Food Sci. Biotechnol. Vol. 18, No. 2, pp. 519 ~ 525 (2009)

Food Science and Biotechnology

Structural Characterization of Non-reducing Oligosaccharide Produced by *Arthrobacter crystallopoietes* N-08

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Abstract A bacterial strain (Strain N-08) capable of extracellularly producing high level of non-reducing oligosaccharide (NR-OS) isolated from soil. The strain was identified phylogenetically by 16S rDNA sequence analysis and found to be very close to *Arthrobacter crystallopoietes*. The high production of NR-OS was observed in the basal culture medium containing maltose as a sole carbon source. The NR-OS in culture supernatant was purified by glucoamylase treatment and Dowex-1 (OH^-) ion exchange chromatography and its structure was characterized. This oligosaccharide consisted of only glucose. Methylation analysis indicated that this fraction was composed mainly of non-reducing terminal glucopyranoside. Matrix-assisted laser-induced/ionization time-of-flight (MALDI-TOF) and electrospray ionization-mass spectrometry (ESI-MS)/MS analyses suggested that this oligosaccharide comprised non-reducing disaccharide unit with 1,1-glucosidic linkage. When this disaccharide was analyzed by ^{1}H-NMR and ^{13}C-NMR, it gave the same signals with α-D-glucopyranosyl-(1,1)-α-D-glucopyranoside. These results indicated that the NR-OS produced by *A. crystallopoietes* N-08 was α1,α1-trehalose. This is the first report of the trehalose which can be produced directly from maltose by *A. crystallopoietes* N-08.

Keywords: structure, non-reducing oligosaccharide, trehalose, *Arthrobacter crystallopoietes*

Introduction

Generally, non-reducing oligosaccharides (NR-OS) are divided into two groups: cyclic and linear oligosaccharides. Cyclomaltohexaose (α-cyclodextrin), one of the most well-known cyclic oligosaccharides, is produced from linear α-(1,4)-glucans by the intramolecular transglycosylation reaction of a cyclomaltodextrin glucanotransferase (EC 2.4.1.19) (1). The cyclic oligosaccharide consists of 6 glucose units linked by α-(1,4)-bonds, and has a hydrophobic cavity in the center of the structure. Guest molecules with suitable sizes can be accommodated in the cavity, and the formation of the inclusion complex is applied for stabilizing labile materials (2), masking odors (3), and modifying viscosity (4). Trehalose (α-D-glucopyranosyl-1.1-α-D-glucopyranoside) is a typical linear NR-OS occurring in bacteria, yeasts, fungi, plants, and invertebrates (5-8). Due to its particular physical features, trehalose is able to stabilize proteins or fatty acids and function as a bioprotectant against various stresses of desiccation, heat, freezing, or osmotic shock (9,10). Due to its desirable physical and chemical characteristics, investigations have been focused on searching for efficient synthetic processes and abundant raw sources for the production of trehalose (11,12). A mass production of trehalose from starch has been developed using two bacterial enzymes, maltooligosyltrehalose synthase (EC 5.4.99.15) and maltooligosyltrehalose trehalohydrolase (EC 3.2.1.141) (13,14), and now this saccharide is used in the fields of food, cosmetic, and pharmaceutical industries (15-18). However, development of a new enzymatic process which has the advantages such as simple reaction, high substrate specificity, high conversion yield, and low cost, still can be great potential in industrial application. Thus novel NR-OSs (linear or cyclic) with heterogeneous linkages and/or different sizes which are produced from various substrates, are of great interest from the functional and industrial points of view.

Recently, we found that a Gram-positive bacterium, strain N-08 isolated from soil directly produces the NR-OS from maltose. In the present paper, the identification of this strain and the structural characterization of the NR-OS produced by this strain are described.

