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Analysis of Transglucosylation Products of *Aspergillus niger* α-Glucosidase that Catalyzes the Formation of α-1,2- and α-1,3-Linked Oligosaccharides

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Abstract: According to whole-genome sequencing, *Aspergillus niger* produces multiple enzymes of glycoside hydrolases (GH) 31. Here we focus on a GH31 α-glucosidase, AgdB, from *A. niger*. AgdB has also previously been reported as being expressed in the yeast species, *Pichia pastoris*; while the recombinant enzyme (rAgdB) has been shown to catalyze tranglycosylation via a complex mechanism. We constructed an expression system for *A. niger* AgdB using *Aspergillus nidulans*. To better elucidate the complicated mechanism employed by AgdB for transglucosylation, we also established a method to quantify glucosidic linkages in the transglucosylation products using 2D NMR spectroscopy. Results from the enzyme activity analysis indicated that the optimum temperature was 65 °C and optimum pH range was 6.0–7.0. Further, the NMR results showed that when maltose or maltopentaose served as the substrate, α-1,2-, α-1,3-, and small amount of α-1,1-β-linked oligosaccharides are present throughout the transglucosylation products of AgdB. These results suggest that AgdB is an α-glucosidase that serves as a transglucosylase capable of effectively producing oligosaccharides with α-1,2-, α-1,3-glucosidic linkages.

Key words: *Aspergillus*, α-glucosidase, glycoside hydrolase family 31, transglucosylation

INTRODUCTION

The enzyme α-glucosidase (EC 3.2.1.20; α-D-glucoside glucohydrolase) is a typical exo-type glycosidase that hydrolyzes α-1,4-glucosidic linkages and releases α-D-glucose from the non-reducing end. The α-glucosidases are primarily classified into either the glycoside hydrolase (GH) 13 or GH31 families.[1)]

The GH31 family is comprised of various types of enzymes, including α-xylosidases (EC 3.2.1.177), isomaltosyltransferases (EC 2.4.1.-), sulfoquinovosidases (EC 3.2.1.199), and α-glucosidases.[2)] *Aspergillus* species are reported to possess multiple GH31 enzymes; for instance, *Aspergillus nidulans* and *Aspergillus oryzae* have ten GH31 proteins.[2)] Moreover, within the *Aspergillus niger* genome, seven GH31 proteins are encoded, namely, AgdA, AgdB, AgdE, AgdF, AgdG, AxlA, and AxlB.[3)] AgdA is the α-glucosidase that produces oligosaccharides containing α-1,6-glucosidic linkages;[4)5)] while AxlA is an α-xylosidase.[6)] Additionally, Ma *et al.* recently cloned an AgdB gene from *A. niger* strain K1 and successfully expressed recombinant AgdB in *Pichia pastoris* (rAgdB). This recombinant protein was reported to have high hydrolytic specificity toward α-1,3- and α-1,4-glucosidic linkages and produced kojibiose and nigerose via transglucosylation when maltose was used as its substrate.[7)]

Oligosaccharides containing α-1,2- and α-1,3-glucosidic linkages may have practical applications. Kojioligosaccharides are not hydrolyzed by salivary amylase, artificial gastric juice, pancreatic amylase, or small intestinal enzymes.[8)] Nigerooligosaccharides have low cariogenicity and may, therefore, diminish the incidence of dental caries in humans and animals.[9)] Nigerooligosaccharides may also have immunopotentiating activity by inducing an IL-12-dependent, T helper 1-like immune response.[10)] GH31 α-glucosidases have demonstrated transglucosylation activity, with GH31

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Abbreviations: GH, glycoside hydrolase; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; MES, 2-morpholinoethanesulfonic acid monohydrate; MOPS, 3-morpholinopropanesulfonic acid; CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; DS, dissolved substrate; HPLC, high performance liquid chromatography; DP, degree of polymerization; HSQC, heteronuclear single quantum correlation; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

enzymes from certain species, namely *Acremonium* spp. and *Podospora anserina*, capable of generating nigerooligosaccharides.[11)12)13)] In the study by Ma *et al.*,[7)] rAgdB was found to produce kojioligosaccharides and nigerooligosaccharides from maltose via complicated mechanisms. However, structural information for the transglucosylation products was not reported.

Nuclear magnetic resonance (NMR) provides structural information on proteins, nucleic acids, and saccharides. Moreover, NMR has been successfully employed to identify glucosidic linkage compositions in oligosaccharide mixtures generated by enzymatic reactions.[14)] However, that analysis was limited to detection of α-1,4- and α-1,6-glucosidic linkages. Thus, it remains challenging to analyze oligosaccharide mixtures containing various types of glucosidic linkages such as α-1,1-α, α-1,1-β, α-1,2, and α-1,3 using NMR.

Herein, we constructed an overexpression system for the AgdB enzyme of *A. niger,* in *A. nidulans* (hereafter simply described as AgdB) and assessed certain properties of the AgdB produced. We also developed an analytical NMR-based method for the quantitation of glucosidic linkages and evaluation of their compositions in the transglucosylation products of AgdB from maltose and maltopentaose. The corresponding transglucosylation products contained largely α-1,4- and α-1,3-glucosidic linkages as well as α-1,2- and, to a lesser degree, α-1,1-β-glucosidic linkages.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from FUJIFILM Wako Pure Chemical Industries Ltd. (Osaka, Japan), unless otherwise specified. Nigerose was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Isomaltotriose and neotrehalose were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The 3^2-*O*-α-D-glucosyl-maltose and isomaltotriosyl-glucose were prepared as previously described.[12)15)]

