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Note

3-*O*-α-D-Glucopyranosyl-L-rhamnose phosphorylase from *Clostridium phytofermentans*

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ABSTRACT

We found an unreported activity of phosphorylase catalyzed by a protein (Cphy1019) belonging to glycoside hydrolase family 65 (GH65) from *Clostridium phytofermentans*. The recombinant Cphy1019 produced in *Escherichia coli* did not phosphorolyze α-linked glucobioses, such as trehalose (α1–α1), kojibiose (α1–2), nigerose (α1–3), and maltose (α1–4), which are typical substrates for GH65 enzymes. In reverse phosphorolysis, Cphy1019 utilized only L-rhamnose as the acceptor among various sugars examined with β-D-glucose 1-phosphate as the donor. The reaction product was determined to be 3-*O*-α-D-glucopyranosyl-L-rhamnose, indicating strict α1-3 regioselectivity. We propose 3-*O*-α-D-glucopyranosyl-L-rhamnose: phosphate β-D-glucosyltransferase as the systematic name and 3-*O*-α-D-glucopyranosyl-L-rhamnose phosphorylase as the short name for this novel GH65 phosphorylase.

Phosphorylases are exolytic enzymes catalyzing phosphorolysis of particular glycosides to produce monosaccharide 1-phosphates with strict substrate specificity. The reaction is reversible, enabling the practical synthesis of oligosaccharides from a donor monosaccharide 1-phosphate and any of the several carbohydrate acceptors, with strict regioselectivity.[1,2] Combining two phosphorylases can often result in the practical preparation of oligosaccharides from abundantly available natural sugars.[2–7] However, there is little variation among phosphorylases, with only 17 known activities.[2,8–10] This limits their utilization for the production of oligosaccharides. Therefore, it would be beneficial to identify phosphorylases with previously unreported substrate specificities. Screening for new phosphorylases from natural organisms is difficult because of the lack of effective screening methods. Two strategies are often used to enhance the variability of phosphorylases: protein engineering to change the specificity of a known phosphorylase[11–13] and searching for new phosphorylases based on genomic information. Based on their amino acid sequences, phosphorylases are classified as members of glycoside hydrolase families (GHs) 13, 65, 94, 112, or 130 (Henrissat, B. personal communication) or glycosyl transferase families 4 or 35 on Carbohydrate Active Enzymes database (http://www.cazy.org/).[14] Among these families, GH65, GH94, and GH112 are primarily comprised of phosphorylases.

Based on its genomic sequence (GenBank ID: CP000885), we observed that *Clostridium phytofermentans* possesses a variety of phosphorylase homologs belonging to GH65, GH94, and GH112. We characterized the three GH112 proteins from *C. phytofermentans* and found that two of them were 3-*O*-β-D-galactopyranosyl-*N*-acetyl-D-hexosamine phosphorylases (EC 2.4.1.211, Cphy0577 and Cphy3030) and that the third was a 4-*O*-β-D-galactopyranosyl-L-rhamnose phosphorylase (EC 2.4.1.247, Cphy1920), which had not been previously reported.[8] We also reported that one of the four GH65 homologs (Cphy1874) was a novel phosphorylase, 3-*O*-α-D-glucopyranosyl-D-glucose phosphorylase (nigerose phosphorylase).[10] In this study, we characterized Cphy1019, another *C. phytofermentans* GH65 homolog, with unique substrate specificity.

The amino acid sequence deduced from *cphy1019* (756 amino acids; 87,990 Da) shows no predicted N-terminal signal peptide based on a SignalP 3.0 analysis (http://www.cbs.dtu.dk/services/SignalP/),[15,16] suggesting that this protein is located in the cytoplasm. Although the amino acid sequence of Cphy1019 exhibits 24–29% identity with α-glucosyl disaccharide phosphorylases belonging to GH65, such as trehalose,[17] kojibiose,[18] nigerose,[10] maltose,[19] and trehalose 6-phosphate phosphorylases,[20] this protein could not be categorized into any of the known GH65 enzymes based on phylogenetic tree analysis (Fig. 1). Therefore, recombinant Cphy1019 with additional His_6 tag sequence at the C terminal was produced in *Escherichia coli* BL21 (DE3) to investigate its enzymatic properties. Approximately 3 mg of purified protein was obtained from a 200-mL culture. The purified Cphy1019 showed a single band in SDS–PAGE with an estimated size of 88 kDa. Cphy1019 did not

