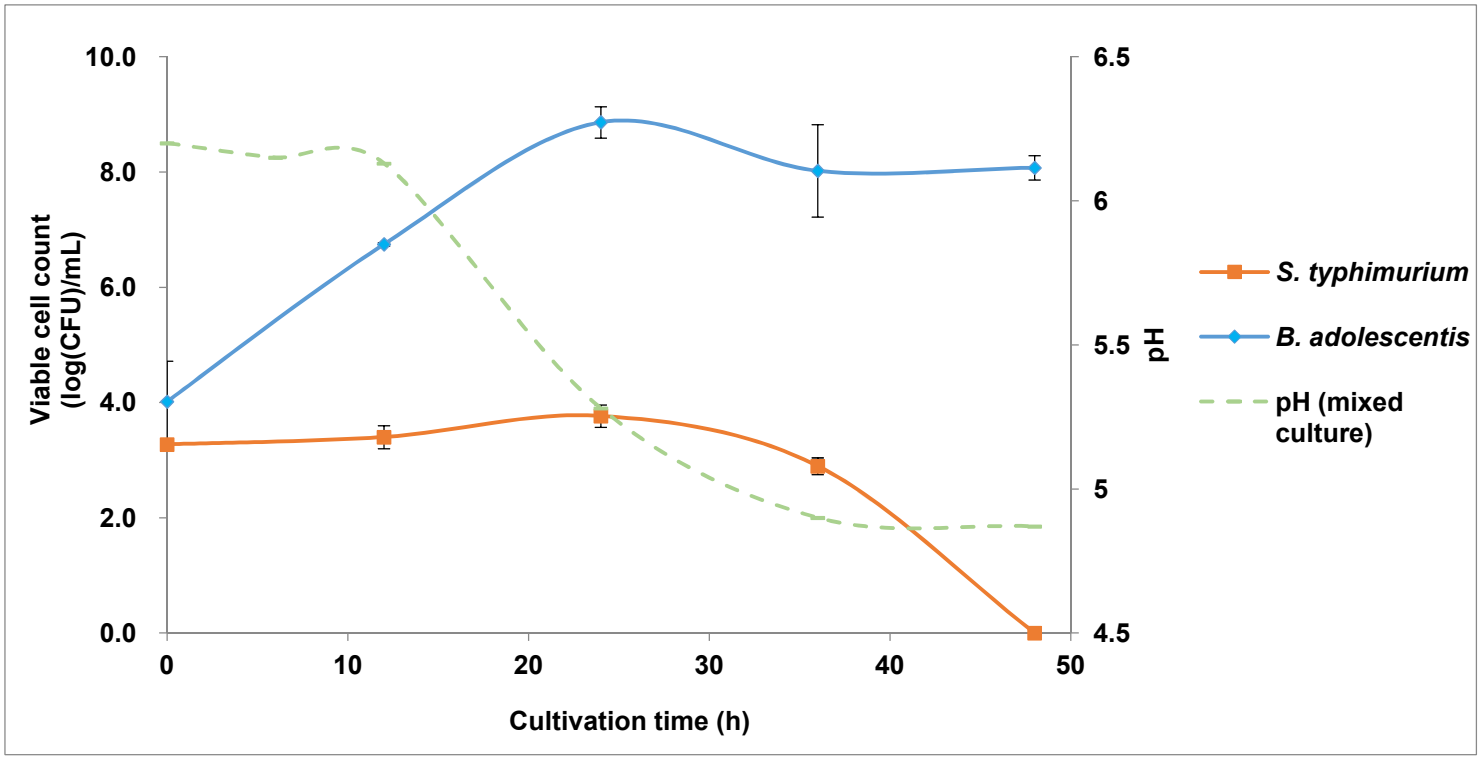


Figure 21. Mixed cultivation of *B. adolescentis* and *S. typhimurium*.