Materials and Methods

Carbohydrates and enzymes Partially hydrolyzed starch, Pinedex #4 (dextrose equivalent 19±2), was purchased from Matsutani Chemical Industry (Hyogo, Japan). Glucose and maltose were obtained from Sigma-Aldrich (St. Louis, MO, USA). Soluble starch was purchased from Duksan Co., Ltd. (Ansan, Korea). Glucoamylase (EC 3.2.1.3) from *Aspergillus niger*, α-glucosidase from (EC 3.2.1.20) from *Saccharomyces cerevisiae*, β-glucosidase (EC 3.2.1.21 from Almond, α-amylase (EC 3.2.1.1) from *Bacillus subtilis*, β-amylase (EC 3.2.1.2) from barley, isoamylase (EC 3.2.1.68) from *Pseudomonas* sp., and pullulanase (EC 3.2.1.41) from *Klebsiella pneumoniae* were purchased from Sigma-Aldrich. The enzyme activities were assayed according to the methods recommended by their suppliers.

Isolation of bacterial strains from soil Various soil samples collected in Suwon, Korea were used as to isolate NR-OS producing bacteria. Soil sample was serially diluted with saline solution, and plated on nutrient agar. Morphologically distinguished colonies were transferred on new nutrient agar, which were incubated at 30°C for 24 hr. One-hundred and twenty-one strains isolated from soil

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Received January 21, 2009; Revised February 22, 2009;
Accepted February 23, 2009

were maintained as frozen stock cultures at −70°C in nutrient broth with 20% glycerol.

Screening of bacterial strains Bacterial strains isolated from soil were incubated with reciprocal shaking at 27°C for 3 days in a liquid medium containing 1.5% maltose, 0.5% peptone, 0.1% yeast extract, 0.1% K_2HPO_4, 0.06% $NaH_2PO_4 \cdot 2H_2O$, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 6.8). Glucoamylase (4 units) was added into 1 mL of each culture supernatant and incubated at 40°C for 16 hr and then boiled for 10 min to stop the enzyme reaction. Non-reducing sugars in the reaction mixture were detected by thin layer chromatography (TLC).

Identification of isolate N-08 The isolate N-08 was identified by 16S rDNA sequencing and phylogenetic analysis (19). DNA was extracted from N-08 using a commercial genomic DNA extraction kit (K-3032; Bioneer Co., Daejeon, Korea). The 16S rDNA was amplified by polymerase chain reaction (PCR, GeneAmp 9700; Applied Biosystems, Foster city, CA, USA) and universal primer pair fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3'). PCR products were then purified by using Multiscreen PCR (Millipore Co., Billerica, MA, USA) and sequencing reactions were carried out by using an automatic DNA sequencer ABI PRISMR 3100-Avant Genetic Analyzer (Applied Biosystem) as described by the manufacturer. The 16S rDNA sequences of strain N-08 was compared to known 16S rDNA sequences in the GenBank database using the BLAST. Multiple alignments were made using Clustal X software version V.1.0.1, and phylogenetic tree was constructed with TreeView program version V.1.6.6. Distances were obtained using the Kimura two-parameter model (20) and clustering was performed using the neighbor-joining algorithm (21). At least 1,000 iterations were performed to calculate bootstrap values and roots of the phylogenetic tree (22).

Isolation of NR-OS Isolate N-08 which showed the highest production of the NR-OS, was cultured at the same condition in screening procedure. Two L of the culture supernatant of strain N-08 were harvested and boiled for 10 min to sterilize the viable bacterial cells in supernatant. Glucoamylase (5,000 units) was added into the reaction mixture and incubated at 40°C for 16 hr and then boiled for 10 min to stop the enzyme reaction. The mixture was passed through a column (2.5×50 cm) of Dowex 1 (OH^- form, 50-100 mesh, Sigma-Aldrich) to bind reducing sugars. As it will be shown below, this also served as the sole step toward purification of NR-OS produced by strain N-08. Then the eluate was concentrated to 200 mL using vacuum rotary evaporator (Eyela, Tokyo Rikakikai Co., Tokyo, Japan) and lyophilized to prepare the NR-OS for structural analysis. The reaction mixtures in each step were monitored by TLC.

