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Simultaneous Biocatalytic Synthesis of Panose During Lactate Fermentation in Kimchi

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Abstract As a functional additive for intestinal microflora, panose (6^2-α-D-glucopyranosylmaltose) was synthesized during kimchi fermentation using the glucose transferring reaction of glucansucrase from *Leuconostoc mesenteroides*. For the glucose transferring reaction, sucrose and maltose were added (2% each, w/v) to dongchimi-kimchi as the glucosyl donor and acceptor molecule, respectively. After five days of incubation at 10°C, referring to the initial phase for the production of lactic acid in kimchi, over 60% (w/v) of the total sugars were converted into panose and other branched oligosaccharides. Thereafter, the kimchi was stored at 4°C and the amount of panose remained at a constant level for three weeks, thereby indicating the stability of panose to microbial degradation during the period of kimchi consumption. The use of maltose as the acceptor molecule in the kimchi also facilitated a lower viscosity in the kimchi-juice by preventing the synthesis of a dextran-like polymer which caused an unfavorable taste. Accordingly, the application of this new method of simultaneous biocatalytic synthesis of oligosaccharides during lactate fermentation should facilitate the extensive development of new function-added lactate foods.

Key words: Kimchi, fermentation, *Leuconostoc mesenteroides*, glucansucrase, dextran, panose, maltosyl-isomaltooligosaccharides, acceptor reaction

Panose, the major constituent of isomaltooligosaccharides, is used as a functional food additive due to its low energy (≤2 kcal/g) and low cariogenic properties, and thus is known to improve the intestinal microflora [14]. In addition, its physical properties, including a high hygroscopicity, low viscosity, and high freezing point, enable its wide application in food products [19].

Kimchi is a general term given to a group of fermented vegetables in Korea, which are traditionally served as side dishes at meals. Many types of kimchi are available, however, they are mainly made of Chinese cabbage or radish and differ according to the raw ingredients, processing methods, seasoning, and locality [10]. Kimchi is characterized by its sour, sweet, and carbonated taste, which is distinct from sauerkraut [1, 11].

Leuconostoc sp., a hetero-fermentative type bacterial species, is the major bacterial population in kimchi from the initial to the middle stages of fermentation [6]. During these stages, this bacterium produces various constituents, such as lactic acid, acetic acid, alcohol, CO_2, mannitol, and dextran, which are all associated with the taste of kimchi, and the number of this bacterium is highest during the optimum ripening period [2]. The number of *Leuconostoc* sp. then starts to decrease when the pH of the kimchi is lowered to 4.0. At this point, a homo-fermentative type lactic acid bacterium, *Lactobacillus* sp., and yeast, both of which have a strong pH tolerance under a high organic acid concentration, continuously increase in number during the kimchi fermentation in the last stage [12]. When *Leuconostoc* sp. strains, isolated from the liquid of ripened kimchi, were previously cultured in a sucrose medium, a glucansucrase activity was detected in the medium and a strain showing a high glucansucrase activity was selected [4]. Dextran is an α-(1→6)-linked D-glucan with α-linked branches [3] that is synthesized from the glucansucrase from *Leuc. mesenteroides* using sucrose as the reactant. Dextran was

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already identified in several kimchis containing sucrose as a sweetener [5]. Glucansucrase (EC 2.4.1.5) elaborated by various species of *Leuconostoc* and *Streptococcus* [15] transfers the glucosyl moiety of sucrose to form dextran. In addition to catalyzing the synthesis of dextran from sucrose, glucansucrase also catalyzes the transfer of glucose from sucrose (donor) to other carbohydrates (acceptors) by the cross linking α-(1→6)-glucosyl bond. When the acceptor is a monosaccharide or disaccharide, a series of oligosaccharide acceptor-products is usually produced [16, 8, 9], and maltose has been recognized as the best acceptor molecule based on an experiment using *Leuc. mesenteroides* NRRL B-512F [16].

The current study utilized the glucose transferring reaction of glucansucrase to synthesize panose as a functional carbohydrate in kimchi. In addition, this report presents a new procedure which can be explored as a possibility of adding a new functionality to lactic-acid fermented foods.

