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Isolation and Structural Characterization of an Oligosaccharide Produced by *Bacillus subtilis* in a Maltose-Containing Medium

Kwang-Soon Shin

Department of Food Science and Biotechnology, Kyonggi University, Gyeonggi 16227, Korea

ABSTRACT: Among 116 bacterial strains isolated from Korean fermented foods, one strain (SS-76) was selected for producing new oligosaccharides in a basal medium containing maltose as the sole source of carbon. Upon morphological characterization using scanning electron microscopy, the cells of strain SS-76 appeared rod-shaped; subsequent 16S rRNA gene sequence analysis revealed that strain SS-76 was phylogenetically close to *Bacillus subtilis*. The main oligosaccharide fraction B extracted from the culture supernatant of *B. subtilis* SS-76 was purified by high performance liquid chromatography. Subsequent structural analysis revealed that this oligosaccharide consisted only of glucose, and methylation analysis indicated similar proportions of glucopyranosides in the 6-linkage, 4-linkage, and non-reducing terminal positions. Matrix-assisted laser-induced/ionization time-of-flight/mass spectrometry and electrospray ionization-based liquid chromatography-mass spectrometry/mass spectrometry analyses suggested that this oligosaccharide consisted of a trisaccharide unit with 1,6- and 1,4-glycosidic linkages. The anomeric signals in the ^{1}H-nuclear magnetic resonance spectrum corresponded to α-anomeric configurations, and the trisaccharide was finally identified as panose (α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,4-D-glucose). These results suggest that *B. subtilis* SS-76 converts maltose into panose; strain SS-76 may thus find industrial application in the production of panose.

Keywords: glucooligosaccharide, bacterial fermentation, structural characterization, *Bacillus subtilis*, acceptor reaction

INTRODUCTION

As consumers become increasingly conscious of their health, foods that promote healthy living and reduce the risk of diseases, such as low-calorie foods and functional foods have become popular (1). In this sense, there is a growing commercial interest in developing new oligosaccharides to be used as prebiotics to improve intestinal function or maintain a balanced intestinal microbiota. According to International Union of Biochemistry-International Union of Pure and Applied Chemistry, oligosaccharides are defined as low molecular weight saccharides containing between 3 and 10 residues. Industrial production of oligosaccharides from natural sources typically relies on hydrolysis of polysaccharides, or on enzymatic or chemical synthesis from disaccharide substrates (1). Among oligosaccharide categories, isomaltooligosaccharide (IMO) is a general term for oligosaccharide such as isomaltose, isomaltotriose, isomaltotetraose, and panose, all of which contain the 1,6-glycosidic linkage (2). As in the case of maltooligosaccharide (*i.e.*, oligosaccharides derived from glucose monomers joined by 1,4-linkage, as in maltose), IMOs are produced using starch as the raw material, but they require a combination of immobilized enzymes in a two-stage reactor. In the first stage, starch is liquefied using α-amylase. The second-stage involves reactions catalyzed by both β-amylase, which hydrolyses the liquefied starch to maltose, and α-glucosidase, which possesses transglucosidase activity. The two stages combined convert maltose into IMO (2).

In this context, a suitable method for the immobilization of dextransucrase is necessary in order to make enzymatic oligosaccharide synthesis a commercially viable option (3). Acceptor reaction represents a successful method for dextransucrase immobilization, and was first reported by Schwengers and Zeuner (4), who immobilized dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F by entrapment in a calcium alginate matrix. Acceptor reactions using dextransucrase from *Leuconostoc* sp. produce α-glucooligosaccharides containing one or more branches with D-glucopyranosyl residues linked *via* α-1,2-glycosidic bonds, when maltose is supplied as an acceptor. Chung (5) reported that α-glucooligosaccharides and IMOs can be produced either by hydrolysis of

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Correspondence to Kwang-Soon Shin, Tel: +82-31-249-9655, E-mail: ksshin@kyonggi.ac.kr

starch, or from sucrose by transglycosylation with a dextransucrase enzyme and an acceptor. Meanwhile, Robyt et al. (6) found that maltose was the most efficient acceptor among 17 common mono- and disaccharides (7), and among a series of methyl-α-D-glucopyranoside analogs (8). Acceptor reactions of dextransucrases, especially those of *Leuconostoc* dextransucrases, have been exploited to synthesize glucooligosaccharides for food- and health-related applications (9-12). However, there are few reports on novel bacterial strains for producing oligosaccharides by acceptor reactions. Furthermore, oligosaccharide production by acceptor reaction using maltose as the sole source of carbon has not been yet reported.

