

Optimal Conditions and Substrate Specificity for Trehalose Production by Resting Cells of *Arthrobacter crystallopoietes* N-08

Yi Seul Seo and Kwang-Soon Shin[†]

Department of Food Science and Biotechnology, Kyonggi University, Gyeonggi 443-760, Korea

Abstract

Recently, we found that *Arthrobacter crystallopoietes* N-08 isolated from soil directly produces trehalose from maltose by a resting cell reaction. In this study, the optimal set of conditions and substrate specificity for the trehalose production using resting cells was investigated. Optimum temperature and pH of the resting cell reaction were 55°C and pH 5.5, respectively, and the reaction was stable for two hours at 37~55°C and for one hour at the wide pH ranges of 3~9. Various disaccharide substrates with different glycosidic linkages, such as maltose, isomaltose, cellobiose, nigerose, sophorose, and laminaribiose, were converted into trehalose-like spots in thin layer chromatography (TLC). These results indicated broad substrate specificity of this reaction and the possibility that cellobiose could be converted into other trehalose anomers such as α,β - and β,β -trehalose. Therefore, the product after the resting cell reaction with cellobiose was purified by β -glucosidase treatment and Dowex-1 (OH⁻) column chromatography and its structure was analyzed. Component sugar and methylation analyses indicated that this cellobiose-conversion product was composed of only non-reducing terminal glucopyranoside. MALDI-TOF and ESI-MS/MS analyses suggested that this oligosaccharide contained a non-reducing disaccharide unit with a 1,1-glucosidic linkage. When this disaccharide was analyzed by ¹H-NMR and ¹³C-NMR, it gave the same signals with α -D-glucopyranosyl-(1,1)- α -D-glucopyranoside. These results suggest that cellobiose can be converted to α,α -trehalose by the resting cells of *A. crystallopoietes* N-08.

Key words: trehalose, resting cell, optimal condition, substrate specificity, cellobiose, *Arthrobacter crystallopoietes*

INTRODUCTION

Trehalose (α -D-glucopyranosyl-1,1- α -D-glucopyranoside) is a typical non-reducing disaccharide of glucose that is widespread in the biological world and may have a variety of functions in living organisms (1-4). Although there are three different anomers of trehalose (i.e. $\alpha 1$ - $\alpha 1$ -, $\alpha 1$ - $\beta 1$ -, and $\beta 1$ - $\beta 1$ -), $\alpha 1$ - $\alpha 1$ -glucosyl-glucose is the only-biologically active form known (2). Trehalose has been isolated from a large number of prokaryotic and eukaryotic cells including mycobacteria, streptomycetes, enteric bacteria, yeast, fungi, insects, slime molds, nematodes, and plants (1-4). 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 (5,6). Many attempts to utilize trehalose are currently underway in several fields of industry, especially foodstuffs, confectioneries, cosmetics and pharmaceuticals, as a heat- and acid-stable sweetening material, with relatively low sweetness compared to sucrose (7,8), stabilizer for proteins, including enzymes (9), and a moisturizer in cosmetics for human skin (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, maltooligosyl trehalose synthase (EC 5.4.99.15) and maltooligosyl trehalose trehalohydrolase (EC 3.2.1.141) (13,14). However, development of a new enzymatic process with advantages, such as a simple reaction, high substrate specificity, high conversion yield, and low cost, can be of great potential in industrial application.

Recently, we found that a gram-positive bacterium, *Arthrobacter crystallopoietes* N-08 isolated from soil, directly produces trehalose from maltose by a resting cell reaction (15). In the present paper, the optimal set of conditions and substrate specificity for the trehalose production using resting cells of this strain are described. Also, a structural analysis was performed on the products converted from cellobiose by the resting cell reaction.