MATERIALS AND METHODS

Materials

The dextran, dextranase, sucrose, and standard chemicals were all purchased from Sigma Inc. (St. Louis, MO, U.S.A.). The maltose was from Duksan Pharmaceuticals (Yongin, Korea). The lactobacilli MRS broth was from DIFCO (Detroit, MI, U.S.A.). Cubic plastic jars with sealing lids were used as the fermentation vessel. The radish, red pepper, and green onion were purchased from a local grocery store.

Kimchi (Dongchimi-Kimchi) Preparation and Fermentation

Dongchimi-kimchi is a popular watery radish kimchi. A whole radish (80 g) was cut into four pieces, then, after washing and peeling the outer layer, the pieces were mixed with salt (40 g) in a plastic jar and incubated at 20°C for 12 h until they became soft. The salted radish and extract solution were mixed with crushed garlic (10 g), ginger (3 g), and chopped green onions (20 g). For a sweet taste, 160 g of sugar (sucrose only or a sucrose/maltose mixture) was added. The jar was then filled with 4 l of drinking water and tightly sealed with a plastic lid. The fermentation temperature was kept at 10°C for the first seven days, during which the growth of *Leuconostoc* sp. and glucansucrase activity reached maximum levels. Then, the temperature was dropped to 4°C to reduce the bacterial growth and sugar consumption. Kimchi samples (10 ml liquid) were harvested periodically to analyze the microbiological and physicochemical changes during fermentation.

Analysis of Sugars

The sugars in the kimchi were analyzed using a high pressure ion-exchange chromatography (HPIC, Dionex Corp., Sunnyvale, CA, U.S.A.) after filtering the kimchi liquid. A CarboPac PA-1 column was used to separate the oligosaccharides, including monosaccharides. After the column was equilibrated with 150 mM of NaOH solution, the samples (50 ml) were loaded with a 1 ml/min flow rate and eluted with 600 mM of Na-acetate solution using a gradient mode with 150 mM of NaOH. A GP40 gradient pump was used for this process. To detect sugars, the pulsed amperometric detection method was used with a ED40 electrochemical detector. For a quantitative and qualitative analysis of the peaks, the software Chromate Window v.3.0 (Interface Engineering Inc. U.S.A.) was used.

Assay of Glucansucrase Activity

The glucansucrase activities in the kimchi samples were measured by assaying the changes in the fructose concentration [13] after modification [17] in 20 mM of an Na-acetate buffer solution (pH 5.2) containing 100 mM of sucrose, 1 mM of $CaCl_2$, and 0.02% of NaN_3. Kimchi samples were centrifuged (1,000 ×g) for 5 min and the liquid fractions recovered for the glucansucrase assay. The reaction was initiated by the addition of 0.1 ml of kimchi liquid in 0.9 ml of the buffer solution at 25°C. Aliquots (100 μl) of the reaction solution were taken every 30 min and mixed with 50 μl of 10% (v/v) pyridine to terminate the reaction. The solution (1 μl) was then put on a Whatman K5 TLC plate and the fructose concentration measured using a thin-layer chromatography (TLC) with four ascents of 85:15 (v/v) acetonitrile:H_2O. The sugars were visualized by dipping the plates into 5% (v/v) H_2SO_4 in ethanol containing 0.5% (w/v) α-naphthol, followed by drying and then heating for 10 min at 110°C. The quantity of each sugar on the TLC plates was measured with a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA, U.S.A.) [17]. One unit of glucansucrase was defined as the amount of enzyme used to produce 1 μmole of fructose per min at 25°C.

Microbial and Physicochemical Analyses of Kimchi

The growth of *Leuconostoc* sp. was measured in colony-forming units (CFU) per ml of kimchi liquid using the culture pouring method on a Lactobacilli MRS agar medium containing 0.002% bromophenol blue [6]. After being diluted with sterile water, the kimchi liquid was poured on the agar plate and incubated at 30°C for 72 h. The dark and blue colonies grown on the plates were counted as *Leuconostoc* sp. The viscosity of the kimchi liquid was measured using an Ostwald micro-viscometer (Schott Gerate, Hofheim, Germany). The specific viscosity of the kimchi liquid was obtained from the densities of the liquid and water in addition to the flow time through the capillary of the viscometer at 50°C. The pH and temperature were measured for each fermentation stage.