Recently, we found that a gram-positive bacterium, *Arthrobacter crystallopoietes* N-08, which extracellularly produces a high level of non-reducing glucooligosaccharide, namely trehalose [α-D-glucopyranosyl-(1,1)-α-D-glucophyranoside], from maltose by a resting cell reaction (13, 14). Based on the previous results, in the present study, we screened diverse bacterial strains from various fermented foods to find a new bacterial strain producing oligosaccharides from maltose containing medium, and identified the structural characteristics of the oligosaccharide produced from the specific bacterium.

MATERIALS AND METHODS

Saccharides and enzymes

Glucose and maltose were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and maltooligosaccharide was purchased from Supelco Inc. (Bellefonte, PA, USA). In order to identify the monosaccharide composition of novel oligosaccharide produced by specific bacteria, the enzymes glucoamylase (EC 3.2.1.3) from *Aspergillus niger* and β-glucosidase (EC 3.2.1.21) from almond were purchased from Sigma-Aldrich Co., while trehalase (EC 3.2.1.28) was purchased from Megazyme (Wicklow, Ireland).

Isolation and cultivation of oligosaccharide-producing bacterial strains

To isolate a bacterial strain that produces novel oligosaccharides, samples from various Korean fermented foods such as soy sauce, soy paste, and highly salted fermented shrimp were collected. The food samples were serially diluted with saline solution and plated on nutrient agar. Morphologically distinguishable colonies were transferred on fresh nutrient agar, and cultured at 30°C for 24 h. A total of 116 strains were discriminated separately, and stored as frozen stock cultures at −70°C in nutrient broth with 20% (v/v) glycerol until use. All bacterial strains were individually cultured in nutrient broth medium for 3 days, and we monitored the potential for production of novel oligosaccharide in their culture supernatant by thin layer chromatographic (TLC) analysis as described by Seo and Shin (13). Among these, 6 bacterial strains (SS-02, SS-21, SS-27, SS-46, SS-65, and SS-76) were selected on the first screening because novel spots that previously did not exist on the TLC plate were expressed in the culture supernatant. To examine the oligosaccharides produced from maltose as the sole source of carbon, 6 isolated strains were individually incubated with reciprocal shaking at 30°C for 3 days in basal medium (0.5% peptone, 0.1% yeast extract, 0.1% K_2HPO_4, 0.06% $NaH_2PO_4 \cdot 2H_2O$, and 0.05% $MgSO_4 \cdot 2H_2O$; pH 6.8) containing 2% maltose. Thereafter, the culture supernatant collected by centrifugation (6,000 rpm for 15 min), was analyzed by TLC.

Purification of a novel oligosaccharide produced by strain SS-76

The bacterial strain SS-76, isolated from traditionally fermented soybean paste, was the only strain that produced a new oligosaccharide. It was cultured in the same medium, and the culture supernatant was harvested and moderately concentrated using a vacuum rotary evaporator (Tokyo Rikakikai Co., Tokyo, Japan). The concentrated culture supernatant was then fractionated using a preparative-high performance liquid chromatography (prep-HPLC) device equipped with a Prevail Carbohydrate ES column (250 mm×4.6 mm, 5 μm; Grace Davison Discovery Sciences, Bannockburn, IL, USA) that used 70% acetonitrile solution as eluent at a flow rate of 0.5 mL/min. The eluates were sequentially collected in test tubes using a fraction collector (1200 series, ELELA, Tokyo, Japan); a total of 80 tubes were obtained. The sample in each tube was monitored by TLC to verify the purity of the oligosaccharide. In addition, to estimate the enzymatic digestion pattern of the new oligosaccharide produced by strain SS-76, the culture supernatant was further incubated for 24 h with each of the following enzymes (20 U/mL): glucoamylase, β-glucosidase, and trehalase. The enzymatic reactions were carried out according to the manufacturer's recommendation, and the resulting enzyme digests were analyzed by TLC.

Morphological characterization

Scanning electron microscopy (SEM) has been widely used in environmental microbiology to characterize the surface structure of biomaterials and to measure cell attachment and changes in the morphology of bacteria (15). To prepare the bacterial specimens for SEM, treatments such as washing, fixation and drying were applied as described by Perevedentseva et al. (16). Gold nanofilms with a thickness of 20∼30 nm were sputtered on bacterial specimens using an ion sputter (JEOL Ltd., To-

kyo, Japan), and the images were obtained using a SEM device (JEOL Ltd.).