Digestibility of NR-OS by various enzymes A reaction mixture (100 μL) containing 1.0 mg of NR-OS and 50 μL of each enzyme solution (20 unit/mL) was incubated for 24 hr. The enzyme reactions were performed according to the methods recommended by their suppliers. Digestibility of NR-OS was detected by TLC.

General analytical methods Total sugar and protein were determined using phenol-H_2SO_4 (23) and Bradford's method (24) with Bio-Rad dye (Bio-Rad Co., Hercules, CA, USA), respectively, using glucose and bovine serum albumin as the respective standards. Reducing sugar was measured by the Somogyi-Nelson method (25,26). The reducing power was calculated as follows: (the amount of reducing sugar as glucose)/(the amount of total sugar as glucose) ×100%. TLC was performed on a Kieselgel 60 plate (E. Merck, Darmstadt, Germany) developed twice with a solvent of buthanol:pyridine:water (6:4:1). Sugar spots were detected by spraying with 20% sulfuric acid in methanol, followed by heating the plates at 120°C for 10 min.

Component sugar analysis The sugar composition of the oligosaccharide samples was determined by gas chromatography (GC) analysis of their alditol acetates. Samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 1.5 hr at 121°C, converted into the corresponding alditol acetates (27,28), and analyzed by GC at 60°C for 1 min, 60°C→220°C (30°C/min), 220°C for 12 min, 220°C →250°C (8°C/min), and 250°C for 15 min, using a GC M600D (Young-Lin Co., Gyeonggi, Korea) equipped with an SP-2330 capillary column (0.25 μm film thickness, 0.32 mm i.d.×30 m, Supelco, Bellefonte, PA, USA). Molar ratios were calculated from the peak areas and response factors on a flame ionization detector (FID).

Methylation analysis Methylation analysis was performed according to the Hakomori method (29,30), and the methylated products were recovered using the modified procedure of Waeghe *et al*. (31). The methylated oligosaccharide was hydrolyzed by 2 M TFA at 120°C for 1.5 hr, and the products were reduced $NaBH_4$ and subsequent acetylation. The resulting partially methylated alditol acetates were analyzed via GC and GC-mass spectrometry (GC-MS) using an SP-2380 capillary column (Supelco). GC-MS was performed with a HP 5890A GC (Hewlett-Packard, Palo Alto, CA, USA) equipped with a 5970B mass-selective detector (Hewlett-Packard). The carrier gas was He (0.5 mL/min), and the temperature program was at 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 250°C for 5 min. The partially methylated alditol acetates were identified by their fragment ions and relative retention times, and their mole percentages were estimated from the peaks and response factors (32).

Matrix-assisted laser-induced/ionization time-of-flight (MALDI-TOF)/MS MALDI-TOF mass spectra were recorded using a Voyager™ DE-STR (Perseptive Biosystem, Framingham, MA, USA) spectrometer operated at an accelerating voltage of 30 kV, an extractor voltage of 9 kV, and a source pressure of 8×10^{-7} Torr (33). The matrix for oligosaccharide analysis was 0.1 M 2,5-dihydroxyacetophenone (DHAP) in 50% aqueous acetonitrile.

Liquid chromatography (LC)/MS The molecular mass of NR-OS was determined by LC-MS. The LC-MS was carried out using Agilent 1200 HPLC system (Palo Alto,

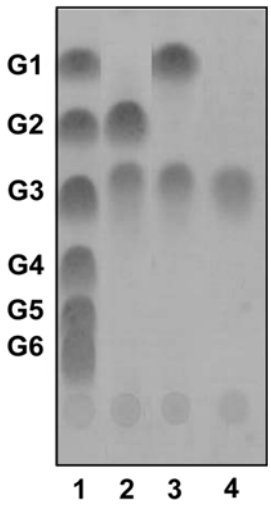

Fig. 1. TLC analysis of non-reducing oligosaccharide produced by an isolated strain N-08. Lane 1, maltooligosaccharides standard solution (G1, glucose; G2, maltose, and so on); lane 2, culture supernatant; lane 3, culture supernatant after glucoamylase digestion; lane 4, reaction mixture (lane 2) after removal of reducing sugars by anion-exchange.