Microorganisms. *Aspergillus niger* NBRC4066 were obtained from National Institute of Technology and Evaluation Biological Resource Center (Chiba, Japan). *Aspergillus oryzae* RIB40 was obtained from the National Research Institute of Brewing (Hiroshima, Japan). *Aspergillus nidulans* ATCC38163 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Gene cloning and expression vector construction for α-glucosidase. The *agdB* gene (GenBank (https://www.ncbi.nlm.nih.gov/genbank/) accession no. LX063802) was cloned from *A. niger* NBRC4066 and used to construct an expression vector. Phuson Hot Start II DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) was used for polymerase chain reaction (PCR) analysis. The oligo nucleotide primers used in this study are shown in Table S1 (see the *J. Appl. Glycosci*. website). To express *agdB* under the control of the *A. oryzae tef1* gene (GeneBank accession no. Q9Y713) promoter, an expression plasmid was constructed as follows. The 0.8-kbp fragment of the *tef1* gene promoter was amplified from the genomic DNA of *A. oryzae* RIB40 using primer No. 1 and 2. The *agdB* gene lacking a stop codon was amplified from the genomic DNA of *A. niger* NBRC4066 using primer No. 3 and 4. The 0.3 kbp fragment from the terminator region of *agdB* containing 10 × His-tag and a stop codon was amplified from the genomic DNA of *A. niger* NBRC4066 using primer No. 5 and 6. The three PCR products and the *Hin*dIII/*Kpn*I-digested pPTRII (Takara Bio Inc., Shiga, Japan) were joined in a four-piece in-fusion reaction using the In-Fusion HD cloning kit (Takara Bio Inc.) and the resulting expression vector was designated pPTRII-AgdB.

Transformation. *A. nidulans* ATCC38163 was transformed with pPTRII-AgdB according to the methods described by Gomi *et al.*[16)]

Purification of recombinant α-glucosidase. To purify the recombinant *A. niger* AgdB, the transformed *A. nidulans* was aerobically cultured in 2-L Erlenmeyer flasks on a rotary shaker at 180 rpm and 37 °C for 4 d. Each flask contained 1 L of Czapek-Dox medium with 0.1 μg mL^{-1} pyrithiamine. The mycelia were separated by filtration through a miracloth (Merck, Darmstadt, Germany). One hundred gram mycelia were suspended in 1 L of 20 mM Tris-HCl buffer (pH 7.4) with 20 mM imidazole and homogenized in a Hiscotron (Nichion Inc., Tokyo, Japan) at 10,000 rpm for 2 min. The mycelia were centrifuged at 15,700 × *G* at 4 °C for 20 min. The supernatant was collected, used as the cell extract, applied to a HisTrap HP column (GE Healthcare, Chicago, IL, USA), and eluted in 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl and 500 mM imidazole. The elution buffer was replaced with 20 mM 2-morpholinoethanesulfonic acid, monohydrate (MES)-NaOH buffer (pH 6.0) using an ultrafiltration membrane and the resulting solution was used as purified enzyme throughout subsequent studies.

Enzyme assay. The α-glucosidase activity of AgdB was determined by incubating the enzymes with 20 mM maltose in 20 mM MES-NaOH buffer (pH 6.0) at 40 °C for 30 min. The reaction was terminated by adding 10 % (w/v) oxalic acid and boiling for 10 min. The glucose product was measured with the Glucose CII Test Wako (FUJIFILM Wako Pure Chemical Industries Ltd.). One unit of α-glucosidase activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol maltose min^{-1}. The effect of temperature on α-glucosidase activity was investigated under the standard conditions with adjusted reaction temperatures of 40–80 °C. The effect of pH on α-glucosidase activity was investigated under the standard conditions with the following buffers; phthalic acid-HCl (pH 3.5), phthalic acid-NaOH (pH 4.0–5.5), MES-NaOH (pH 6.0–7.0), 3-morpholinopropanesulfonic acid (MOPS)-NaOH (pH 7.5–10.0), *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS)-NaOH (pH 11.0–12.0). The thermal and pH stability of AgdB were determined from its residual activity following incubation at various temperatures (40–80 °C) for 1 h and at various pH (3.5–12.0) at 4 °C for 24 h. Phthalic acid-NaOH buffer was used to reach pH 4.0–5.5, MES-NaOH buffer was used for pH 6.1–7.1 and MOPS-NaOH buffer was used for pH 7.6–10.1. For determination of kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m), the initial rates were measured at a minimum of five concentrations per substrate, which were then fit to the Michaelis-Menten equation

using KaleidaGraph version 4.5.3 (Symless, Camberley, UK). AgdB (42 pmol/mL) was incubated with different substrates in 20 mM MES-NaOH buffer (pH 6.0) at 40 °C for 60 min. The reaction was terminated by adding 10 % (w/v) oxalic acid and boiling for 10 min. The glucose product was measured with a Glucose CII Test Wako (FUJIFILM Wako Pure Chemical Industries Ltd.).

Physical measurements. Protein concentration was determined with a Quick Start Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The apparent molecular mass of the enzymes was determined by SDS-PAGE in a 10–15 % polyacrylamide gel gradient as described by Laemmli.[17] A BenchMark Ladder (Invitrogen, Carlsbad, CA, USA) provided the standard protein markers. The molecular mass of the native enzyme was determined by native-PAGE in an 8–25 % polyacrylamide gel gradient as described by Davis.[18] The HMW marker kit (GE Healthcare, Chicago, IL, USA) provided the standard protein markers. The p*I* for thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (66 kDa) in the protein marker mix were 4.5, 4.5, 5.4, 5.0, and 4.9, respectively. The apparent p*I* of the enzyme was determined by isoelectric focusing on a Phastgel IEF 3–9 using the Phastsystem (GE Healthcare, Chicago, IL, USA). The proteins were stained with Rapid CBB KANTO (Kanto Chemical Co. Inc., Chuo-ku, Japan).

Preparation of transglucosylation products. The transglucosylation products were synthesized using purified AgdB enzyme. For the maltose substrate, the enzyme was incubated with 30 % (w/w) maltose and 1 U g^{-1} DS (dissolved substrate) at pH 6.0 and 60 °C for 1, 3, 6, 10, 15, 24, and 48 h. For the maltopentaose substrate, the enzyme was incubated with 30 % (w/w) maltopentaose and 8 U g^{-1} DS at pH 6.0 and 50 °C for 24 h. The reactions were terminated by boiling for 10 min.