RESULTS

Fermentation Profile in Kimchi

During the kimchi fermentation, a low temperature was required to reduce the rate of lactate synthesis by *Leuconostoc* sp. and *Lactobacillus* sp. because an excess accumulation of lactic acid in kimchi caused a fast drop in the pH level, thereby inhibiting the growth of *Leuc. mesenteroides* and producing a strong sour taste. A preliminary experiment with kimchi incubated at room temperature showed a rapid drop in pH below pH 4, just one day after incubation, and a reduction in the cell numbers of *Leuc. mesenteroides*. In contrast, to maximize the glucansucrase activity in the kimchi, the fermentation temperature needed to maintain close to 30°C, which is the optimum temperature for the glucansucrase from *Leuc. mesenteroides*. Accordingly, considering the above constraints, 10°C was chosen as the optimum initial temperature for the growth of *Leuconostoc* sp. and reaction of glucansucrase. Figure 1 shows the fermentation profile in the kimchi, including the pH change, temperature control, cell growth of *Leuconostoc* sp., and glucansucrase activity. After 3 days at 10°C, the glucansucrase activity reached its highest level and the cell number of *Leuconostoc* sp. began to decline; therefore, after 7 days, the fermentation temperature was adjusted to 4°C to reduce any further microbial growth of lactobacilli and yeast and the consumption of sugars.

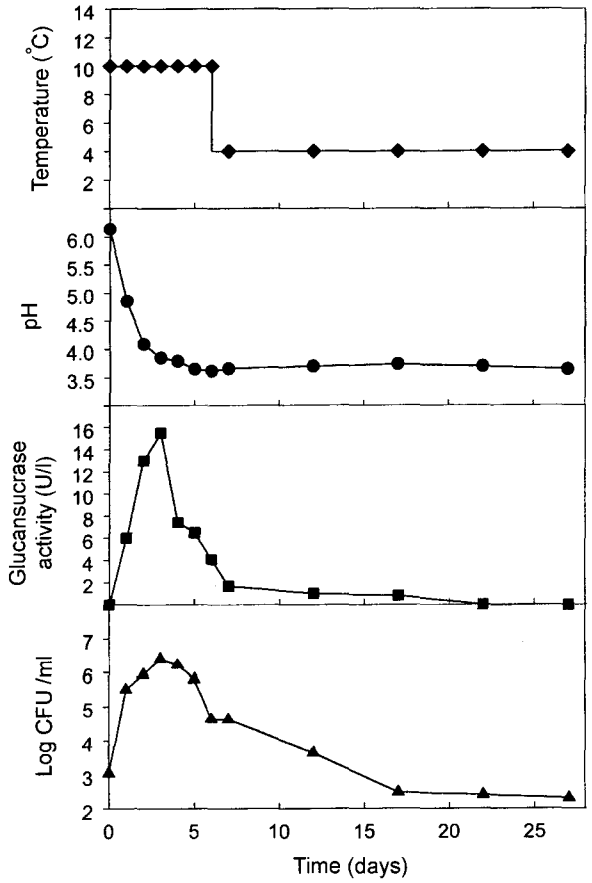

Fig. 1. Profiles of cell growth of *Leuconostoc* sp. lactic acid synthesis, pH change, temperature control, and glucansucrase activity during kimchi fermentation.

Kimchi containing 4% sucrose was incubated at 10°C for 7 days and at 4°C thereafter. During the fermentation period, the cell growth of *Leuconostoc* sp., production of lactate, pH change, and glucansucrase activity in the kimchi liquid were all measured. The detailed methods are described in Materials and Methods.

Polymer Synthesis Reaction in Kimchi Containing 4% Sucrose

As expected from the reaction mode of glucansucrase, when sucrose was the only ingredient added to the kimchi, a glucosyl transfer reaction occurred, which produced polymers and left fructose as the monomer in the kimchi liquid. Figure 2 shows the degradation of sucrose and production of polymers over the entire fermentation period. After 28 days, most of the added sucrose (4%) disappeared