[†]Corresponding author. E-mail: ksshin@kyonggi.ac.kr
Phone: +82-31-249-9655, Fax: +82-31-249-9650

MATERIALS AND METHODS

Carbohydrates and enzymes

Glucose, maltose, trehalose, nigerose, maltitol, cellobiose, gentiobiose and laminaribiose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Isomaltose from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), sophorose from Serva Electrophoresis GmbH (Heidelberg, Germany) and malto-oligosaccharides kit from Supelco, Inc. (Bellefonte, PA, USA) were also used in this study. Glucoamylase (EC 3.2.1.3) from *Aspergillus niger*, and β -glucosidase (EC 3.2.1.21) from almond were purchased from Sigma. Trehalase (EC 3.2.1.28) from a prokaryote obtained from Megazyme Co. (Wicklow, Ireland). The enzyme reactions were performed according to the methods recommended by their suppliers.

Microorganisms and culture conditions

Arthrobacter crystallopoietes N-08 isolated from soil, described previously (15), was incubated with reciprocal shaking at 200 rpm and 27°C for 3 days in a liquid medium containing 1.5% maltose (or cellobiose), 0.5% peptone, 0.1% yeast extract, 0.1% K₂HPO₄, 0.06% NaH₂PO₄·2H₂O, and 0.05% MgSO₄·7H₂O (pH 6.8).

Resting cell reaction

The cells of *A. crystallopoietes* N-08 were harvested by centrifugation (7,000 × g) at 4°C for 30 min. The pellets were washed with 0.9% saline solution and suspended in the standard mixture. The reaction mixture was 2 mL and contained 50 mg/mL (wet weight) of resting cells, 1% substrate (disaccharides such as maltose, cellobiose, etc.) and 100 mM acetate buffer (pH 5.5). The reactions were performed at 30°C for 4 hr with weak shaking (50 rpm). Glucoamylase or β -glucosidase (4 units) was added into 1 mL of the supernatant and incubated at 37°C for 4 hr and then boiled for 10 min to stop the enzyme reaction. The products in the reaction mixture were detected by thin layer chromatography (TLC).

Assay for the activity of the resting cell reaction

The activity of trehalose synthase by the resting cell reaction was assayed by measuring the decrease of reducing power by maltose. The reaction mixture, consisting of 1 mL of 0.1 M sodium acetate buffer (pH 6.0), 1% (w/v) maltose, and 1.0 mL of the resting cells adjusted to optical density (at 600 nm) 2.0, was incubated at 37°C for 3 hr. The reaction was stopped by adding 0.1 mL of 1 N NaOH and the reaction mixture was centrifuged. The decreasing amount of the reducing sugar (maltose) in the supernatant was measured by the

3,5-dinitrosalicylic acid (DNS) method (16). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the decrease of 1 μ mole of maltose in one minute. The relative enzyme activity (%) was defined as the percentage of enzyme activity in the control.

Purification of cellobiose-conversion product by the resting cell reaction

A. crystallopoietes N-08 was cultured using cellobiose as a sole carbon source in the same culture procedure stated previously. Following the resting cell reaction with cellobiose, 200 mL of the reaction supernatants were collected and boiled for 10 min to sterilize the viable bacterial cells. The supernatant was passed through a column (2.5 × 50 cm) of Dowex-1 (OH form, 50~100 mesh, Sigma) to bind reducing sugars. As shown below, this also served as the sole step toward purification of the cellobiose-conversion product by the resting cell reaction. Then, the eluates were concentrated to 50 mL using a vacuum rotary evaporator (Eyela, Tokyo Rikakikai Co., Tokyo, Japan) and lyophilized to prepare the cellobiose-conversion product for structural analysis. The reaction mixtures in each step were monitored by TLC.