Table 1. Chemical properties of the non-reducing oligosaccharide (NR-OS) produced by *A. crystallopoietes* N-08

Chemical composition	NR-OS	(%)
Neutral sugar	99.0	
Protein	0.0	
Reducing power	0.1	
Protein	0.0	
Component sugars[1)]		(mol%)
Glucose	99.9	
Other sugars	Not detected	
Methylation analysis[2)]		(mol%)
T-Glc*p*[3)] (2,3,4,6-tetra-*O*-methyl-1,5-di-acetylated glucitol)	99.9	
Other linkages	Not detected	

[1)]Monosaccharides were analyzed using alditol acetates. Mol% was calculated from the detected total carbohydrate.
[2)]Calculated from the peak areas and molecular response factors of each partially methylated alditol acetate in GC and GC-MS.
[3)]T-Glc*p* means non-reducing terminal glucopyranoside.

CA, USA) interfaced to a Triple quadrupole tandem mass spectrometer (Agilent) fitted with an electrospray-ionization (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 (NMR) spectroscopy The solutions of NR-OS in D_2O (0.6 mL, 99.9%; Cambridge Isotope Laboratories, Andover, MA, USA) were analyzed at 25°C using Adavance II 900 spectrometer (^{1}H 900.2 MHz, ^{13}C 226.3 MHz: Bruker, Karlsruhe, Germany).

Results and Discussion

Screening and identification of NR-OS producing bacterial strains About 120 bacterial strains from soil were tested for production of NR-OS from maltose. After glucoamylase digestion of the reaction mixtures, the samples were passing through a Dowex 1 (OH^-) anion-exchange column to remove reducing saccharides and were analyzed by TLC. As shown in Fig. 1, an isolate N-08 produced an unknown oligosaccharide whose Rf value was 0.46 in addition to maltose (lane 2). This oligosaccharide remained after glucoamylase digestion (lane 3) and passing on Dowex 1 (OH^-) (lane 4). The Rf value of this oligosaccharide was different from those of other reducing oligosaccharides, such as maltose, isomaltose, and maltotriose (lane 1). This oligosaccharide was hydrolyzed by not only glucoamylase (lane 3) but also by various amylases, such as α-amylase, β-amylase, isoamylase, pullulanase, and α-glucosidase (data not shown), and its reducing power was negligible (Table 1). These results indicated that this saccharide should be a non-reducing oligosaccharide. The isolate N-08 was identified by 16S rDNA sequencing and phylogenetic analysis (Fig. 2). The 16S rDNA sequence of strain N-08 revealed a high degree of similarity with that of the genus *Arthrobacter*. Multiple sequences alignment of 16S rDNA sequences revealed close relationship of N-08 to *Arthrobacter crystallopoietes* (more than 99% similarity).