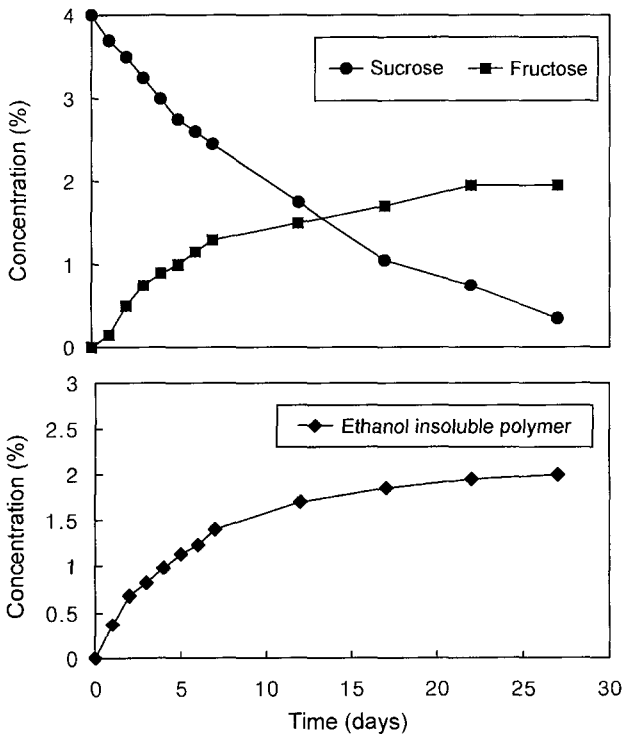

Fig. 2. Profiles of sugar changes in kimchi containing 4% sucrose.

During the fermentation of kimchi containing 4% sucrose, the glucosyl transferation reaction was monitored by assaying the sucrose (●), fructose (■), and ethanol-insoluble polymers (◆). The sucrose and fructose were measured using HPIC, as described in Materials and Methods. To analyze the polymer synthesis, kimchi samples were centrifuged for 5 min at 5,000 ×g. Supernatants (0.7 ml) were then transferred to eppendorf tubes. The same volume of ethanol was added, then the polymers in the kimchi liquid were precipitated by centrifugation for 10 min at 10,000 ×g. After decanting the supernatants, the ethanol-insoluble precipitates were dried under a vacuum and weighed on a balance.

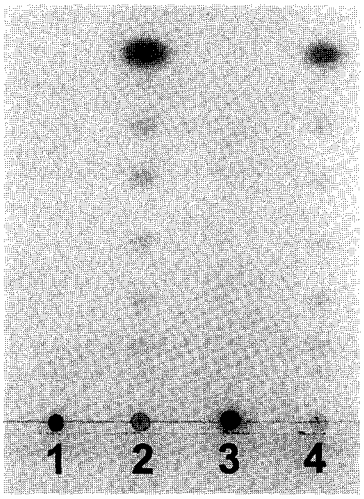

Fig. 3. TLC analysis of ethanol-insoluble polymer and its reaction products using dextranase.

The ethanol-insoluble polymers (0.5%, w/v) were dissolved in a citrate phosphate buffer (0.05 M, pH 5.5, containing 0.02% sodium azide) and incubated with *Penicillium* dextranase (0.15 U/ml) for 48 h at 28°C. The carbohydrate composition of the digest was analyzed by thin-layer chromatography (TLC), using Whatman K5 plates with two ascents of 1:2:3:4:5 (v/v) nitroethane/ nitromethane/ ethanol/ water/ 1-propanol. The carbohydrates were visualized by dipping the plates into 5% (v/v) H_2SO_4 in ethanol containing 0.5% (w/v) α-naphthol, followed by drying, and then heating for 10 min at 110°C. Lane 1 - Ethanol-insoluble polymer obtained from kimchi. Lane 2 - Reaction products of dextranase and ethanol-insoluble polymer. Lane 3 - Commercial dextran from *Leuc. mesenteroides* (Sigma). Lane 4 - Reaction products of dextranase and commercial dextran.