High performance liquid chromatography analysis and transglucosylation product fractionation. The transglucosylation products were analyzed and fractionated by high performance liquid chromatography (HPLC) as previously described.[19] To determine the degree of polymerization (DP), the HPLC system was run in the MCI GEL CK04S column (Mitsubishi Chemical Corp., Tokyo, Japan) with a refractive index detector (RID-10A; Shimazu Corp., Kyoto, Japan). The transglucosylation products were eluted with distilled water. For structural isomer analysis, the HPLC system was run with a Unison UK-Amino column (Imtakt, Kyoto, Japan) and the Nano quantity analyte detector (NQAD; Asahi Techneion Co., Ltd., Kyoto, Japan). The transglucosylation products were separated using a linear acetonitrile/water gradient. To fractionate the transglucosylation products, the HPLC system was run with a Bio-Gel P2 column (Bio-Rad Laboratories) and a refractive index detector (RI704P; GL Sciences Inc., Tokyo, Japan). The products were eluted with distilled water.

NMR analysis. Each sample was dissolved in D_2O and incubated at 4 °C for > 48 h to achieve equilibrium between the α- and β-anomers at the reducing end of the oligosaccharide. The NMR experiments were performed on a Bruker Advance III (Bruker BioSpin, Billerica, MA, USA). The ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) spectra were recorded at 400 MHz and 25 °C. Trimethylsilylpropanoic acid was used as the internal standard. Topspin v. 3.2 (Bruker BioSpin) was used to process and analyze the data.

MALDI-TOF MS analysis. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) mass spectra were obtained on an Autoflex Speed IC TOF/TOF (Bruker BioSpin) using 2,5-dihydroxybenzoic acid as a matrix. A 1-μL aliquot was mixed with 1 μL of 2,5-dihydroxybenzoic acid in TA30 (30:70 [v/v] acetonitrile:TFA 0.1 % in water) and dried at 20–25 °C room temperature. The molar masses were measured in positive reflectron mode.

RESULTS AND DISCUSSION

Purification and biochemical characterization of AgdB.

Amino acid sequence of AgdB was predicted by comparing gene sequences with unnamed protein product of *A. niger* CBS513.88 (GenBank accession no. CAK37273). As determined using Signal-5.0 (http://www.cbs.dtu.dk/services/SignalP/), the predicted amino acid sequence contains 865 amino acids with a 23 amino acid signal peptide. The sequence was 100 % identical to rAgdB and differed by one amino acid (Thr676→Ser676) from GenBank accession no. CAK37273. The difference in the amino acid was the same as that for rAgdB.[7] The sequence of AgdB exhibited 35 % homology with *A. niger* AgdA (GenBank accession no. CAK44692, hereafter simply described as AgdA).

Although rAgdB expressed in *P. pastoris* was previously collected from culture supernatants,[7] within our study enzyme activity was not detected in the supernatant but rather in cell extracts. Similarly, a previous study reported that *A. nidulans* AgdB appeared to be an extracellular enzyme present in the cell wall.[20] It was indicated that AgdB has also been described as extracellular enzyme present in the cell wall when expressed in *A. nidulans*.

Recombinant AgdB was purified by His-Trap affinity chromatography. AgdB purified on SDS-PAGE consisted of two polypeptide chains with apparent molecular masses of 64 and 77 kDa, respectively (Fig. 1A). Although the gel signal was smeared during the isoelectric focusing, the p*I* for AgdB was determined to approximately 5.2 (Fig. 1C). This value closely approaches the molecular mass standards for native-PAGE. Similarly, the p*I* calculated by the ExPASy ProtParam tool (https://web.expasy.org/protparam/) from the amino acid sequence was 5.0. Native-PAGE analysis of AgdB showed a single polypeptide chain, 124 kDa in size (Fig. 1B). Thus, AgdB is a heterodimeric protein of 64 and 77 kDa that has its single polypeptide chain cleaved during heterologous expression in *A. nidulans*. In ExPASy ProtParam tool, molecular masses of AgdB and AgdB without signal peptide are predicted to be 97 and 95 kDa, respectively, indicating that AgdB is glycosylated. Similarly, *P. anserina* α-glucosidases PAG and *A. nidulans* AgdB underwent proteolysis and separated into two components during SDS-PAGE.[13][20] During native-PAGE analysis, rAgdB was found to contain two segments, while

Table 1. Kinetic parameters of *A. niger* AgdB and *A. niger* AgdA for hydrolysis of various substrates.

Substrates	***A. niger*** AgdB			***A. niger*** AgdA[a]		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	(mM)	(s^{-1})	(mM^{-1} s^{-1})	(mM)	(s^{-1})	(mM^{-1} s^{-1})
Maltose (Glcp-α-1,4-Glcp)	4.3±1.0	46.5±5.9	10.8	3.2±0.8	52.3±6.1	16.5
Kojibiose (Glcp-α-1,2-Glcp)	3.7±0.5	34.7±1.9	9.3	5.2±0.1	16.8±0.1	3.2
Nigerose (Glcp-α-1,3-Glcp)	4.6±0.6	45.6±2.0	9.9	13.0±0.3	34.0±0.4	2.6
Isomaltose (Glcp-α-1,6-Glcp)	2.1±0.9	2.7±0.3	1.3	5.0±0.1	20.6±0.2	4.1
Maltotriose (Glcp-α-1,4-Glcp-α-1,4-Glcp)	4.0±0.8	98.5±12.1	24.4	2.0±0.4	62.8±5.8	31.7
Maltotetraose (Glcp-(-α-1,4-Glcp-)$_2$-α-1,4-Glcp)	2.5±0.6	85.9±11.9	34.7	1.2±0.2	47.4±3.3	39.4
Maltopentaose (Glcp-(-α-1,4-Glcp-)$_3$-α-1,4-Glcp)	2.5±0.5	96.1±12.9	38.4	1.1±0.1	40.4±1.3	36.3
Maltohexaose (Glcp-(-α-1,4-Glcp-)$_4$-α-1,4-Glcp)	1.5±0.3	71.3±8.8	47.3	1.1±0.1	43.1±2.0	38.7
Soluble starch[b] (Glcp-(-α-1,4-Glcp-)$_n$-α-1,4-Glcp)	0.7±0.1	47.4±3.1	67.7	3.0±0.5	184.3±16.5	62.0

K_m and k_{cat} are shown with standard errors (SE). [a]Kinetic parameter of *A. niger* AgdA cited from a previous report.[19] [b]Weighted average molecular weight of soluble starch was 8,778 determined by gel permeation chromatography.