Identification of bacterial strain

Strain SS-76 was isolated from the samples of Korean fermented foods and identified using 16S rRNA gene (16S rDNA) sequencing and subsequent phylogenetic analysis. DNA was extracted using a commercial genomic DNA extraction kit (Bioneer Co., Daejeon, Korea), and 16S rDNA was amplified by polymerase chain reaction (PCR) (Applied Biosystem, Foster City, CA, USA). The PCR-products were sent to Solgent Co. (Daejeon, Korea) for sequencing. The sequencing results were aligned using Clustal X (v1.0.1) and the phylogenetic tree was constructed with TreeView (v1.6.6). Phylogenetic distances were obtained based on the Kimura two-parameter model (17), and clustering was performed using the neighbor-joining algorithm (18). At least 1,000 bootstrap iterations were performed to find the roots of phylogenetic tree (19).

Analytical methods for determining composition

The total carbohydrate content was measured by the phenol-sulfuric acid method (20) using glucose as reference, while the total protein content was determined by the Bradford method using protein assay dye reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (21) with bovine serum albumin as reference. The monosaccharide composition of the oligosaccharide was analyzed by a modified alditol acetate method (22), using an ACME-6100 gas chromatography system (Young-Lin Co., Anyang, Korea) equipped with an SP-2380 capillary column (film, 0.2 μm; internal diameter, 0.25 mm; length, 30 m; Supelco Inc.). The analysis was performed with the following temperature program: 60°C→220°C (30°C/min), 220°C for 8 min, 220°C→250°C (8°C/min), and finally 250°C for 15 min. The molar ratios of monosaccharides were determined from the peak areas and response factors, using a flame ionization detector.

Determination of glycosidic linkage by methylation analysis

Methylation analysis was performed according to the methods described by Hakomori (23) and Waeghe et al. (24). The methylated oligosaccharide was hydrolyzed at 120°C using 2 M trifluoroacetic acid for 90 min; the products were reduced with NaBH4, and subsequently acetylated. The resulting partially methylated alditol acetates (PMAAs) were analyzed by gas chromatography-mass spectrometry (GC-MS) using a 6890 GC/5975 MSD device (Agilent Technologies, Santa Clara, CA, USA) equipped with an SP-2380 capillary column. The analysis was performed according to the following temperature program: 60°C for 1 min, 60°C→150°C (30°C/min), 150°C →180°C (1°C/min), 180°C→231°C (1.5°C/min), 231°C→250°C (30°C/min), and finally 250°C for 10 min. PMAAs were identified by their fragment ions and relative retention times, and their molar ratios were estimated from the peak areas and response factors, using a flame ionization detector.

Mass spectrometry analysis

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and electrospray ionization-based liquid chromatography-mass spectrometry/mass spectrometry (ESI-LC-MS/MS) were utilized to determine the precise molecular mass of the novel oligosaccharide. Mass spectra from MALDI-TOF were obtained on a 4700 Proteomics Analyzer/Voyager DE STR workstation (Applied Biosystems, Framingham, MA, USA) operated at an accelerating voltage of 30 kV, an extractor voltage of 9 kV, and a source pressure of approximately 8×10^{-7} Torr. The matrix for oligosaccharide analysis was 0.1 M 2,5-dihydroxyacetophenone in 50% aqueous acetonitrile. ESI-LC-MS/MS was carried out using an Agilent 1200 HPLC system interfaced with a triple quadrupole tandem mass spectrometer (Agilent Technologies) fitted with an ESI interface. The mass spectrometer was operated in the positive-ion mode; the ESI voltage was set at 5 kV, and the gas temperature was 350°C.

Nuclear magnetic resonance analysis

Nuclear magnetic resonance (NMR) spectroscopy was utilized to obtain the structural and dynamic details of the novel oligosaccharide. A solution containing the novel oligosaccharide (1∼2 mg) was exchanged three times with D_2O (Cambridge Isotope Laboratories, Andover, MA, USA), filtered, then placed in NMR tubes. Measurements were performed at 25°C using the Advance II 900 spectrometer (Bruker Corporation, Karlsruhe, Germany) operating at 900.2 MHz for ^1H.

RESULTS AND DISCUSSION

Screening and identification of a new oligosaccharide-producing bacterial strain

In the present study, we investigated a novel bacterial strain from Korean fermented foods that produces a new oligosaccharide using maltose the sole source of carbon. Out of 116 bacterial strains isolated from Korean fermented foods, 6 strains (SS-02, SS-21, SS-27, SS-46, SS-65, and SS-76) were selected in the first screening using TLC analysis, because they produced novel spots expecting a new oligosaccharide during 3 days incubation in the nutrient broth medium (data not shown). Therefore, they were monitored for production of a novel oligosaccharide from maltose. After each strain was individually in-

cubated with basal medium containing 2% maltose, the culture supernatants were spotted and developed on TLC plates. With one exception, all strains showed spots corresponding to maltose (Fig. 1). Only strain SS-76 produced an unknown oligosaccharide as well as an additional spot presumably corresponding to a glucose (lane 7). This demonstrates that strain SS-76 may be able to convert maltose into a novel oligosaccharide and an additional monosaccharide expected the glucose.