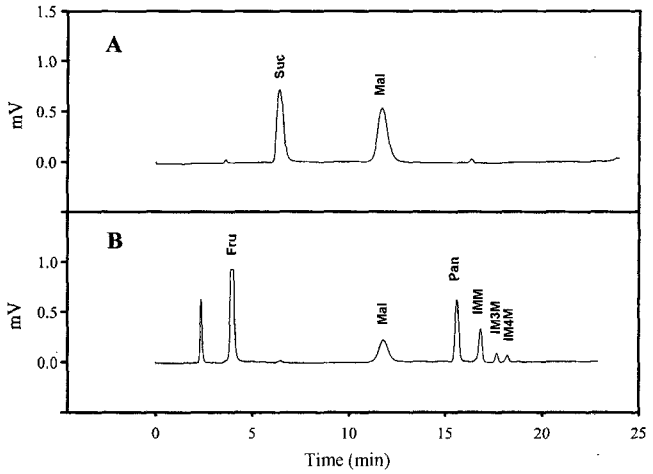

Fig. 4. Analysis of sugar concentration in kimchi using High Pressure Ion-Exchange Chromatography (HPIC).

A carboPac PA-1 column was used to separate the oligosaccharides. After the column was equilibrated with 150 mM of an NaOH solution, the kimchi samples (50 ml) were loaded and then eluted with 600 mM of a Na-acetate solution using a gradient mode with 150 mM of NaOH. The pulsed amperometric detection method and Chromate Window v.3.0 were used to detect and analyze the sugars. Graph A shows the peaks in kimchi samples containing 2% sucrose and 2% maltose on day 0. Graph B shows peaks of graph A kimchi after 4 days fermentation at 10°C. Based on the acceptor reaction with sucrose and maltose, several oligosaccharides synthesized in the kimchi are shown (Suc: sucrose; Mal: maltose; Fru: fructose; Pan: panose; IMM: isomaltosyl maltose; IM3M: isomaltotriosyl maltose; IM4M: isomaltotetraosyl maltose).

from the kimchi liquid; meanwhile, about 2% fructose and 2% of a polymer were synthesized. From the mass balance of sucrose and fructose, it would appear that the microorganisms in the kimchi did not utilize much sucrose or fructose for their growth.

To identify the polymer synthesized in the kimchi with sucrose, the polymer was recovered by ethanol precipitation (50% v/v) and digested by *Penicillium* dextranase (Fig. 3). TLC analysis of the reaction products of the polymer with dextranase was identical with a commercial dextran made from *Leuc. mesenteroides* NRRL B-512F. This result suggested that the major glucan polymer synthesized in dongchimi-kimchi showed very similar linkage structure with the dextran from *Leuc. mesenteroides* NRRL B-512F, which consists of 95% α-(1→6) glycosyl backbone linkages and 5% α-(1→3) glucosyl branches [15].

Production of Panose in Kimchi Containing 2% Sucrose and 2% Maltose

The glucosyl transfer reaction to an acceptor molecule of glucansucrase was used for the synthesis of panose in the kimchi. Accordingly, maltose was added as the acceptor molecule with the ratio of sucrose to maltose as 1:1. The previous work using glucansucrase from *Leuc. mesenteroides* NRRL B-512F suggested the equimolar addition of sucrose and maltose gave the best condition for higher production of panose, the major product of the acceptor molecule [16]. To maintain the same total sugar concentration as in the above experiment, 2% sucrose and 2% maltose were added to the kimchi during preparation. The changes in the sugar concentration in the kimchi during fermentation were analyzed by HPIC (Fig. 4), and the results are summarized in Fig. 5. As expected, an acceptor reaction by glucansucrase which transferred the glucose residue from sucrose to maltose occurred, thereby producing panose, isomaltosyl maltose, isomaltotriosyl maltose, and isomaltotetraosyl maltose. The sucrose (2%) was rapidly consumed within 4 days, while during the same period about half of the maltose (1%) was used as acceptor molecules. The concentrations of oligosaccharides were highest after 4–7 days, and these levels were maintained for the next 3 weeks at 4°C without any significant deterioration. However, when the kimchi was kept at 10°C without lowering the temperature, approximately 20% of the oligosaccharides were digested within 3 weeks, thus suggesting that the refrigeration of kimchi at 4°C after the production of isomaltooligosaccharides is recommended to preserve the sugars in the kimchi. Dextran-like ethanol-insoluble polymers were also produced. However, this amount was dramatically lower at 0.1% when compared with the fermentation containing only 4% sucrose.