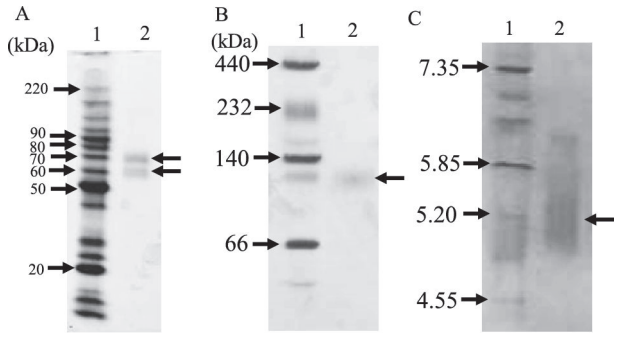

Fig. 1. Molecular mass and isoelectric point of purified AgdB.
(A) SDS-PAGE of BenchMark Ladder (lane 1) and purified AgdB (lane 2). (B) Native-PAGE of HMW marker kit (lane 1) and purified AgdB (lane 2). (C) Isoelectric focusing of Broad p*I* kit (lane 1) and purified AgdB (lane 2).

SDS-PAGE detected three (I-Nt, II-Nt, and I- and II-Ct) . These three were subsequently converted into two, following Endo H treatment (I- and II-Nt; and I- and II-Ct).[7] It is suggested that the 74 kDa polypeptide of AgdB corresponds to I- and II-Nt of rAgdB, the 64 kDa polypeptide corresponds to I- and II-Ct, and differences in glycosylation between the two host strains, *A. nidulans* and *P. pastoris,* caused the difference in the number of components observed with SDS-PAGE.

Next, we examined the effects of pH and temperature on AgdB activity and stability (Fig. S1; see the *J. Appl. Glycosci*. website). AgdB had an optimum pH range of 6.0–7.0, with less than 20 % of its activity lost between pH 6.5 and 7.5. Further, its optimum temperature was determined to be 65 °C with less than 20 % of its activity lost after incubation 60 °C for 1 h, whereas 95 % activity was lost at 75 °C after incubation for 1 h. Alternatively, for rAgdB[7] the optimum pH was neutral, with a narrow stable pH range. These differences between AgdB and rAgdB may have been caused by the different expression systems.

Kinetic analysis of AgdB.

To elucidate the enzymatic properties of AgdB, its kinetic parameters were determined for various saccharides. The results of these assays were compared with those previously reported for AgdA.[19] The activities of AgdB and AgdA were determined based on the relative amounts of glucose released from their substrates. No trehalose-hydrolyzing activity was detected. The enzymatic activities for maltooligosaccharides, kojibiose, nigerose, isomaltose, and soluble starch are shown in Table 1. At the substrate concentrations tested, the enzymatic reaction fit well with the Michaelis-Menten kinetic model (Fig. S2; see the *J. Appl. Glycosci*. website).

Both enzymes exhibited broad oligosaccharide substrate specificity yet different substrate preferences. The k_{cat}/K_m values of AgdB for maltose, kojibiose, and nigerose were approximately 10 mM^{-1} s^{-1}. In contrast, the k_{cat}/K_m for isomaltose was 1.3 mM^{-1} s^{-1}. On the other hand, AgdA preferred maltose for which the k_{cat}/K_m value was 16.5 mM^{-1} s^{-1}; yet AgdA exhibited low enzymatic activity on kojibiose, nigerose, and isomaltose (k_{cat}/K_m approximately 3 mM^{-1} s^{-1}). AgdB and AgdA were highly specific for maltooligosaccharides with the k_{cat}/K_m values for maltotriose to maltohexaose all approximately 30 mM^{-1} s^{-1}. AgdB and AgdA also acted on soluble starch. Additionally, the α-glucosidase from *Acremonium implicatum* had low k_{cat}/K_m values for kojibiose relative to those for nigerose and maltose. This enzyme produces oligosaccharides containing α-1,3-glucosidic linkages.[12] Thus, AgdB exhibited substrate specificities that differed from those of AgdA and the *A. implicatum* α-glucosidase. Within our study AgdB acted on α-1,2- and α-1,3-glucosidic linkages as previously reported by Ma *et al*.[7] However, each kinetic parameter differed from that of the Ma *et al* report. The apparent differences in the expression systems or procedures employed for enzyme purification may have been the cause of these discrepancies.

NMR signal assignment.

The enzymatic reaction of AgdB produces transglucosylation products that may contain α-1,1-, α-1,2-, α-1,3-, α-1,4-, and/or α-1,6-glucosidic linkages. Here, we employed 2D NMR spectroscopy to identify the specific glucosidic linkages in the transglucosylation products. We first estimated the theoretical values of the chemical shifts for the anomeric protons and carbon atoms expected to be in

the transglucosylation products. The chemical shifts of the anomeric protons and carbon atoms are highly dependent on the types of glucosidic linkages present at the reducing and non-reducing ends of a sugar unit. Here, a structural combination of glucose and glucosidic linkages at the reducing and non-reducing ends is considered a "sugar unit".[14] Specific examples of the sugar unit are shown in Fig. 2. Thus, the theoretical values for the chemical shifts of anomeric proton and carbon in 54 sugar units (g1–54; Table S2; also see the *J. Appl. Glycosci*. website) were estimated based on those presented in the CASPER database (http://www.casper.organ.su.se/casper/) and described by Roslund *et al*.[21] The theoretical ^{1}H-^{13}C HSQC spectra (Fig. 3A) showed that the ^{1}H- and the ^{13}C signals were located in an area delineated by the ranges 4.4–5.6 and 85–110 ppm, respectively. The ^{1}H-^{13}C signals were not uniformly distributed in the aforementioned area, but rather many occurred at 12 sites designated S1–S12. The relationships between the sugar units g1–g54 and the sites S1–S12 are listed in Table S2. Signals derived from g4, g23, g24, g25, g26, g27, g28, g29, and g30 were not expected in S1–S12. However, the ^{1}H-^{13}C signals for these sugar units were not observed in the subsequent experimental NMR measurements of ten standard saccharides or the transglucosylation products described below.