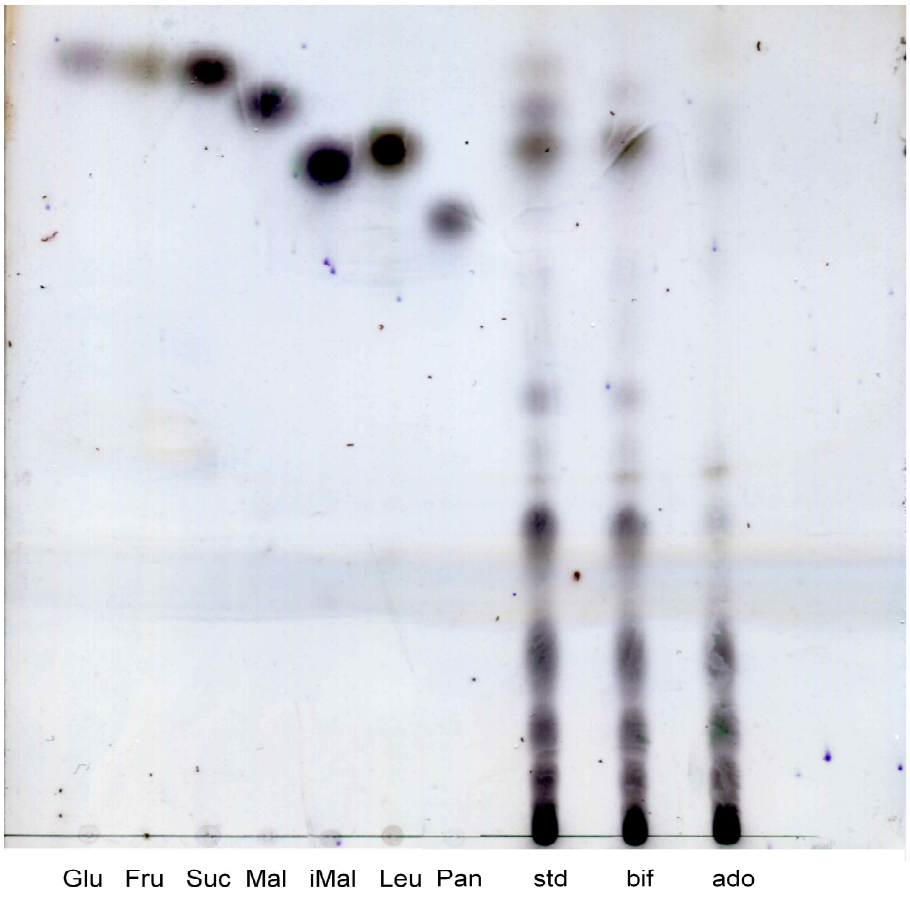


Figure 22. Change of the IDMOS components by cultivation of *Bifidobacteria* strains (72 h). Lane Glu: glucose, lane Fru: fructose, lane Suc: sucrose, lane Mal: maltose, lane iMal: isomaltose, lane Leu: leucrose, lane Pan: panose, lane std: MRS-IDMOS broth not cultivated, lane bif: cultivated MRS-IDMOS broth of *B. bifidum*, lane ado: cultivated MRS-IDMOS broth of *B. adolescentis*.

Discussion

IDMOS was firstly synthesized from original food bacteria strain, not recombinant strain.

A main goal of this study was to synthesize non- or in-digestible isomaltooligosaccharides because indigestibility is a key factor of prebiotics. To do so, two different enzymes which form different alpha glycosidic bonds were introduced. Our laboratory has isolated bacteria strains, *Leuconostoc mesenteroides* B-512FMCM and NRRL B-1355CF10 that produce high-activated enzymes (glucansucarses) mutation strain from Kimchi, one of Korean tratidional fermented food.

Almost *Leuconostoc* strains need sucrose in media to induce enzyme production. However, these mutated strains can produce enzymes using glucose or sucrose. Using glucose rather than sucrose, glucansucrase can be easily purified. Because the glucansucrases easily bond with dextran, so procedure for removal of dextran should be needed to purify enzymes. Through fermentation and concentration, enzymes were produces in large scale.

After consuming glucose perfectly, enzymes production is suspended and pH is lowering so enzymes activity is reduced or lost. So at the point of small amount of glucose existed, fermentation should be suspended. To find this point, TLC analysis was used to detect glucose in media. If fermentation was suspended, media was kept at 4 and centrifugation was carried out to remove bacterial cells. Supernatant was reacted with sucrose. Colonies were selected which have high activity of enzymes under the same condition of sucrose and reaction time and finally they were used 14 L fermentation. Verification for alternansucrase of $GSase_{1355}$ activity had one more step that is heating at 45°C. Because the $GSase_{1355}$ have dextransucrase and alternansucrase

simultaneously. So by heat treatment, dextransucrase was inactivated and only alternansucrase activity was checked. Finally colonies of the B-1355CF10 strain that have high alternansucrase activity were chosen. Alternan and fructose production were verified at the same time and especially colonies that produce a lot of alternan were stressed on (**Figure 4**). The growth pattern of B-1355CF10 strain on 14 L fermentation showed in **Figure 5**.