General analytical methods

Total sugar and protein were determined using phenol-H₂SO₄ (17) and Bradford's method (18) 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 (19,20). The reducing power was calculated as follows: reducing power (%) = (amount of reducing sugar in sample) / (amount of total sugar in sample) × 100. TLC was performed on a Kieselgel 60 plate (E. Merck, Darmstadt, Germany) developed twice with a 6:4:1 butanol : pyridine : water solvent. 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) analyses of their alditol acetates (21,22). Samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 1.5 hr at 121°C, converted into the corresponding alditol acetates, 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 M600D GC (Young-Lin Co., Gyeonggi-do, Korea) equipped with an SP-2330 capillary column (0.25 μ m film thickness, 0.32 mm I.D. × 30 m, Supelco). 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 (23) and Choi et al. method (24), and the methylated products were recovered using the modified procedure of Waeghe et al. (25). The methylated oligosaccharide was hydrolyzed by 2 M TFA at 120°C for 1.5 hr, and the products were reduced with NaBH₄ and subsequently acetylated. 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 (26).

Matrix-assisted laser-induced/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

MALDI-TOF mass spectra were recorded using a Voyager™ DE-STR 4700 proteomics analyzer (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 approx. 8×10^{-7} Torr (27). The matrix for oligosaccharide analysis was 0.1 M 2,5-dihydroxybenzoic acid (DHB) in 50% aqueous acetonitrile.

Liquid chromatography-mass spectrometry (LC-MS)

Molecular mass of the sample was determined by LC-MS. The LC-MS was carried out using the Agilent 1200 HPLC system (Palo Alto, 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 sample in D₂O (0.6 mL, 99.9%; Cambridge Isotope Laboratories, Andover, MA, USA) were analyzed at 25°C using a Varian VI500 FT-NMR spectrometer (¹H 500.2 MHz, ¹³C 125.8 MHz; Varian Inc., Palo Alto, CA, USA)

RESULTS AND DISCUSSION

Properties of the resting cell reaction by *A. crystallopoietes* N-08

In our previous work, we found that a gram-positive bacterium, *A. crystallopoietes* N-08 isolated from soil, directly produces trehalose from maltose by the resting cell reaction (15). In this work, the optimal conditions of the resting cell reaction for trehalose production were investigated.

The effects of pH and temperature on the resting cell reaction for trehalose production were tested using 0.1 M citrate (pH 3.0~6.0), acetate (pH 5.0~7.0) and sodium phosphate (pH 6.5~8.0) buffers. Maximum activity was observed at pH 5.5, but the reaction activity did not deviate considerably in the pH range of 5.0~6.0 (Fig. 1A). At pH 3.0 and 8.0, the reaction activity was maintained up to about 50% of the maximal value. Most of the trehalose synthase, purified from several sources, exhibits pH optima ranging from 6.5 to 7.5 (28,29). At temperatures ranging from 25 to 55°C, the reaction activity increased gradually, achieving the maximum at 55°C (Fig. 1B). However, at 60°C the detected activity dropped to only about 60% of the maximal value.

To examine the pH stability, the resting cell was incubated at various pHs using 0.1 M glycine-HCl (pH 2.5~3.5), acetate (pH 3.5~5.5), sodium phosphate (pH 5.5~8.0) and NaOH-boric acid (pH 8.0~10.0) buffers