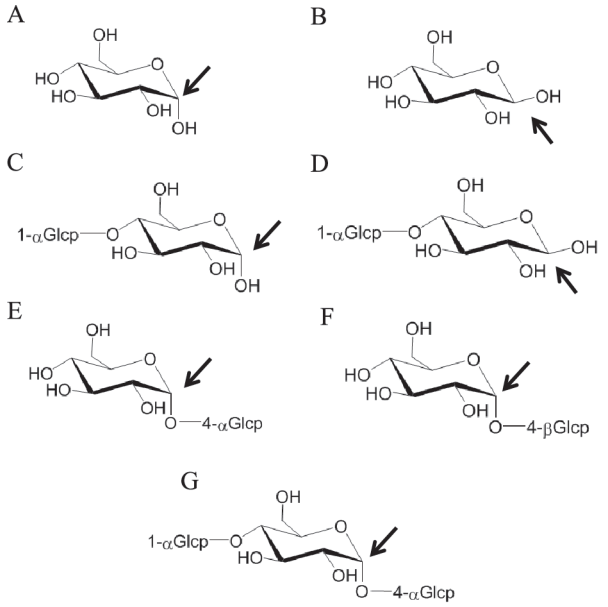

Fig. 2. Molecular structures of sugar units.

The positions of the anomeric carbons on the sugar units are indicated by arrows. (A) αGlcp, (B) βGlcp, (C) αGlcp-(1,4)-αGlcp, (D) αGlcp-(1,4)-βGlcp, (E) αGlcp-(1,4)-αGlcp, (F) αGlcp-(1,4)-βGlcp, (G) αGlcp-(1,4)-αGlcp-(1,4)-αGlcp. Underlines indicate glucopyranoside with the targeted anomeric carbon.

To determine whether the experimental NMR signals were observed at the same positions as those for the theoretical values, we measured the ^{1}H-^{13}C HSQC spectra for glucose, trehalose, neotrehalose, kojibiose, nigerose, maltose, isomaltose, maltotriose, isomaltotriose, and panose. Their detected signals (Table S3; see the *J. Appl. Glycosci*. website) were plotted on a single graph (Fig. 3B). Save for S9, the observed experimental values were nearly identical to the theoretical values. In S9, the ^{1}H-signal had a shift of approximately 0.1 ppm from the theoretical value.

NMR estimation of the sugar unit compositions.

The compositions of the sugar units were determined based on their signal intensities in the HSQC spectra as described in Supplemental Method S1 (see the *J. Appl. Glycosci*. website). The compositions of the sugar units for glucose, trehalose, neotrehalose, kojibiose, nigerose, maltose, isomaltose, maltotriose, isomaltotriose, 3^{2}-*O*-α-D-glucosyl-maltose, panose, maltotetraose, isomaltotetraose, and isomaltotriosyl-glucose were estimated using the peak areas (Tables 2 and 3). These estimated compositions were in agreement with the actual compositions of the sugar units deduced from their saccharide structures. Panose (Glcp-α-1,6-Glcp-α-1,4-Glcp) contains one α-1,6-glucosidic linkage, one α-1,4-glucosidic linkage, and one reducing end. The ratio of the α-reducing ends to the β-reducing ends is 0.36:0.64 at 25 °C.[22] Thus, the expected ratio of the peak areas of S1:S2:S11:S12 is 0.36:0.64:1:1; while the actual observed ratio was 0.12:0.22:0.31:0.36, which contains a minor error; however, nevertheless, is similar to that of

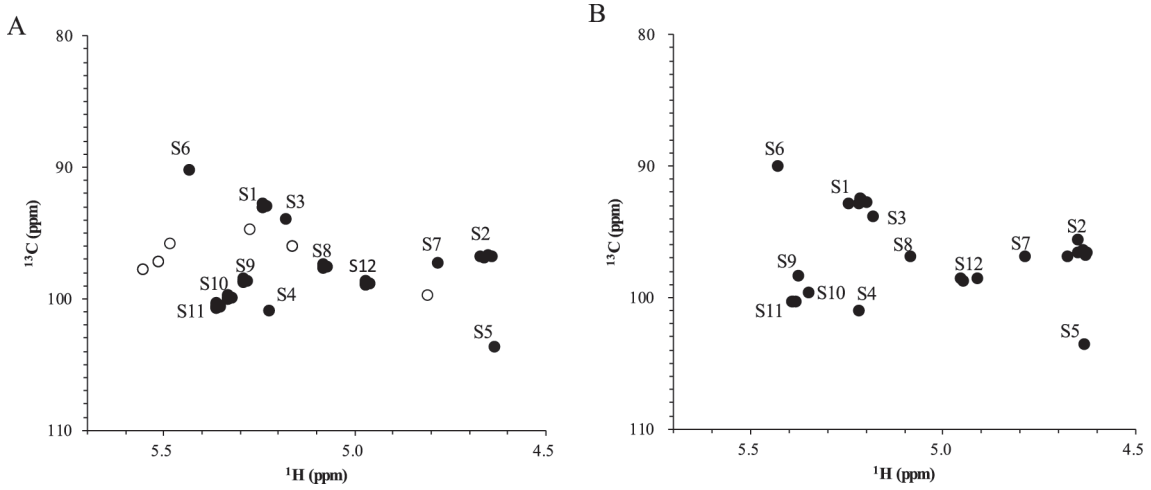

Fig. 3. (A) Simulated ^{1}H-^{13}C HSQC signal assignments of the sugar units. (B) Experimental ^{1}H-^{13}C HSQC spectra for standard substances.

Closed circle: sugar units detected in this study and used to calculate glucosidic linkage compositions. Open circle: sugar units not detected in this study.

Table 2. Compositions of sugar units of standard substances (monosaccharide and disaccharides) estimated from ^{1}H-^{13}C HSQC spectra.