Structure of NR-OS produced by *A. crystallopoietes* N-08 NR-OS mainly contained neutral sugar, whereas protein and any other materials were not detected. Sugar analysis indicated that NR-OS consisted of only one kind of sugar, glucose (Table 1). The molecular weight of NR-OS was found to be 342 by measuring the $[M+Na]^+$ ion (*m/z* 365) by MALDI-TOF/MS (Fig. 3) and LC-MS (Fig. 4A). The value was consistent with that of maltose anhydride, indicating that NR-OS consisted of 2 glucose residues [$M=162\times n$ (n=2)]. Major pseudo-molecular ion of the disaccharide (*m/z* 365) was analyzed by ESI-MS-MS. The MS-MS spectrum gave fragment ion at *m/z* 203 due to elimination of glucosyl (minus 162 mu) unit from disaccharide (Fig. 4B). Methylation analysis gave a sole peak of 2,3,4,6-tetra-*O*-methyl-1,5-di-acetylated glucitol (Table 1). This result indicated that NR-OS comprised only non-reducing terminal glucosyl linkages. To confirm this structure, NMR spectroscopy measurements were performed. The ^{13}C NMR spectrum contained only 6 signals (Fig. 5A) in agreement with the stereochemically symmetrical structure of the oligosaccharide in solution. No NMR peaks attributable to a reducing end C-1 were detected. Similarly, the ^{1}H NMR spectrum showed only 1 anomeric proton signal (Fig. 5B). Signals revealed the α-configurations of glucose residues in NR-OS that was confirmed by the C-1 signal at 93.2 ppm and H-1 signal at 5.14 ppm (d, $J_{1,2}$ 3.6 Hz). These NMR data were in good agreement with those of authentic trehalose (Table 2). Thus, we determined the structure of NR-OS, a α1, α1-trehalose whose structure was α-D-glucopyranosyl-(1.1)-α-D-glucopyranoside.

Trehalose is a unique sugar capable of protecting biomolecules against environmental stress (9,10). Its relative sweetness is 45% of sucrose. Trehalose has high thermostability and a wide pH-stability range. Therefore, it is one of the most stable saccharides. When 4% trehalose

Fig. 2. Phylogenetic tree showing relationships between the isolate N-08 and related strains on the basis of 16S rDNA sequences. The neighbor-joining method was used. Scale bar corresponds to 0.1 substitutions/ nucleotide position.

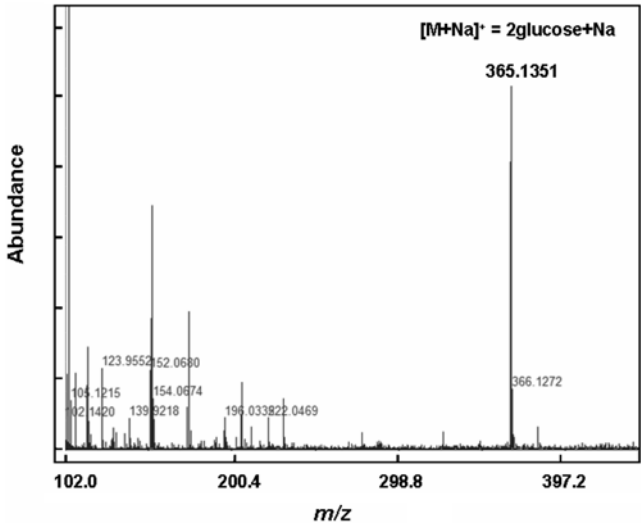

Fig. 3. MALDI-TOF-MS spectrum of the non-reducing oligosaccharide produced by *A. crystallopoietes* N-08.