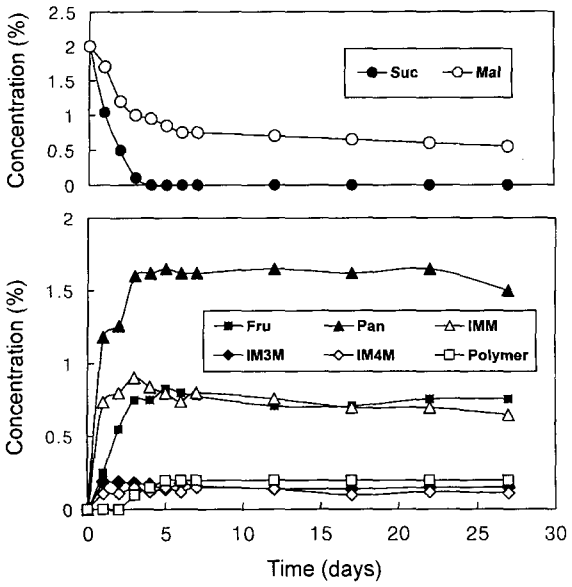

Fig. 5. Profiles of sugar changes in kimchi containing 2% sucrose and 2% maltose.

During the fermentation of kimchi containing 2% sucrose and 2% maltose, the acceptor reaction of glucansucrase in the kimchi liquid was monitored by assaying the sugar concentrations. HPIC was used to analyze the sugars, including the oligosaccharides. The polymers were weighed after 50% ethanol precipitation. Sucrose (●) and maltose (○) are presented in the upper section. Fructose (■), panose (▲), isomaltosyl maltose (IMM △), isomaltotriosyl maltose (IM3M ◆), isomaltotetraosyl maltose (IM4M ◇), and dextran-like ethanol-insoluble polymer (□) are presented in the lower section.

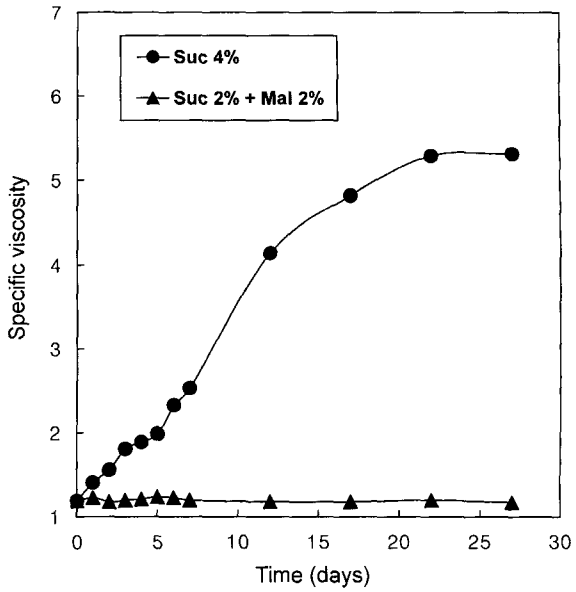

Fig. 6. Comparison of specific viscosity of kimchi during fermentation.

The viscosities of kimchi containing 2% sucrose (●) and kimchi containing 2% sucrose and 2% maltose (▲) were measured using an Oswald micro-viscometer and the specific viscosity was calculated by comparison with water.

Analysis of Glucansucrase Reaction in Kimchi

The concentrations of the products synthesized from the two experiments above are summarized in Table 1. During the kimchi fermentation process, using only sucrose as the sweetener, a dextran-like polymer was synthesized by the polymerization reaction of glucansucrase. Almost 50% of the sucrose was converted into polymers and the other 50% into fructose after the reaction was completed. In contrast, in the kimchi fermentation containing 2% sucrose and 2% maltose, a glucose transfer reaction occurred, which produced a spectrum of isomaltosyl-maltooligosaccharides instead of a polymer synthesis. The sucrose (2%) and maltose (2%) were converted into panose (1.6%), isomaltosyl maltose (0.7%), isomaltotriosyl maltose (0.2%), isomaltotetraosyl maltose (0.1%), a dextran-like polymer (0.1%), and fructose (0.7%). The relative concentrations of the products in the kimchi were 40, 18, 3, 2, 2, and 18% (w/v), respectively, and the branched oligosaccharides exceeded 60% of the total sugar.

The polymer synthesis in the kimchi containing 4% sucrose was attributed to a change in the physical properties, thereby the specific viscosity was increased to 5.3 (Fig. 6). However, in the kimchi containing 2% sucrose and 2% maltose, the polymer synthesis reaction was substantially inhibited by a competitive acceptor reaction, resulting in the synthesis of 0.1% of a polymer and essentially no change in viscosity.