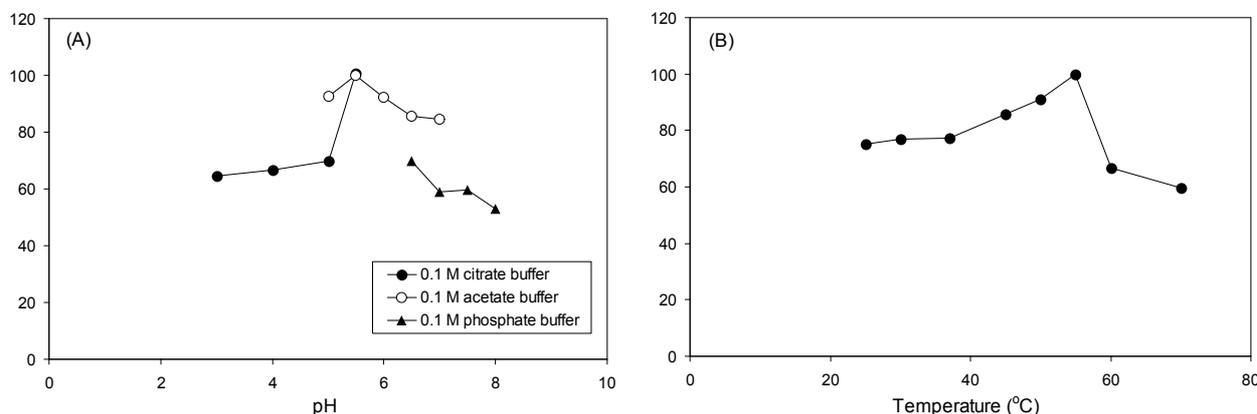


Fig. 1. Optimal pH (A) and temperature (B) of resting cell reaction by *A. crystallopoietes* N-08.

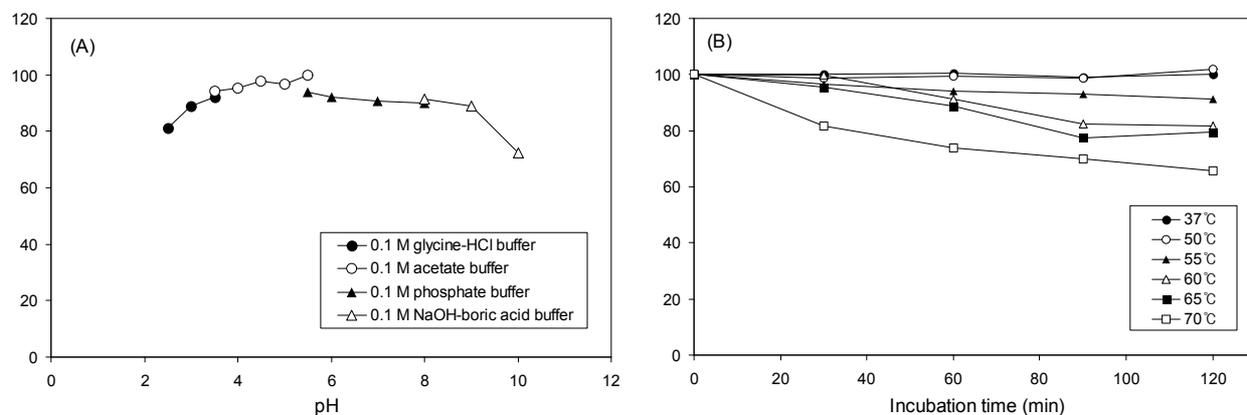


Fig. 2. Effect of pH (A) and temperature (B) on the stability of resting cell reaction by *A. crystallopoietes* N-08.

for 1 hr (37°C), followed by assaying the residual activities at pH 5.5. As shown in Fig. 2A, the activity was relatively stable (higher than 90%) in a wide range of pHs (3.0~9.0). To access the thermostability of the reaction, each reaction was incubated at the designated temperature for 0~2 hr and subsequently cooled down to 4°C prior to activity analysis. The residual activities were assayed at 37°C. As shown in Fig. 2B, the resting cell reaction exhibited high thermostability and activity was retained up to more than 90% of maximal activity at 37~55°C. Compared with previous studies (28,29), the trehalose synthase activity from *A. crystallopoietes* N-08 is not as thermostable as that from *Thermus aquaticus* ATCC 33923, but higher compared to *Pimelobacter* sp. R48. *T. aquaticus* ATCC 33923 lives in hot springs, suggesting the enzyme is thermostable. However, the optimum pHs of the enzyme from the three strains are close.