	Glucose	Trehalose	Neo-trehalose	Kojibiose	Nigerose	Maltose	Iso-maltose
αGlcp, αGlcp-(X)-αGlcp	0.34	-	-	-	0.17	0.18	0.15
βGlcp, αGlcp-(X)-βGlcp	0.66	-	-	-	0.33	0.32	0.35
αGlcp-(1,2)-αGlcp	-	-	-	0.20	-	-	-
αGlcp-(1,2)-βGlcp	-	-	-	0.30	-	-	-
αGlcp-(1,1)-αGlcp	-	1.00	-	-	-	-	-
βGlcp-(1,1)-αGlcp	-	-	0.47	-	-	-	-
αGlcp-(1,1)-βGlcp	-	-	0.53	-	-	-	-
αGlcp-(1,2)-αGlcp, αGlcp-(X)-αGlcp-(1,2)-αGlcp	-	-	-	0.22	-	-	-
αGlcp-(1,2)-βGlcp, αGlcp-(X)-αGlcp-(1,2)-βGlcp	-	-	-	0.28	-	-	-
αGlcp-(1,3)-Y, αGlcp-(X)-αGlcp-(1,3)-Y	-	-	-	-	0.50	-	-
αGlcp-(1,4)-Y, αGlcp-(X)-αGlcp-(1,4)-Y	-	-	-	-	-	0.50	-
αGlcp-(1,6)-Y, αGlcp-(X)-αGlcp-(1,6)-Y	-	-	-	-	-	-	0.50

X represents 1,3, 1,4, or 1,6. Y represents αGlcp or βGlcp. Glucopyranoside for which signal assignment is derived from its anomeric protons and carbons are underlined.

Table 3. Compositions of sugar units of standard substances (trisaccharides and tetrasaccharides) estimated from ^{1}H-^{13}C HSQC spectra.

	Malto-triose	Isomalto-triose	3^{2}-*O*-α-D-glucosyl-maltose	Panose	Malto-tetraose	Isomalto-tetraose	Isomalto-triosyl-glucose
αGlcp, αGlcp-(X)-αGlcp	0.12	0.11	0.12	0.12	0.09	0.08	0.09
βGlcp, αGlcp-(X)-βGlcp	0.21	0.22	0.21	0.22	0.16	0.17	0.16
αGlcp-(1,2)-αGlcp	-	-	-	-	-	-	-
αGlcp-(1,2)-βGlcp	-	-	-	-	-	-	-
αGlcp-(1,1)-αGlcp	-	-	-	-	-	-	-
βGlcp-(1,1)-αGlcp	-	-	-	-	-	-	-
αGlcp-(1,1)-βGlcp	-	-	-	-	-	-	-
αGlcp-(1,2)-αGlcp, αGlcp-(X)-αGlcp-(1,2)-αGlcp	-	-	-	-	-	-	-
αGlcp-(1,2)-βGlcp, αGlcp-(X)-αGlcp-(1,2)-βGlcp	-	-	-	-	-	-	-
αGlcp-(1,3)-Y, αGlcp-(X)-αGlcp-(1,3)-Y	-	-	0.34	-	-	-	-
αGlcp-(1,4)-Y, αGlcp-(X)-αGlcp-(1,4)-Y	0.67	-	0.33	0.31	0.75	-	0.25
αGlcp-(1,6)-Y, αGlcp-(X)-αGlcp-(1,6)-Y	-	0.67	-	0.36	-	0.75	0.50

X means 1,3, 1,4, or 1,6. Y means αGlcp or βGlcp. Glucopyranosides for which signal assignment is derived from its anomeric protons and carbons are underlined.

the expected ratio. Moreover, the observed and expected ratios of the peak areas were similar for all saccharides tested (Tables 2 and 3). The sugar unit ratios were, therefore, measured using NMR. Results show that no α-1,1-α-glucosidic linkages were detected in the transglucosylation product analysis. We constructed a formula to determine the compositions of the α-1,1-β-, α-1,2-, α-1,3-, α-1,4-, and α-1,6-glucosidic linkages (Supplemental Method S2; see the *J. Appl. Glycosci*. website).

Transglucosylation by AgdB with maltose as substrate.

AgdB was incubated with 30 % (w/w) maltose and its product generation time course was monitored by HPLC using the MCI GEL CK04S column (Mitsubishi Chemical Corp., Tokyo, Japan). In the early stage of the reaction (0–15 h; Fig. 4), the disaccharides (DP 2) (primarily maltose) decreased rapidly, whereas the monosaccharides (DP 1), trisaccharides (DP 3), tetrasaccharides (DP 4), and oligosaccharides larger than tetrasaccharide (DP ≥ 5) were found to accumulate. By the late stage of the reaction (15–48 h; Fig. 4), the DP composition was virtually unchanged. Further, DP 3 accumulated most rapidly during the early stage of the reaction; while DP 1, DP 4, and a small amount of DP ≥ 5 also accumulated during this stage. Alternatively, within the late stage of the reaction, DP 3 gradually decreased, while DP 1 gradually accumulated possibly as the result of AgdB hydrolytic activity.

Next, the DP 2 fraction was analyzed with the Unison UK-Amino column (Imtakt, Kyoto, Japan), as shown in Fig. 5. The DP 3, DP 4, and DP ≥ 5 fractions were analyzed from HSQC spectra (Fig. 6). The compositions of the glucosidic linkages in each DP fraction are shown in Fig. 7. The values were defined as follows: if same amount of nigerose (DP 2, Glcp-α-1,3-Glcp), maltose (DP 2, Glcp-α-1,4-Glcp), maltotriose (DP 3, Glcp-α-1,4-Glcp-α-1,4-Glcp), and panose (DP 3, Glcp-α-1,6-Glcp-α-1,4-Glcp) were present, the result of this analysis would be described as α-1,3-glucosidic linkage and DP 2 = 25 % (half the glucosidic linkages in DP 2), α-1,4-glucosidic linkage and DP 2 = 25 % (half the glucosidic linkages in

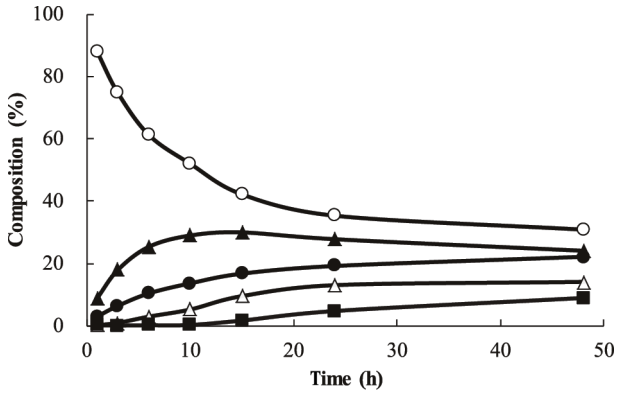

Fig. 4. Time courses for DP compositions of transglucosylation products of AgdB using maltose as substrate.
Closed circle, DP 1; open circle, DP 2; closed triangle, DP 3; open triangle, DP 4; closed square, DP ≥ 5.