Table 1. Comparison of sugar concentrations in kimchi after completion of glucansucrase reaction.

Types of kimchi	Sugar concentration (%) after completion of glucansucrase reaction (Relative concentration, %)							
	Suc	Mal	Fru	Pan	IMM	IM3M	IM4M	Dex
Kimchi containing 4% of sucrose	0.3 (8)	0	1.8 (45)	0	0	0	0	1.9 (48)
Kimchi containing 2% of suc and 2% of mal	0	0.6 (15)	0.7 (18)	1.6 (40)	0.7 (18)	0.2 (3)	0.1 (2)	0.1 (2)

The sugar concentrations in the kimchi were assayed after 28 days of fermentation. The abbreviations used are as follows: Suc, sucrose; Mal, maltose; Fru, fructose; Pan, panose; IMM, isomaltosyl maltose; IM3M, isomaltotriosyl maltose; IM4M, isomaltotetraosyl maltose; Dex, dextran.

DISCUSSION

Leuc. mesenteroides is one of the major bacteria in kimchi, which provides the appropriate conditions for lactate fermentation that gives the unique taste to kimchi. This bacterium grows fast during the initial stage of kimchi fermentation and scavenges oxygen from the liquid to create anaerobic conditions for lactic acid fermentation. The bacterium also produces lactic acid and CO_2 through the hetero-lactic acid fermentation pathway, giving a sour and carbonated taste to kimchi. Furthermore, the strain elaborates an extracellular glucansucrase when sucrose exists in the medium so that the enzyme synthesizes a dextran polymer. Therefore, this enzyme was used to develop a method for synthesizing panose in a high concentration based on the addition of sucrose and maltose. Once accumulated in kimchi, panose can be maintained at a constant level during an extended period of storage at a low temperature and shows resistance to microbial degradation. Yun *et al.* [20] have confirmed that isomaltooligosaccharides exhibit the highest stability among fructo-, soybean-, and isomalto-oligosaccharides in kimchi. This fact suggests that isomaltooligosaccharides including panose can be recommended as an effective ingredient in kimchi due to their beneficial functionalities.

When sucrose was added during the dongchimi-kimchi preparation to give a sweet taste, the synthesis of a dextran polymer was frequently observed, which is generally considered to be an unfavorable effect, since this polymer produces a viscous and sticky feeling in the mouth. In order to prevent this occurrence, alternative sweeteners such as saccharin and fructose are often used, which in turn raise issues of safety and cost. Accordingly, the method developed in the current study solves the problem of the polymer, while also meeting the requirement for a sweetener; maltose inhibits the synthesis of a dextran polymer, since it acts as a strong acceptor molecule that synthesizes panose competitively, while sucrose releases an equivalent amount of fructose as a free molecule after a glucose transferring reaction, thereby providing the sweet taste to the kimchi. As an economically feasible source of maltose, a preliminary experiment showed that the maltose-syrup, which can be easily obtained from a market containing about 50% maltose, produced a suitable yield of panose.

Robyt and Eklund [16] carried out a series of reactions with *Leuc. mesenteroides* B-512F glucansucrase using different acceptors at a 1:1 ratio of acceptor-to-sucrose, then they measured the amount of dextran formed in the reaction. Among sixteen other acceptors that were compared on a relative scale, with maltose as 100%, the next best acceptor was isomaltose (89%), followed by nigerose (58%), methyl-α-D-glucopyranoside (52%), D-glucose (17%), turanose (13%), lactose (11%), cellobiose (9%), and D-fructose (6.4%). Thus, these results indicate a way of producing various oligosaccharides in kimchi based on the glucose transferring reaction of glucansucrase with the above acceptor compounds.

In conclusion, the proposed method, which uses the glucose transferring reaction of *Leuc. mesenteroides* glucansucrase, enables the production of beneficial oligosaccharides while both maintaining an appropriate sweetness level and preventing an unfavorable polymer synthesis in the kimchi. The application of this method, referred to as the simultaneous biocatalytic synthesis of panose during lactate fermentation, should permit the development of new function-added lactate foods.

Acknowledgments

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