From centrifuged broth, concentration procedure was carried out. Taken supernatant of broth was flowed in hollow fiber. Targeted protein has 180 kD, so 100 kD cut off hollow fiber was used and part of low molecular weight was removed. And then concentrated supernatant was lyophilized. Change of $GSase_{1355}$ activity through concentration procedure is described in **Table 1**. $GSase_{1355}$ powder from concentration procedure was quantized and calculated enzyme unit (U) under fixed sucrose concentration with buffer solution. This unit continuously was utilized all this experiment.

To make the indigestible isomaltooligosaccharides (IDMOS), first maximization was carried out. Using too much sucrose concentration has some problems such as high viscosity interrupting reaction and handling. But a major problem is economic efficiency. So synthesis of oligosaccharides went along under 2 M sucrose concentration. The $DSase_{512}$, the $GSase_{1355}$, maltose concentration and reaction time were factors synthesizing the IDMOS. Maximal conditions were set when treating two digestion enzymes, α-amylase and α-amyloglucosidase, the most amount of oligosaccharides were existed. First, results of maximization for the $GSase_{1355}$ was shown in **Table 1** and **Figure 2**. In statistical analysis results, superscript ^{a} means statistically not different groups. In the $GSase_{1355}$ concentration ranges shown in **Table 2**, the maximal concentration of the $GSase_{1355}$ was 0.75 U/mL. However, as the $GSase_{1355}$ was increased, remaining the IDMOS was also showed growing trend. So the maximal concentration of $GSase_{1355}$ could not be decided. Because of this problem, conversion yield (%) was applied and 1 U/mL of the $GSase_{1355}$ concentration could be decided as maximal condition. This

maximization procedure was judged important so HPLC analysis was additionally done and compared the TLC results. Putting together the analysis results they showed the same pattern.

Secondly the maximal concentration of the $GSase_{512}$ was 1 U/mL according to statistical analysis. And in case of maltose concentration, the highest amount of the IDMOS was remained at 250 mM concentration and the IDMOS was lowered upper or lower concentration of 250 mM. With 2 M sucrose concentration as substrate the maltose concentration as acceptor was needed 250 mM. Ratio of sucrose over maltose was 8.

Last factor of IDMOS synthesis was reaction time. Sucrose concentration was set fiducially. Results showed after 4 h reaction sucrose concentration was constantly held. Integrating the maximization results, for the IDMOS synthesis 1 U/mL of the $GSase_{1355}$, 1 U/mL of the $GSase_{512}$, 250 mM maltose and 4 h reaction time should be needed.

And with this condition the IDMOS was compared with industrial oligosaccharides (Ottugi Co., Korea). After treating digestion enzymes, synthesized the IDMOS showed indigestibility by TLC analysis (**Figure 15**).

To raise the IDMOS content, selective removal of monosaccharides went along. Yeast beads were introduced for food safety and application for industrialization. Yeast, *Saccharomyces cerevisiae*, one of GRAS (generally recognized as safe) material, can selectively ferment monosaccharides and the yeast beads can be withdrawn so they can use continuously. After removal of monosaccharides, components were verified by TLC analysis (**Figure 10**).

As prebiotics oligosaccharides should have functions such as indigestibility, carbon source of probiotics, low calorie, low GI (glycemix index) and GL (glycemic load) etc. So prebiotic tests were carried out to confirm characteristics of the IDMOS.

First, experiment for too decay was carried out. Under fixed sucrose concentration, amount of insoluble glucan that causes tooth decay was checked using mutansucrase from *Streptococcus mutans* at specific the

IDMOS concentration. Produced insoluble glucan was solubilized by treating NaOH and quantified them using phenol-sulfuric acid method. Results showed at fixed sucrose concentration (1, 3, 5 %, w/v), as the IDMOS concentration increased, produced insoluble glucan was decreased.

Originally mutansucrase produces insoluble glucan from sucrose by introducing α-(1,3) glycosidic bonds. However, in presence of the IDMOS, α-(1,3) bonds is not introduced perfectly at polymer of α-(1,3) glycosidic bonds. Finally the IDMOS impede synthesis of insoluble glucan. So insoluble polymer was reduced. Like this, the IDMOS contribute to prevent tooth decay.