Based on the SEM image (Fig. 2), strain SS-76 was revealed to be a rod-shaped bacterium; the 16S rRNA gene sequence of strain SS-76 exhibited a high degree of similarity with sequences of the genus *Bacillus*. Multiple sequence alignment of 16S rRNA gene sequences revealed a close phylogenetic relationship between strain SS-76 and *Bacillus subtilis* (>99% similarity). *B. subtilis* is a gram-positive and catalase-positive bacterium, which has historically been classified as an obligate aerobe, though evidence exists that it is a facultative aerobe. Furthermore, there have not been any reports regarding the production of oligosaccharides by acceptor reaction using *B. subtilis*.

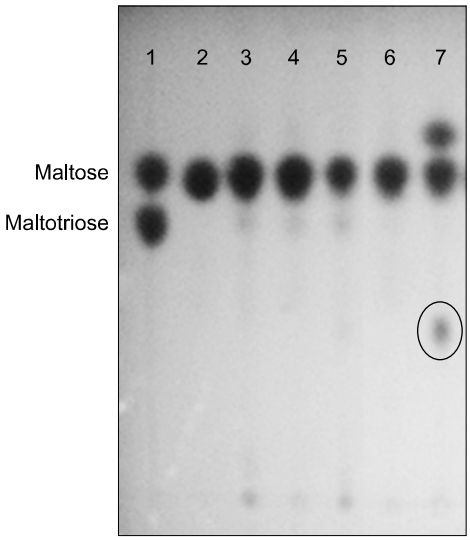

Fig. 1. Thin layer chromatographic (TLC) analysis of culture supernatants from six bacterial strains cultured in basal medium containing 2% maltose. Culture supernatants were spotted onto a TLC plate, and developed twice with a solvent containing butanol : pyridine : water (6:4:1). Saccharide spots were detected by spraying the plates with 20% sulfuric acid in methanol, followed by heating the plates at 120°C for 10 min. Lane 1, maltose and maltotriose standard; lane 2, culture supernatant of strain SS-02; lane 3, culture supernatant of strain SS-21; lane 4, culture supernatant of strain SS-27; lane 5, culture supernatant of strain SS-46; lane 6, culture supernatant of strain SS-65; lane 7, culture supernatant of strain SS-76.

Purification of the new oligosaccharide produced by *B. subtilis* SS-76

To monitor the oligosaccharides produced by strain SS-76, the culture supernatant of strain SS-76 was loaded onto an HPLC equipped with a Prevail Carbohydrate ES column. As shown in Fig. 3A, the supernatant of strain SS-76 produced mainly four peaks at a retention time of 19.7 min, 25.0 min, 28.3 min, and 30.9 min. Compared with certified reference materials of glucose and maltose (data not shown), peaks at 19.7 min and 25.0 min were identified as glucose and maltose, respectively (data not shown), however, peaks at 28.3 min and 30.9 min were not. In order to identify the unknown materials produced from strain SS-76, eluates that had passed through the detector were sequentially collected using a fraction collector; a total of 80 fractions (250 μL per tube) were obtained, and were further analyzed by TLC. As shown in Fig. 3B, ten fractions (lane 2~11) were obtained, with retention times ranging from 30 min to 35 min. Time difference by two min of retention time between Fig. 3A (from 28.3 min to 30.9 min) and 3B (from 30 min to 35 min) comes from volume margin from detector to test tube in fraction collector.

In order to obtain pure compounds, the oligosaccharide labeled as "fraction A" (corresponding to peak at 28.3 in the HPLC analysis) was collected from lane 2 to 4, and