Substrate specificity of the resting cell reaction by *A. crystallopoietes* N-08

The resting cell reaction for trehalose production was tested with various possible substrates and the productivity relative to maltose was monitored by TLC (Table 1). The resting cell reaction could convert a trehalose-like substance from various disaccharides, such as maltose, nigerose, isomaltose, cellobiose, laminaribiose and sophorose, but not with others such as starch and maltooligosaccharides (degree of polymerization (DP) = 3~4). These results showed that maltose (α -1,4) and cellobiose (β -1,4) were utilized as favorable substrates by resting cells for trehalose production.

At present, many trehalose synthesizing enzyme systems have been reported in microorganisms. The maltooligosyl trehalose synthase and hydrolase derived from *Sulfolobus shibatae* (30), *Arthrobacter* sp. Q36 (13,14) and several other bacteria are involved in production of trehalose from starch and dextrans. Furthermore, trehalose synthase catalyzing the conversion of maltose into trehalose by intramolecular transglucosylation has very

Table 1. Substrate specificity of the resting cell reaction by *A. crystallopoietes* N-08

Carbohydrate	Glucosidic linkage	Relative reactivity ¹⁾	
Maltooligosaccharides	Maltose	α -1,4	+++
	Maltotriose	α -1,4	-
	Maltotetrose	α -1,4	-
	Soluble starch	α -1,4	-
Disaccharides with α -linkages	Trehalose	α 1- α 1	-
	Nigerose	α -1,3	++
	Maltose	α -1,4	+++
	Maltitol	α -1,4	-
	Isomaltose	α -1,6	+
Disaccharides with β -linkages	Sophorose	β -1,2	+
	Laminaribiose	β -1,3	+
	Cellobiose	β -1,4	+++
	Gentiobiose	β -1,6	-

The resting cell reaction for trehalose production was tested with various possible substrates. Each standard disaccharide and its resting cell reaction product were spotted onto a thin layer chromatography (TLC) plate, developed twice, and then detected by spraying with 20% sulfuric acid in MeOH.

¹⁾Relative reactivity represents as color strength in TLC.

useful industrial applications (28,29,31). Trehalose synthase (TS; EC 5.4.99.16) converts a simple disaccharide into trehalose in the absence of a coenzyme, making this enzymatic process advantageous as a simple reaction with low cost and increased potential in industrial applications. These results suggest that the trehalose production by *A. crystallopoietes* N-08 was probably mediated by the TS enzyme system with broad specificity to several disaccharides, and cellobiose could be converted into other trehalose anomers such as isotrehalose (β 1- β 1-trehalose) and neotrehalose (α 1- β 1-trehalose) by this system.

Cellobiose is a valuable product that can be obtained from enzymatic hydrolysis of cellulose (32). However, trehalose production from cellobiose has not been attempted until now. Therefore, the products converted from cellobiose by the resting cell reaction were purified

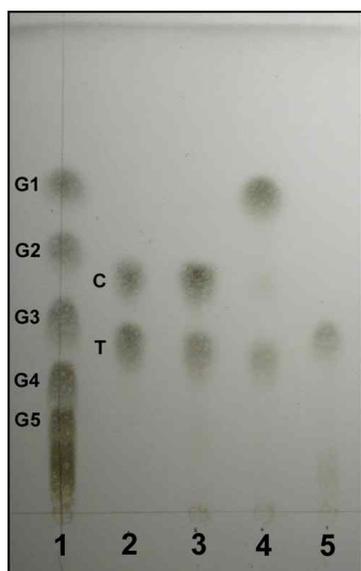


Fig. 3. TLC analysis of cellobiose-conversion product by resting cell reaction of *A. crystallopoietes* N-08. Samples were spotted onto a TLC plate, developed twice, and then detected by spraying with 20% sulfuric acid in MeOH. Lane 1, standard maltooligosaccharides (G1, glucose; G2, maltose, and so on); Lane 2, standard cellobiose (C) and trehalose (T); Lane 3, reaction supernatant; Lane 4, a sample after β -glucosidase digestion of lane 3; Lane 5, a sample after removal of reducing sugars by anion-exchange chromatography of lane 4.

and their chemical properties were examined.