Secondly, experiment that whether the IDMOS can be used for growth of *Bifidobacteria* was carried out. *B. bifidum*, *B. infantis*, *B. longum* and *B. adolescentis*, 4 strains were tested. In case of media, three types, not carbohydrate added media (cfMRS), glucose as carbon source (MRS-glucose), and the IDMOS as carbon source (MRS-IDMOS), were used. Growth pattern of *Bifidobacteria* were observed in three different media.

pH was measured as a indicator of utilization of carbon source and this shows in **Table 7**. As growing carbon source metabolize organic acids like acetic acid, propionic acid etc. and they lower pH value. Comparing pH of MRS-IDMOS media with cfMRS media, all 4 strains could use the IDMOS. And especially pH of *B. adolescentis* media was the lowest. This means *B. adolescentis* is the best one that consumes the IDMOS. By TLC analysis, consumption of the IDMOS cultivating *Bifidobacteria* was verified. While *B. bifidum* used monosaccharides part of the IDMOS, *B. adolescentis* consumed monosaccharides and around DP 7 part of the IDMOS. This result was regarded the enzymatic characteristics of *Bifidobacteria*.

B. adolescentis is known having their enzyme system which includes genes named aglB and aglA [31]. AglB (EC 3.2.1.20) had ability to hydrolyse maltose (α-1,4-linkage) and isomaltose (α-1,6-linkage), but not isomaltotriose. AglA (EC 3.2.1.10) showed no activity to maltose but had high activity toward isomaltose and isomaltotriose. AglA, the main factor to utilize for

isomaltooligosaccharides could play a role by *B. adolescentis*.

Thirdly, experiment was done to verify inhibition of growth of food pathogens as growing *Bifidobacteria*. *Salmonella typhimurium* was selected as a pathogen. Non-typhoidal human salmonellosis is characterized by the acute onset of fever, abdominal pain, nausea, diarrhea, and sometimes vomiting [42]. In this part of experiment, I tried to check the growth pattern of *Salmonella* with *Bifidobacteria* to see inhibitory effect of pathogen growth. *B. bifidum* and *S. typhimurium* was cultivated in MRS-IDMOS broth. Another *Bifidobacteria* strain, *B. adolescentis*, also cultivated with *S. typhimurium*. Comparing with single culture, differences of mixed culture were observed. Two *Bifidobacteria* strains showed a pattern of inhibition from *Salmonella* growth. And in mixed culture between *B. adolescentis* and *S. typhimurium*, death time of *Salmonella* was put forward. These results were regarded lowered pH because of organic acids generating carbohydrate metabolizing process by *Bifidobacteria* [32].

Salmonella was known it is sensitive to pH. To get over this, some *Salmonella* strains have pH regulating systems. If *Salmonella* lay in low pH condition, they try to keep intracellular pH relatively constant at pH 7.6-7.8, even as extra pH dramatically changes. The intracellular pH is maintained by pumps that exclude protons from the cytoplasm in low-pH environments [33]. In addition, it has become clear that inducible lysine decarboxylase and arginine decarboxylase systems play an important role in the maintenance of intracellular pH in *Salmonella* [34, 35, 36, 37, 38]. However, if some *Salmonella* strains don't have these systems or don't endure proton concentration because of too low pH, they eventually die out. So pH condition is important to *Salmonella* growth.

Bifidobacterium adolescentis has antiviral anticancer activity [39] and is used in probiotics and "therapeutic milk", often mixed with *Streptococcus thermophilus*. It synthesizes various B vitamins including thiamin (B1), pyridoxine (B6), folic acid (B9), nicotine, cyanocobalamin (B12), ascorbic

acid (Vitamin C), biotin, and riboflavin.

Dietary consumption of selected *Bifidobacteria* strains, especially *B. adolescentis***,** in a yogurt-based diet may offer benefit to elderly individuals to prevent some of the harmful effects of immunosenescence [40].

B. adolescentis could decrease immunity to opportunistic pathogens such as *Bacteroides thetaiotaomicron***,** the second most common infectious anaerobic gram-negative bacteria, frequently associated with peritonitis, septicemia, and wound infections.