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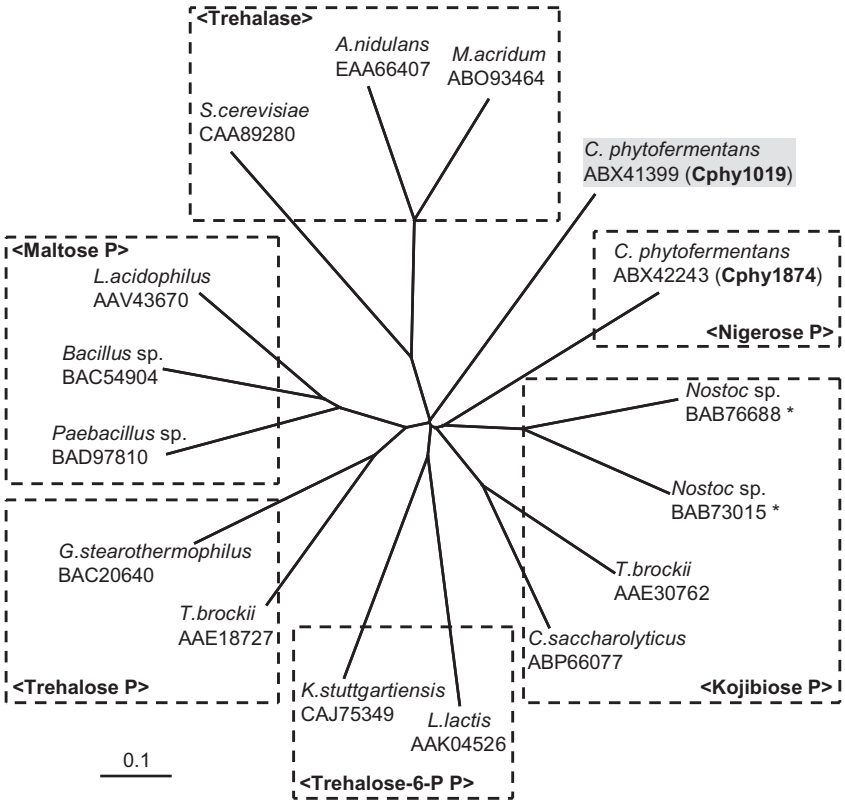

Figure 1. Phylogenetic tree of characterized GH65 enzymes. Multiple alignments were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed using TreeView version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The gene cloned in this study is represented by the organism name and GenBank™ accession number with a gray background. The other genes encoding characterized GH65 enzymes (http://www.cazy.org), represented by the organism names and GenBank™ accession numbers, are categorized in boxes framed with broken lines according to their substrate specificities. *Characterization of these proteins were presented by Watanabe et al. and Yamamoto et al. at the annual meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry on March 28, 2010. The abstracts are available on page 23 of the meeting abstracts under paper numbers 2AFa14 and 2AFa15 (in Japanese).

Table 1
NMR analysis of the product

Sugar ring	Position	α-Anomer (62%)				β-Anomer (38%)			
		^{13}C NMR	^{1}H NMR			^{13}C NMR	^{1}H NMR		
		δ (ppm)	δ (ppm)	Pattern	J (Hz)	δ (ppm)	δ (ppm)	Pattern	J (Hz)
I (L-rhamnose)	1	95.31	5.14	d, 1H	$J_{1,2}$ = 1.2	95.14	4.85	d, 1H	$J_{1,2}$ = 0.7
	2	69.52	4.11	dd, 1H	$J_{2,3}$ = 3.1	69.16	4.13	dd, 1H	$J_{2,3}$ = 3.1
	3	**77.08***	**3.85***	dd, 1H	$J_{3,4}$ = 9.7	**79.22***	**3.68***	dd, 1H	$J_{3,4}$ = 9.2
	4	72.11	3.53	t, 1H	$J_{4,5}$ = 9.7	71.79	3.46	dd, 1H	$J_{4,5}$ = 10.1
	5	70.11	3.89	dq, 1H	$J_{5,6}$ = 5.7	73.72	3.43	dq, 1H	$J_{5,6}$ = 5.8
	6	18.58	1.28	d, 3H		18.55	1.30	d, 3H	
II (D-glucose)	1	97.28	5.07	d, 1H	$J_{1,2}$ = 3.8	96.99	5.09	d, 1H	$J_{1,2}$ = 3.8
	2	73.08	3.57	dd, 1H	$J_{2,3}$ = 9.9	73.08	3.57	dd, 1H	$J_{2,3}$ = 9.9
	3	74.60	3.78	m	$J_{3,4}$ = 9.2	74.60	3.78	m	$J_{3,4}$ = 9.2
	4	71.00	3.46	dd, 1H	$J_{4,5}$ = 9.9	71.00	3.46	dd, 1H	$J_{4,5}$ = 9.9
	5	73.36	3.96	m, 1H		73.36	3.96	m, 1H	
	6	61.89	3.78	m, 2H		61.89	3.78	m, 2H	