Purification and chemical properties of cellobiose-conversion product of resting cell reaction by *A. crystallopoietes* N-08

After β -glucosidase digestion of the reaction mixtures, the samples were passed through a Dowex-1 (OH^-) anion-exchange column to remove reducing saccharides and were analyzed by TLC. As shown in Fig. 3, the reaction mixture contained a spot whose R_f value was 0.35, in addition to cellobiose (lane 3). This spot remained after β -glucosidase digestion (lane 4) and passing through the Dowex-1 (OH^-) (lane 5). The R_f value of this spot was different from those of other reducing oligosaccharides, such as glucose, maltose and maltotriose (lane 1), but similar to trehalose. This product was neither hydrolyzed by α -glucoamylase nor various amylases, such as α -amylase, β -amylase and isoamylase (data not shown), and its reducing power was negligible (Table 1). These results indicated that this saccharide is probably a non-reducing oligosaccharide (NROS).

Structural analysis of cellobiose-conversion product of resting cell reaction by *A. crystallopoietes* N-08

NROS converted from cellobiose mainly contained a neutral sugar, whereas protein and any other materials were not detected. NROS consisted of only glucose by sugar analysis (Table 2). The molecular weight of NROS

Table 2. Chemical properties of cellobiose-conversion product by the resting cell reaction of *A. crystallopoietes* N-08

Chemical composition	Non-reducing oligosaccharide (%)
Neutral sugar	99.1
Protein	0.0
Reducing power	0.1
Protein	0.0
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Component sugars ¹⁾	(Mole %)
Glucose	99.9
Other sugars	Not detected
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Methylation analysis ²⁾	(Mole %)
T-Glcp ³⁾	99.9
(2,3,4,6-tetra-O-methyl-1,5-di-acetylated glucitol)	
Other linkages	Not detected

¹⁾Monosaccharides were analyzed using alditol acetates. Mole % 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-Glcp means non-reducing terminal glucopyranoside.

was found to be 342 Da by measuring the $[\text{M} + \text{Na}]^+$ ion (m/z 365) by MALDI-TOF-MS (Fig. 4) and LC-MS (Fig. 5A). The value was consistent with that of maltose anhydride, indicating that NROS consisted of two glucose residues [$\text{M} = (180 \times 2) - 18 = 342$]. The major pseudo-molecular ion of the disaccharide (m/z 365) was analyzed by ESI-MS-MS. The MS-MS spectrum gave a fragment ion at m/z 203 due to elimination of a glucosyl (minus 162 mu) unit from disaccharide (Fig. 5B). Methylation analysis gave a sole peak of 2,3,4,6-tetra-O-methyl-1,5-di-acetylated glucitol (Table 2). This result indicated that NROS contained 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. 6A) in agreement with the stereochemically symmetrical structure of the oligosaccharide in solution. No NMR peaks attrib-

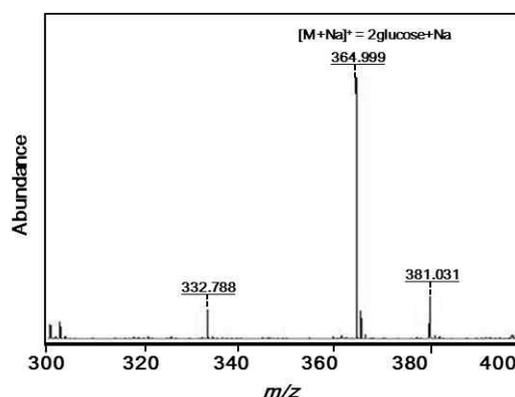


Fig. 4. MALDI-TOF-MS spectrum of cellobiose-conversion product by resting cell reaction of *A. crystallopoietes* N-08.