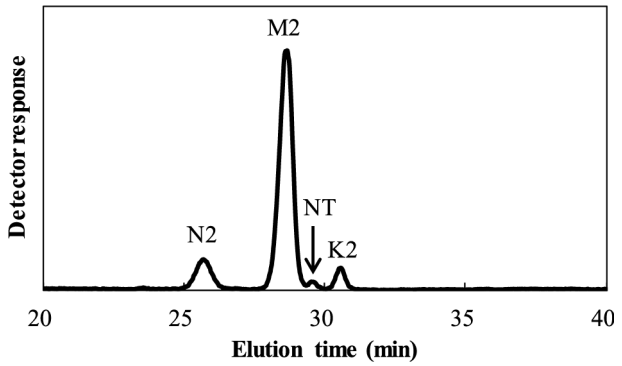

Fig. 5. HPLC chromatogram (Unison UK-Amino column) of transglucosylation products of AgdB using maltose as substrate at 24 h reaction time.
N2, nigerose; M2, maltose; NT, Neotrehalose: K2, kojibiose.

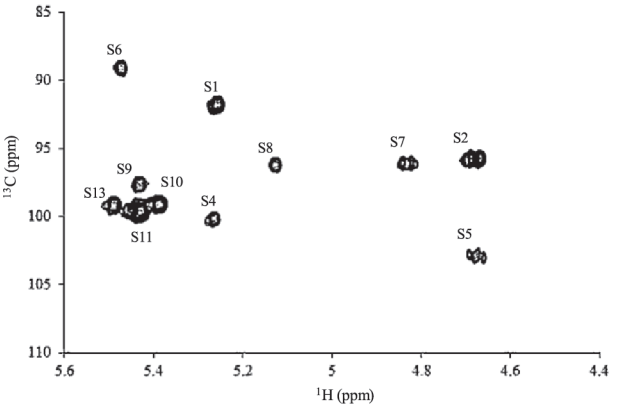

Fig. 6. ^{1}H-^{13}C HSQC spectrum of DP 3 fraction of transglucosylation products of AgdB using maltose as substrate at 24 h reaction time.

DP 2), α-1,4-glucosidic linkage and DP 3 = 37.5 % (3/4 of the glucosidic linkages in DP 3) as well as α-1,6-glucosidic linkage and DP 3 = 12.5 % (1/4 of the glucosidic linkages in DP 3). The total glucosidic linkages = 100 %.

Analysis of DP 2 (Figs. 7A and B) showed that the α-1,4-glucosidic linkages decreased throughout the reaction whereas the α-1,2- and α-1,3-glucosidic linkages, together with small amounts of the α-1,1-β-glucosidic linkage

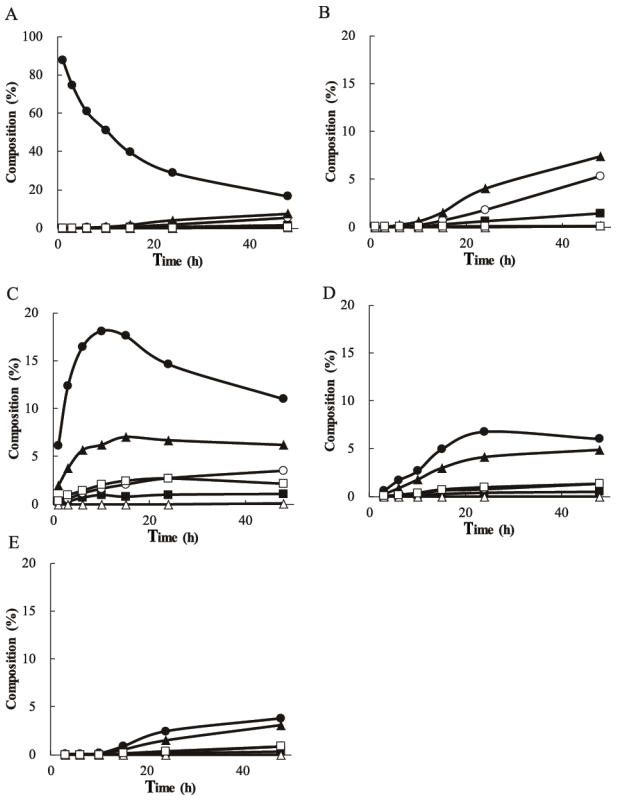

Fig. 7. Time courses for all DPs of linkage compositions in transglucosylation products of AgdB using maltose as substrate.
Closed circle, α-1,4-glucosidic linkage; open circle, α-1,2-glucosidic linkage; closed triangle, α-1,3-glucosidic linkage; open triangle, α-1,6-glucosidic linkage; closed square, α-1,1-β-glucosidic linkage; open square, unknown signal. (A) DP 2, (B) DP 2 except for α-1,4-glucosidic linkage, (C) DP 3, (D) DP 4, (E) DP ≥ 5.

appeared in the late stage of the reaction. In DP 3, the α-1,4-glucosidic linkages accumulated in the early stage and decreased after 10 h; whereas the α-1,3-glucosidic linkages, and small amounts of the α-1,2- and α-1,1-β-glucosidic linkages, appeared in the late stage of the reaction. In DP 4 and DP ≥ 5, the α-1,4- and α-1,3-glucosidic linkages predominated with small amounts of the α-1,2- and α-1,1-β-glucosidic linkages appearing in the late stage of the reaction. Hence, AgdB catalyzes the formation of α-1,4- and α-1,3-glucosidic linkages as major products and α-1,2- and α-1,1-β-glucosidic linkages as minor products. In DP 3, during the early stage of reaction, α-1,4-glucosidic linkages accounted for more than the sum of all other linkages. Hence, if AgdB had not synthesized α-1,4-glucosidic linkages their number would be lower than the sum of all other glucosidic linkages. It was, therefore, concluded that AgdB produced α-1,4-glucosidic linkages in addition to α-1,3- and small quantities of α-1,2-glucosidic linkages. However, saccharides containing α-1,4-glucosidic linkages may serve as glucosyl donors. As a result, the ratio of α-1,4-glucosidic linkages decreased during the latter stage of the reaction. In DP 4 and DP ≥ 5, α-1,4-, α-1,3-, the small amounts of α-1,2-glucosidic linkages that appeared in the later stage of the reaction support the hypothesis that AgdB produced α-1,4-, α-1,3-, as well as small amounts of α-1,2-glucosidic linkages. Additionally, small quantities of α-1,1-β-glucosidic linkages were detected throughout the reaction. This