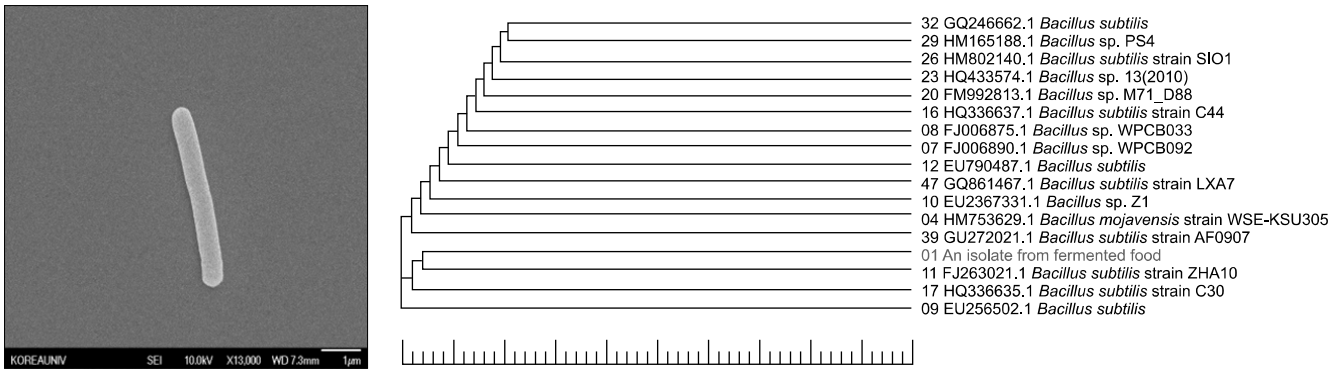

Fig. 2. Phylogenetic tree showing the relationship between bacterial strain SS-76 and related microorganisms in the *Bacillus* genus based on 16S rRNA gene sequences; morphology of strain SS-76 visualized by scanning electron microscope. The tree branch pattern was generated by the neighbor-joining method. Bootstrap value are expressed as percentages per 1,000 replications. Soil-1 fasta screen contigs (bold letter) refer to the 16S rRNA gene sequences from strain SS-76.

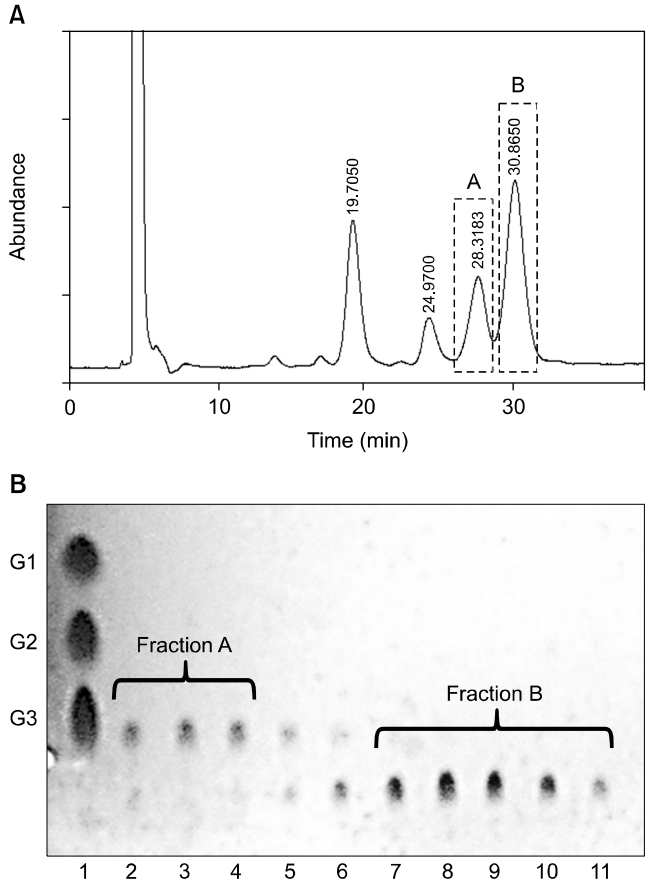

Fig. 3. (A) Fractionation profile of the supernatant of bacterial strain SS-76 cultured in basal medium containing 2% maltose by high performance liquid chromatography (HPLC). The culture supernatant was injected into the column of an HPLC device equipped with a Prevail Carbohydrate ES column with 70% acetonitrile solution as eluent, and 250 μL of eluate were collected sequentially in tubes. (B) Thin layer chromatographic (TLC) analysis of the fractions obtained by preparative (prep)-HPLC. The fractions were spotted onto a TLC plate, and developed twice with a solvent containing butanol : pyridine : water (6:4:1). The saccharide spots were detected by spraying the plate with 20% sulfuric acid in methanol, followed by heating the plate at 120°C for 10 min. Lane 1, glucose (G1), maltose (G2), and maltotriose (G3) standard; lane 2~11, fractions obtained by pre-HPLC with retention times ranging from 30 min to 35 min.

the oligosaccharide labeled as "fraction B" (corresponding to peak B at 30.9 min in the HPLC analysis) was collected from lanes 7 to 11. We chose fraction B, being assumed the highest degree of polymerization, for further analysis to identify the new oligosaccharide produced by *B. subtilis* SS-76.