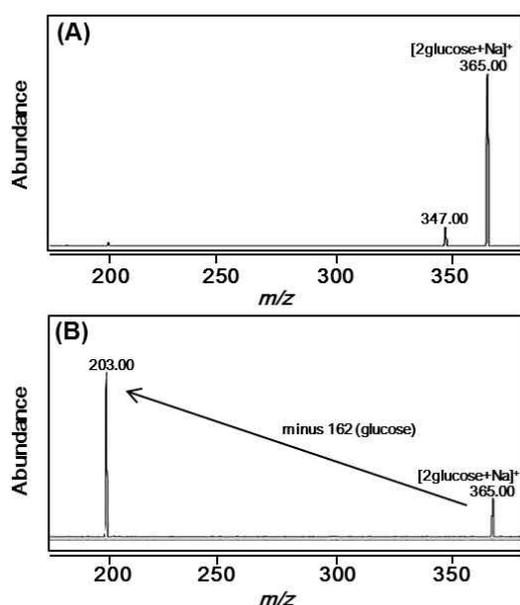


Fig. 5. LC-MS (A) and ESI-MS/MS spectrum (B) of cellobiose-conversion product by resting cell reaction of *A. crystallopoietes* N-08.

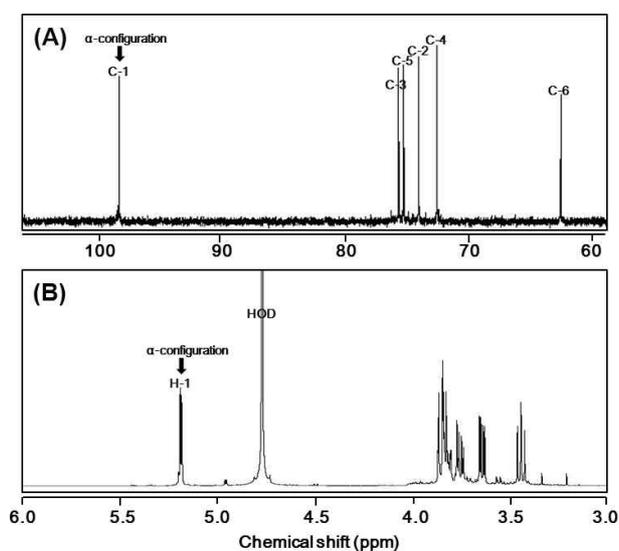


Fig. 6. ^{13}C -NMR (A) and ^1H -NMR (B) spectra of cellobiose-conversion product by resting cell reaction of *A. crystallopoietes* N-08. ^{13}C -NMR (A) and ^1H -NMR spectra of cellobiose-conversion product were recorded at 125.8 MHz and 500.2 MHz, respectively. Arrow indicates characteristic alpha-configuration. The proton and carbon positions designated in NMR spectra are represented by, for example, H-1 and C-1, respectively.

able to a reducing end C-1 were detected. Similarly, the ^1H NMR spectrum showed only one anomeric proton signal (Fig. 6B). These signals revealed the α -configurations of glucose residues in NROS that was confirmed by the C-1 signal at 96.1 ppm and the H-1 signal at 5.19 ppm (d, $J_{1,2}$ 3.6 Hz). These NMR data were in good agreement with those of authentic trehalose. Thus, we determined the structure of NROS as an $\alpha 1, \alpha 1$ -trehalose

from the original structure of α -D-glucopyranosyl-(1,1)- α -D-glucopyranoside.

Trehalose is a unique sugar capable of protecting biomolecules against environmental stress (5,6). Its relative sweetness is 45% of sucrose. Trehalose has high thermostability and a wide pH-stability range, making it one of the most stable saccharides. When 4% trehalose 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 the non-reducing sugar, this saccharide does not show a Maillard reaction with amino compounds such as amino acids or proteins (7). The physical features of trehalose particularly 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 FDA gave a letter of no objection to a GRAS Notice (GRN 000045) (33).

Therefore, we predicted that the resting cell reaction by *A. crystallopoietes* N-08 could be a candidate technology for trehalose production from both maltose and cellobiose.

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