The population of these bacteria in the colon appears to be relatively stable until late adulthood when it appears to decline. Numbers are higher in elderly and younger adults [41]. Therefore, by intaking the IDMOS, *B. adolescentis* will be able to grow selectively and can help health intestine for adults and maintain human body functions.

So far, the IDMOS as a material, maximization for synthesis, functions as prebiotics such as prevention of tooth decay, carbon source of *Bifidobacteria* and inhibition of pathogen's growth were done. If the IDMOS has functions like low calorie, GI and GL *in vitro*, the IDMOS can surely be prebiotics. And structural analysis of the IDMOS should also be needed.

Conclusion

In this study, I synthesized the novel branched and indigestible isomaltooligosaccharides. To do so, glucansucrases were needed and I obtained them from *L. mesenteroides* fermentation. From small volume to large volume *L. mesenteroides* strains were cultivated. Finally large-volume of crude enzymes were obtained using fermentation. And then concentration step was proceeded to reduce enzyme volume. After lyophilization, about 4,000 U/g of the $GSase_{1355}$ was obtained.

With 4 different factors, I maximized for synthesis of the IDMOS. The maximal condition was 1 U/mL of $GSase_{1355}$, 1 U/mL of $DSase_{512}$, 250 mM of maltose concentration and 4 h reaction time.

Using this maximal condition, I synthesized the large-scale of IDMOS to 500 mL. 500 mL of reaction sample was treated with yeast bead to remove monosaccharide. So I got more purified the IDMOS.

I check inhibition of tooth decay by the IDMOS. By treating the IDMOS, formation of insoluble glucan was reduced.

I also checked the prebiotic activity of the IDMOS. The result was 4 *Bifidobacteria* strains could use the IDMOS as carbon source. Furthermore, under mixed cultivation condition, *Salmonella* as a food pathogen was died out because of growth of *Bifidobacteria*.

Integrating all of the results, IDMOS has indigestibility, inhibitory effect of tooth decay and prebiotic functions.

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국문 초록

새롭게 합성된 난소화성 이소말토올리고당 (IDMOS)은 한국의 전통 식품 발효균인 류코노스톡 메센테로이즈에서 개량한 후 발현된 류코노스톡 메센테로이즈 B-512FMCM (덱스트란수크라아제$_{512}$)와 류코노스톡 메센테로이즈 NRRL B-1355CF10 (글루칸수크라아제$_{1355}$) 두 효소의 조합에 의해 최초로 합성되었다.

포도당 (공여체)이 맥아당 (수용체)에 알파-형태로 공유결합하는 수용체 반응을 이용하였다.

그리고 난소화성 이소말토올리고당 합성의 최적 조건을 찾기 위해 최적화 과정을 진행하였다. 합성된 난소화성 이소말토올리고당은 얇은 막 크로마토그래피와 고성능 액체 크로마토그래피를 통해 분석되었다. 그 결과 약 85%의 올리고당이 장 소화 효소 (알파-아밀라아제와 아밀로글루코시다아제) 처리 후에 남아있음을 확인하였다.

효모가 포집된 구슬을 사용하여 난소화성 이소말토올리고당에서 단당 부분만을 선택적으로 제거하였다.

난소화성 이소말토올리고당의 기능 중 하나로 불용성 다당인 뮤탄의 합성을 저해하는 기능을 보았다. 5 %의 난소화성 이소말토올리고당을 3 %의 설탕 농도 조건에서 처리하였을 때, 뮤탄의 생성량이 절반으로 감소하였다.

난소화성 이소말토올리고당은 비피도박테리아의 성장에 이용되는 탄소원으로 사용되었다. 비피도박테리아의 네 균주 모두 난소화성

이소말토올리고당을 부분적으로 이용함을 확인하였다. 특히 비피도박테리움 아돌레센티스 균주는 얇은 막 크로마토그래피로 확인한 결과 난소화성 이소말토올리고당을 가장 많이 이용하는 것으로 나타났다. 그리고 비피도박테리아의 성장에 따라 식품 병원균 중 하나인 살모넬라의 성장은 저해됨을 알 수 있었다.

안전한 균주로부터 최초로 합성된 난소화성 이소말토올리고당은 비피도박테리아의 활성 효과를 보여 산업적으로 이용 가치가 충분한 물질이라 할 수 있다.

주요어 : 글루칸수크라아제, 덱스트란, 얼터난, 난소화성,
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