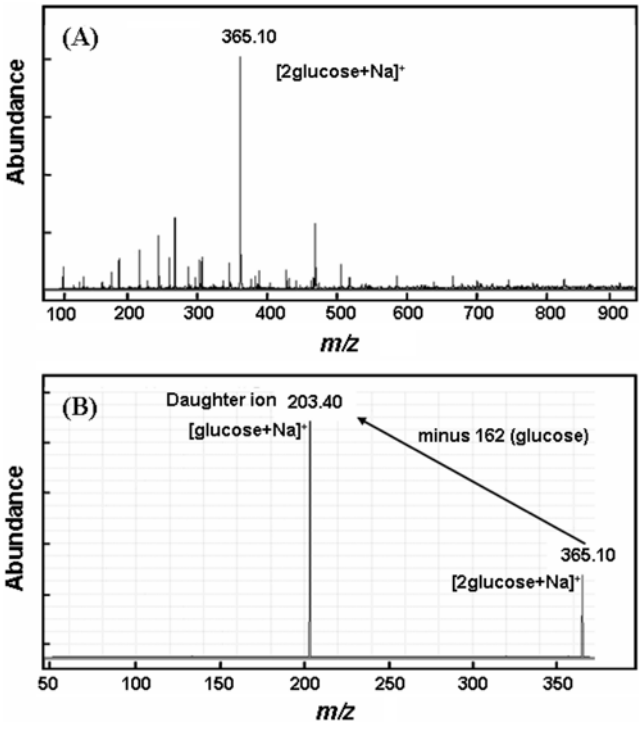

Fig. 4. LC-MS (A) and ESI-MS/MS spectrum (B) of the non-reducing oligosaccharide produced by *A. crystallopoietes* N-08.

solutions with 3.5 to 10 pH were heated at 100°C for 24 hr, no degradation of trehalose was observed in any case. Because of nonreducing sugar, this saccharide does not show Maillard reaction with amino compounds such as amino acids or proteins (34). Its particular physical features make it an extremely attractive substance for industrial applications. Furthermore, trehalose was approved as a food ingredient in Korea and Taiwan in 1998 with no use limits. In October 2000, the US Food and Drug Administration (FDA) gave a letter of no objection to a generally recognized as safe (GRAS) Notice (GRN 000045) (35).

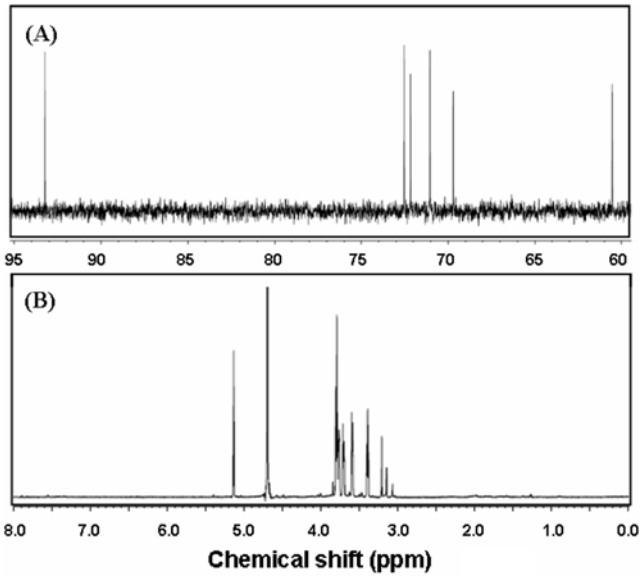

Fig. 5. ^{13}C-NMR (A) and ^{1}H-NMR (B) spectra of the non-reducing oligosaccharide produced by *A. crystallopoietes* N-08. ^{13}C-NMR and ^{1}H-NMR spectra of the non-reducing oligosaccharide were recorded at 900.2 and 226.3 MHz, respectively.

Table 2. Chemical shifts for non-reducing sugar produced by *A. crystallopoietes* and authentic trehalose

Carbon number	Chemical shifts (ppm)			
	δ_H		δ_C	
	Unknown	Authentic trehalose	Unknown	Authentic trehalose
1	5.138	5.061	93.236	93.136
2	3.598	3.512	71.045	70.961
3	3.807	3.623	72.523	72.450
4	3.393	3.315	69.699	69.621
5	3.773	3.690	72.162	72.064
6 (H-6a)	3.807	3.731	60.534	60.471
6 (H-6b)	3.712	3.637		

Therefore, it was predicted that *A. crystallopoietes* N-08 screened in this study could be a candidate to develop new production technology of trehalose.