Chemical and structural characteristics of new oligosaccharide

Chemical composition analysis and component sugar analysis indicated that fraction B contained 99.0% neutral saccharides, and no protein (Table 1). Monosaccharide composition analysis by the alditol acetate derivative method indicated that fraction B consisted of only one saccharide (glucose). This demonstrates that oligosaccharide fraction B produced by strain SS-76 using maltose as a sole source of carbon corresponds to a glucooligosaccharide, not to maltose. Methylation analysis is an essential step for determining the position of the glycosidic linkages in carbohydrates (25). This step showed that fraction B contains 3 types of glucosyl linkages in equal ratios: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol; and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol. These types of linkages correspond, respectively, to: non-reducing terminal glucopyranose; 1,6-glucopyranose; and 1,4-glucopyranose.

Table 1. Information regarding composition and linkage for the oligosaccharide fraction B generated by *Bacillus subtilis* SS-76

Chemical composition (%)	
Neutral carbohydrate	99.0
Protein	ND[1)]
Monosaccharide composition (mole%)[2)]	
Glucose	99.9
Other sugars	ND
Linkage information (mole%)	
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (T-Glc*p*)[3)]	34.9
1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (6-linked Glc*p*)	31.9
1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (4-linked Glc*p*)	33.2

Monosaccharide composition of fraction B was analyzed using the alditol acetate method, and linkage information was determined using a methylation analysis.
[1)]ND: not detected.
[2)]Calculated from the detected neutral sugar.
[3)]T, non-reducing terminal linkage; Glc*p*, glucopyranoside.

In order to measure the molecular mass and degree of polymerization, fraction B was analyzed using MALDI-TOF/MS and ESI-LC-MS/MS (Fig. 4). When fraction B was introduced to MALDI-TOF/MS, two signals (*m/z* 527 and 543) were detected (Fig. 4A). The pseudomolecular ion at *m/z* 527 corresponds to three glucosyl residues with one sodium adduct [(3 glucose+Na)$^+$], while the ion at *m/z* 543 corresponds to the same residues with one potassium adduct [(3 glucose+K)$^+$]. This was confirmed using an ESI-LC-MS/MS analysis, as shown in Fig. 4B. The peaks in the triplet of signals at *m/z* 527, 365, and 203 were separated by 162 mass units, suggesting the presence of a trisaccharide. ^{1}H-NMR analysis of fraction B showed two anomeric proton signals (δ_H 5.33 and δ_H 5.12 ppm), which were assigned the α-anomers of the reducing end (Fig. 5). Although ^{13}C-NMR analysis was not performed in this study, our results revealed α-configurations for two glucose residues in fraction B. For a more precise elucidation of the anomeric configuration, we treated fraction B with specific enzymes: glucoamylase, β-glucosidase, and trehalase (Fig. 6). The results indicated that fraction B was hydrolyzed only by glucoamylase (lane 5), resulting in the loss of ex-