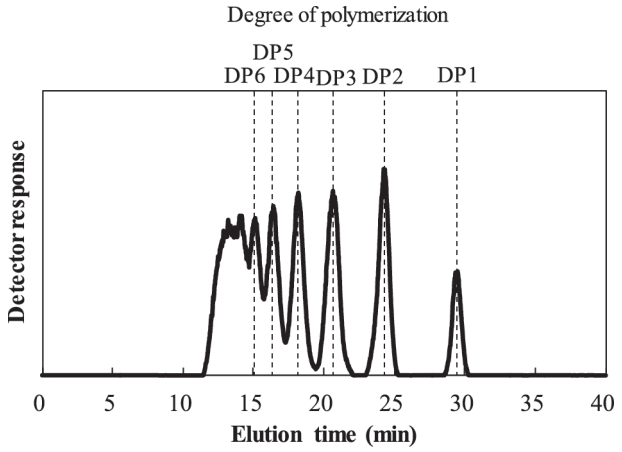

Fig. 8. HPLC chromatogram (MCI GEL CK04S column) of transglucosylation products of AgdB at 24 h reaction time using maltopentaose as substrate.

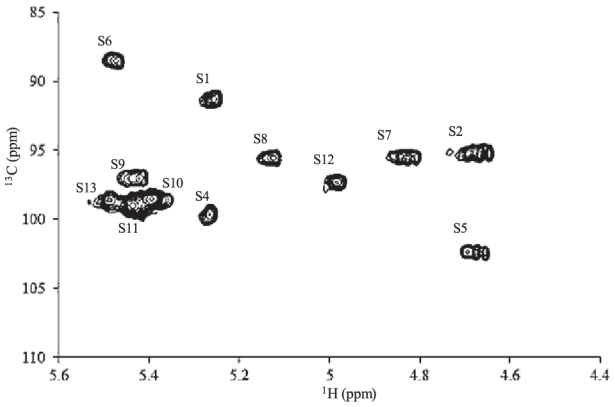

Fig. 9. ^{1}H-^{13}C HSQC spectrum of transglucosylation product of AgdB using maltopentaose as substrate at 24 h reaction time.

was similarly observed following reactions with A GH31 α-glucosidase from *Bacillus* sp,[23] and, to date, no other fungal α-glucosidases have been reported to produce this type of linkage.

Transglucosylation by AgdB with maltopentaose as substrate.

In the DP analysis of the transglucosylation products produced by the reaction of AgdB with maltopentaose, oligosaccharides were detected that had higher molecular weights than that of the original substrate. The HPLC chart at 24 h is shown in Fig. 8. MS analyses were performed to estimate the DPs for the peaks. Those at retention times 29, 24, 21, 18, 16, and 15 min were identified as DP 1, DP 2, DP 3, DP 4, DP 5, and DP 6, respectively. Moreover, the molecular weights of 39 % of the transglucosylation products were larger than DP 5; while DP 16 accounted for the maximum molecular weight of the transglucosylation products according to the MALDI-TOF MS analysis (Fig. S3; see the *J. Appl. Glycosci*. website). In the HSQC analysis, the transglucosylation products consisted of 14 % α-1,2-glucosidic linkages, 39 % α-1,3-glucosidic linkages, 30 % α-1,4-glucosidic linkages, 5 % α-1,6-glucosidic linkages, 5 % α-1,1-β-glucosidic linkages, and 7 % unknown signal assignments (Fig. 9). Unlike the transglucosylation products derived from the maltose substrate, here AgdB was found to produce small amounts of α-1,6-glucosidic linkages. Thus, AgdB can also produce α-1,6-glucosidic linkages depending on the substrate chain length.

In the present study, we devised an analytical method to identify oligosaccharide glucosidic linkages by NMR. We found that α-1,2-, α-1,3-, and small amount of α-1,1-β-linked oligosaccharides are present throughout the transglucosylation products of AgdB from maltose and maltopentaose. Similarly, Ma *et al*.[7] detected four oligosaccharides consisting of α-1,2- and α-1,3-glucosidic linkages, namely, kojibiose, nigerose, 3^{2}-*O*-α-D-glucosyl-maltose, and centose in the transglucosylation products from maltose. However, these products constituted a portion of the observed transglucosylation products of rAgdB. The k_{cat}/K_m values for maltose, kojibiose, and nigerose were higher than those for isomaltose in AgdB reactions. Moreover, AgdB transglucosylation products contained a higher number of α-1,4-, α-1,2- and α-1,3-glucosidic linkages compared to α-1,6-glucosidic linkages. These results suggest that there may be a relationship between hydrolytic specificity and transglucosylation activity; however, further investigation is required to confirm this.

Oligosaccharides produced by α-glucosidase from *Acremonium* spp. and containing α-1,3-glucosidic linkages are manufactured at industrial scales and are commercially available. However, those including either α-1,2- or both α-1,2- and α-1,3-glucosidic linkages are not yet available on this same scale. *Aspergillus* spp. have often been used as industrial enzyme sources with the α-glucosidases from *A. niger*, *A. sojae*, *A. nidulans*, and *A. oryzae* demonstrating the capacity to catalyze transglucosylation resulting in products with primarily α-1,6-glucosidic linkages.[5)19)20)24)] Alternatively, AgdB can produce both α-1,2- and α-1,3-linked oligosaccharides and may be capable of doing so on an industrial scale. However, the physiological functions of the oligosaccharides produced by AgdB have not yet been characterized, and thus require further investigation.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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