NMR spectra are shown in Figures S1–S5. The structure of the compound is shown in Figure 2.
* Interring HMBC cross peaks were observed with the anomeric position of glucose.

phosphorolyze the α-linked glucobioses, such as trehalose (α1–α1), kojibiose (α1–2), nigerose (α1–3), and maltose (α1–4), which are typical substrates for GH65 enzymes. In reverse phosphorolysis using various carbohydrate acceptors (see Section 1.3) in the presence of β-D-glucose 1-phosphate as the donor, Cphy1019 showed activity only with L-rhamnose as the acceptor. The disaccharide product was isolated and identified as 3-*O*-α-D-glucopyranosyl-L-rhamnose based on ^{1}H and ^{13}C NMR spectroscopic analyses (Table 1, Figs. S1–S5). Cphy1019 did not utilize any of the disaccharides examined as acceptors in the reverse phosphorolysis. These results indicate that Cphy1019 is a novel phosphorylase showing strict acceptor specificity for L-rhamnose with strict α-1,3-regioselectivity. We propose 3-*O*-α-D-glucopyranosyl-L-rhamnose: phosphate β-D-glucosyltransferase (3-*O*-α-D-glucopyranosyl-L-rhamnose phosphorylase) as the systematic name for Cphy1019 and suggest that a new EC number is required for this phosphorylase (Fig. 2).

Cphy1019 was stable up to 30 °C for 10 min, and the optimum temperature for the reverse phosphorolysis was 30 °C (Fig. S6). This corresponds to the optimum growth temperature of mesophilic *C. phytofermentans*.[21] Cphy1019 was stable in the range of pH 6.0–9.0, and the optimum pH for the reverse phosphorolysis was pH 6.5 (Fig. S6). These temperature and pH profiles suggest

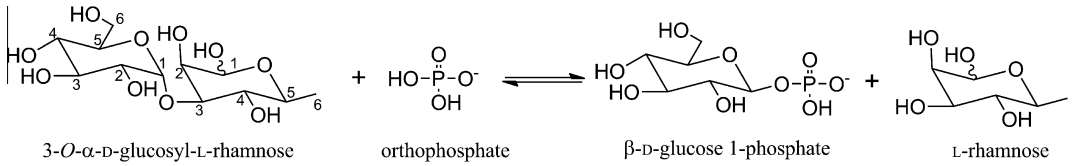

Figure 2. Schematic representation of the reactions catalyzed by 3-*O*-α-D-glucosyl-L-rhamnose phosphorylase.

that Cphy1019 can function in the cytosol of *C. phytofermentans*, as predicted from the amino acid sequence analysis.

Kinetic parameters for Cphy1019 with L-rhamnose as the acceptor and β-D-glucose 1-phosphate as the donor were determined with 10 mM of the other substrates by fitting the data to the Michaelis–Menten equation (Fig. S7). The apparent K_m and k_{cat} values for L-rhamnose were 2.7 (±0.2) mM and 1.6 (±0.1) s^{-1}, respectively, and those for β-D-glucose 1-phosphate were 9.6 (±1.9) mM and 2.5 (±0.3) s^{-1}, respectively. The K_m value for L-rhamnose is in the same range as that of other disaccharide-specific phosphorylases for their specific acceptors. Although the k_{cat} values of Cphy1019 are much smaller than that of nigerose phosphorylase from the same organism,[10] they are in the range of k_{cat} values reported for some phosphorylases.[22,23]

Because phosphorylases have very strict substrate specificities, the substrates are presumed to play important roles in organisms possessing these phosphorylases. Although *C. phytofermentans* was isolated from forest soil, no possible source of 3-*O*-α-D-glucopyranosyl-L-rhamnose was found among plant saccharides. A search of the bacterial carbohydrate structural database (http://www.glyco.a-c.ru/bcsdb3/)[24] found 154 compounds among 9071 registered structures. Most of these were in repeating units of lipopolysaccharides or capsule polysaccharides. Although no structures of polysaccharides produced by *C. phytofermentans* have been identified, the enzyme may be involved in salvaging such polysaccharides. In conclusion, we have identified a novel *C. phytofermentans* 3-*O*-α-D-glucopyranosyl-L-rhamnose phosphorylase, which is a suitable catalyst for the efficient synthesis of 3-*O*-α-D-glucosyl-L-rhamnose with strict regioselectivity.