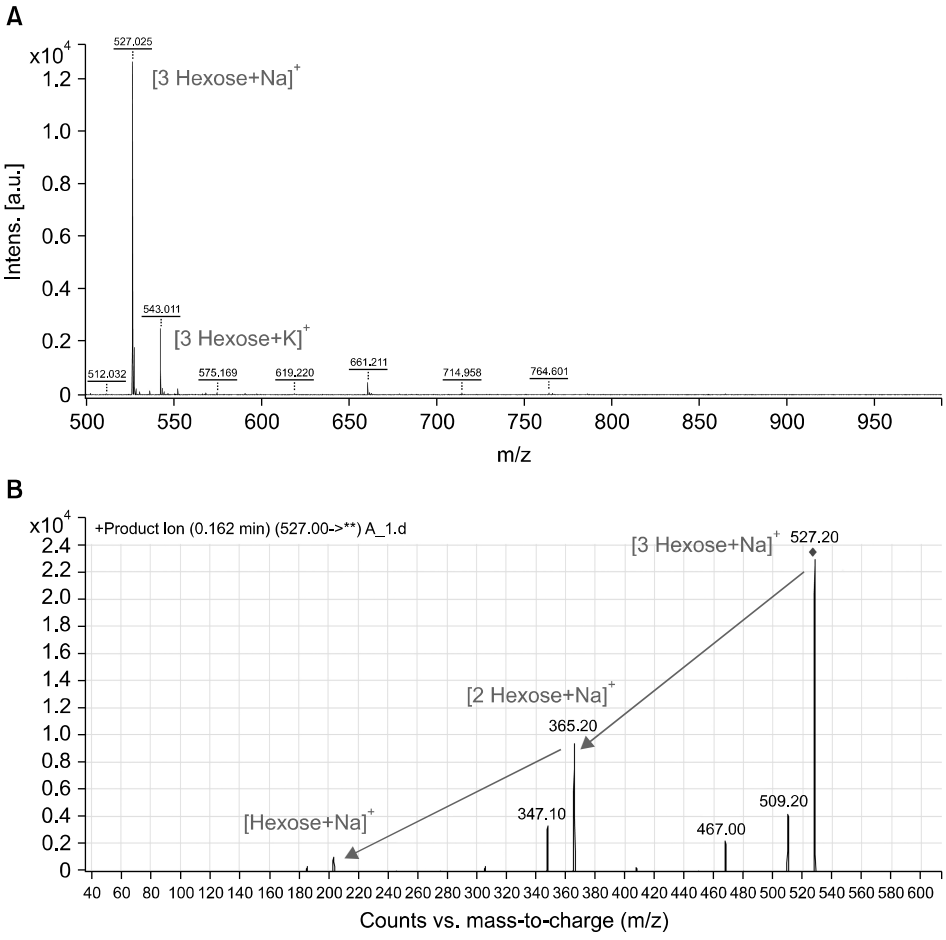

Fig. 4. Spectra obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (A) and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (B) analyses of the oligosaccharide fraction B produced by *Bacillus subtilis* SS-76. The major pseudomolecular ion obtained by MALDI-TOF/MS at *m/z* 527 corresponds to three glucosyl residues with one sodium adduct; the ion obtained by LC-MS/MS at *m/z* 527, 365, and 203 are separated by 162 mass units, suggesting th presence of glucotriose.

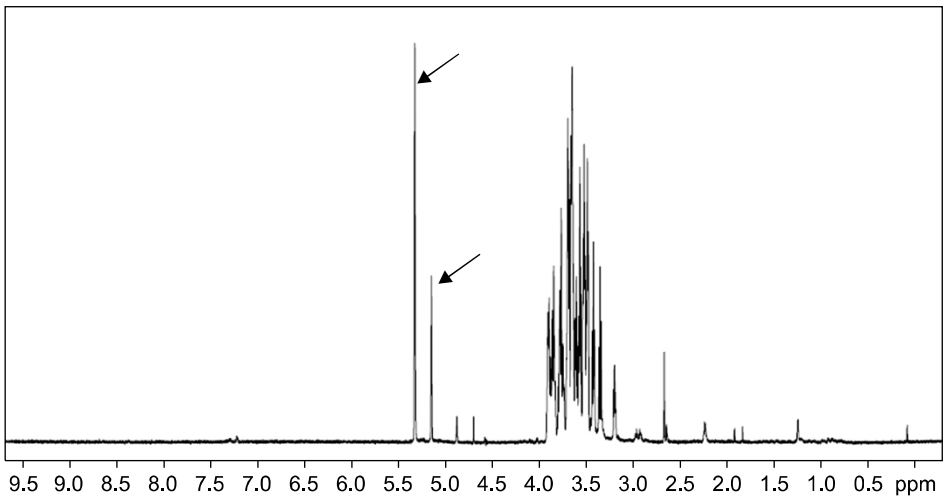

Fig. 5. ^{1}H spectrum of the oligosaccharide fraction B produced by *Bacillus subtilis* SS-76. Measurements were carried out in D_2O at 900.2 MHz and 25°C. The chemical shift at δ_H 5.33 ppm and δ_H 5.12 ppm, shown as arrows, were assigned to the α-anomeric configuration of the reducing end.

isted spot (G5) as well as the release glucose residues (G1). Since glucoamylases (1,4-α-D-glucan glucohydrolase) catalyze the hydrolysis of α-1,4- and α-1,6-glycosidic linkages (26), fraction B should contain α-1,4- and/or α-1,6-glycosidic linkages.

Putative structure of oligosaccharide fraction B produced by *B. subtilis* SS-76

Based on the chemical and structural characteristics, there are two possible trisaccharides with α-1,4- and/or α-1,6-glycosidic bonds. One is pullulan, and the other is panose. Pullulan is a polysaccharide consisting of maltotriose units with α-1,4- and α-1,6-glucose. However, in its structure, the three glucose residues of maltotriose are connected by an α-1,4-glycosidic bond, whereas consecutive maltotriose units are connected to each other by an α-1,6-glycosidic bond. Meanwhile, panose is a trisaccharide composed of one maltose unit and one glucose unit linked by an α-1,6-glycoside. Therefore, the ratio of α-1,4 to α-1,6 linkages in panose is approximately 1:1. Based