Optimal carbon source for trehalose production The selection of a suitable carbon source for a fermentation process is a critical factor and thus involves the screening of several carbon sources for trehalose formation. In the present studies, 4 carbon sources such as soluble starch, Pinedex #4, maltose, and glucose were used for trehalose production by *A. crystallopoietes* N-08, and their productivities were detected by TLC. The results were shown in Fig. 6. Maltose produced the higher amount of trehalose (lane 4 and 8) whereas soluble starch and glucose did not produce any trehalose (lane 2, 5, 6, and 9). Even if Pinedex #4 also produced small amount of trehalose (lane 3 and 7), it did not give rise to higher trehalose production compared to maltose. These results indicated that trehalose could be produced directly from maltose as a sole carbon source by *A. crystallopoietes* N-08.

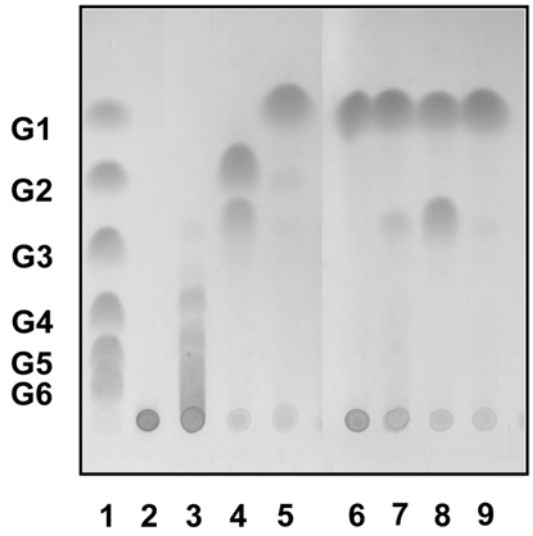

Fig. 6. TLC analysis of culture supernatants by *A. crystallopoietes* N-08 and their enzyme-digested products. Lane 1, maltooligosaccharides standard solution (G1, glucose; G2, maltose, and so on); lane 2, culture supernatant with soluble starch; lane 3, culture supernatant with Pinedex #4; lane 4, culture supernatant with maltose; lane 5, culture supernatant with glucose; lane 6, reaction mixture after glucoamylase digestion of lane 2; lane 7, after glucoamylase digestion of lane 3; lane 8, after glucoamylase digestion of lane 4; lane 9, after glucoamylase digestion of lane 5.

Due to its desirable physical and chemical characteristics, investigations have been focused on searching for efficient synthetic processes and abundant raw sources for the production of trehalose (11,12). At present, many trehalose synthesizing enzyme systems have been reported in microorganisms (14, 36-39). It is mainly accepted that trehalose can be metabolized in several ways, which involve different enzyme systems including trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase (40), trehalose synthase (38,41), maltose phosphorylase and trehalose phosphorylase (41,42), glycosyltransferase (43), and maltooligosyltrehalose trehalohydrolase and maltooligosyltrehalose synthase (14). Trehalose synthase (TS; EC 5.4.99.16) uses a very simple disaccharide as its substrate and converts it into trehalose in the absence of a coenzyme. This enzymatic process has the advantages of simple reaction, high substrate specificity, high conversion yield, and low cost. Therefore, it has great potential in industrial application.

From above results, it supposed that the trehalose-production by *A. crystallopoietes* N-08 was probably via TS enzyme system. So far, the TS pathway has been identified in *Pimelobacter* sp. R48 (38), *Pseudomonas* sp. F1 (39), and a *Thermus* strain (44), and only TS from the *Thermus* strain was characterized. Three trehalose synthase genes from *T. thermophilus*, *Pimelobacter* sp. R48, and *Picrophilus torridus* have been cloned (38,45,46). We are now cloning and sequencing the gene of this enzyme. Finally, this enzyme should prove especially useful in the industrial-scale preparation of trehalose, a disaccharide incorporated into foods, cosmetics and pharmaceutical products.

Acknowledgments

This work was financially supported by the 2006 Kyonggi University specialization program.

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