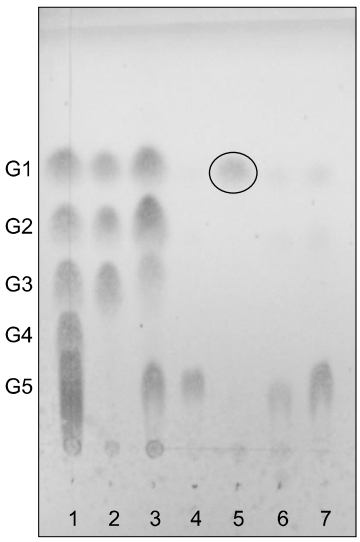

Fig. 6. Thin layer chromatographic (TLC) analysis of enzymatic hydrolysates of the oligosaccharide fraction B produced by *Bacillus subtilis* SS-76. Enzyme digests were spotted onto a TLC plate, and developed twice with a solvent containing butanol: pyridine:water (6:4:1). Saccharide spots were detected by spraying the plate with 20% sulfuric acid in methanol, followed by heating the plate at 120°C for 10 min. Lane 1, mixture of certificated standard refernces including glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), and maltopentose (G5); lane 2, certificated standard references of glucose (G1), maltose (G2), and maltotriose (G3); lane 3, culture supernatant of *B. subtilis* SS-76; lane 4, oligosaccharide fraction B obtained from culture supernatant of *B. subtilis* SS-76 by purification using preparative-high performance liquid chromatography; lane 5~7, products of enzymatic digestion of oligosaccharide fraction B with glucoamylase (lane 5), β-glucosidase (lane 6), and trehalase (lane 7).

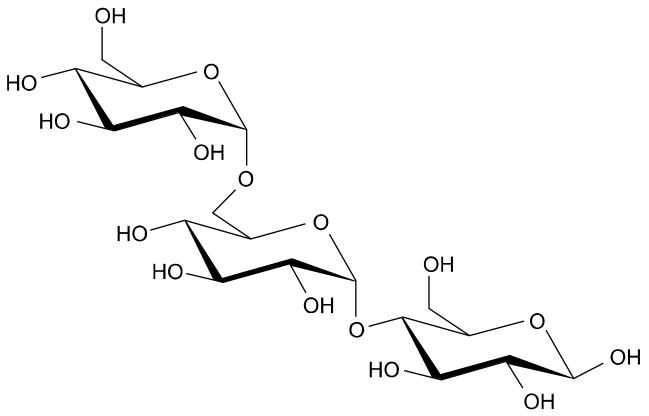

Fig. 7. Proposed structure of oligosaccharide fraction B produced by *Bacillus subtilis* SS-76. The trisaccharide structure corresponds to panose (α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,4-D-glucose).

on this information, the novel oligosaccharide produced by *B. subtilis* SS-76 using maltose as the sole source of carbon should be panose (α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,4-D-glucopyranose) (Fig. 7). Panose contains 1,4- and 1,6-glycosidic linkages, and is classified as an IMO (27). IMOs are commonly produced using starch as the raw material, but they require a combination of immobilized enzymes, such as α- and β-amylase as well as α-glucosidase (2). In particular, it is well known that the transglucosidase activity of α-glucosidase plays a pivotal role in the production of IMOs, containing panose. It is thus possible that strain SS-76 had the enzyme α-glucosidase and utilizes the transglycosylation systems in panose production. Similarly, Heincke et al. (3) reported that panose is the prime acceptor product formed when maltose is used as acceptor, and using a high ratio of maltose to sucrose results in high panose yields.

Panose has potential applications for foods and drinks because it is non-fermentable by human oral microorganisms, which grants panose an anti-cariogenic property (28). Furthermore, panose can be used as an anti-fading agent for food pigments and as a food antioxidant (29). It can also be considered a prebiotic agent for stimulating the growth of useful microorganisms such as *Lactobacillus* and *Bifidobacterium*, and for inhibiting the growth of noxious microorganisms such as *Escherichia* and *Salmonella* (9,30-32). Despite these potential applications, panose is still not produced in large-scale for industrial purposes (33). Moreover, few studies have reported the production of panose using enzymatic biotransformation such as acceptor reaction (3,7,34). Panose has the potential to be used in the food industry as a non-cariogenic sweetener (28) and is considered a new candidate prebiotic carbohydrate (30); however, a large-scale process for panose production, such as bacterial fermentation, cannot be implemented yet because of the lack of information regarding suitable panose-producing strains. In this context, our results suggest the possible application of panose for industrial production by producing the trisaccharide using bacterial fermentation with maltose as substrate. However, more studies regarding the purification and identification of the enzyme involved in the panose production are needed. Furthermore, more studies are also needed to evaluate the optimal conditions for panose production, and the enzyme substrate specificity in order to allow industrial applications of panose.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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