1. Experimental

1.1. Construction of expression plasmid

A gene (*cphy1019*; GenBank ID: ABX41399.1) encoding a GH65 homolog (Cphy1019) was amplified by PCR with genomic DNA of *C. phytofermentans* ATCC700394 as the template. KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was used with the following oligonucleotides based on the genomic sequence (GenBank ID: CP000885): 5′-gatatacatatgttaattcatgaagataat-3′ as the forward primer containing an *Nde*I site (underlined) and 5′-ggtgctcgaggaaggatacctccaatctgt-3′ as the reverse primer containing a *Xho*I site (underlined). The amplified *cphy1019* was purified with a MinElute Cleanup Kit (Qiagen, Hilden, Germany) and inserted into pET24a (Novagen, Madison, WI, USA). The expression plasmid *cphy1019*/pET24a was propagated in *E. coli* XL1 Blue (Agilent Technologies, Waldbronn, Germany), purified by illustra plasmidPrep Mini Spin Kit (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK), and verified by sequencing (ABI 3730xl sequencer, Applied Biosystems, Foster City, CA, USA).

1.2. Recombinant Cphy1019 preparation

An *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) transformant harboring *cphy1019*/pET24a was grown at 25 °C in 200 mL Luria–Bertani medium containing 33 μg/mL of kanamycin. Expression was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) when Abs_{600} reached 0.6 and continued for 6 h. Cells were harvested by centrifugation at 4000*g* for 5 min and suspended in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH (pH 7.5) containing 500 mM NaCl (buffer A). The suspended cells were disrupted by sonication, and the supernatant collected by centrifugation at 10,000*g* for 20 min was applied to an immobilized metal affinity chromatography column equilibrated with buffer A containing 5 mM imidazole using a Profinia protein purification system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After washing the column with buffer A containing 10 mM imidazole, Cphy1019 was eluted with 50 mM MOPS–NaOH buffer (pH 7.5) containing 500 mM NaCl and 500 mM imidazole, and desalted with 10 mM MOPS–NaOH (pH 7.5) containing 100 mM NaCl using a Bio-Gel P-6 column (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of $\varepsilon = 131{,}560\ cm^{-1}\ M^{-1}$.

1.3. Phosphorolysis and reverse phosphorolysis

Phosphorolysis of α-linked glucobioses (trehalose, kojibiose, nigerose, maltose, and isomaltose) was examined at 30 °C in 25 mM MOPS–NaOH (pH 6.5), 10 mM phosphate, 10 mM substrates, and 1.5 μM Cphy1019 by detection of the released glucose as described previously.[10]

The reverse phosphorolysis was performed with Cphy1019 (1.5 μM) in 25 mM MOPS–NaOH (pH 6.5) containing 10 mM β-D-glucose 1-phosphate as the donor and 10 mM putative carbohydrate acceptors [L-rhamnose, D-glucose, D-mannose, D-allose, D-galactose, D-xylose, L-arabinose, D-lyxose, L-fucose, D-fructose, 2-deoxy-D-arabino-hexopyranose (2-deoxy-D-glucose), D-glucal, D-glucosamine, α-D-glucose 1-phosphate, β-D-glucose 1-phosphate, D-glucose 6-phosphate, D-glucuronic acid, methyl α-D-glucoside, methyl β-D-glucoside, 1,5-anhydro-D-glucitol, 3-*O*-methyl-D-glucose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, trehalose, kojibiose, nigerose, maltose, isomaltose, sophorose, laminaribiose, cellobiose, gentiobiose, xylobiose, lactose, and sucrose] at 30 °C for 2 h. The reaction mixture (1 μL) was spotted on a thin layer chromatography (TLC) plate (Kieselgel 60 F_{254}; Merck, Darmstadt, Germany), and the sample was developed with a solution of acetonitrile/water (4:1, v/v). The TLC plates were briefly soaked in 5% sulfuric acid-methanol solution and heated in an oven until bands were sufficiently visible. The reverse phosphorolytic activity was determined by measuring the increase in phosphate in the reaction mixture containing 10 mM β-D-glucose 1-phosphate and 10 mM L-rhamnose in 25 mM MOPS–NaOH (pH 6.5) at 30 °C by following the method of Lowry and Lopez[25] as described previously.[10]

1.4. Temperature and pH profile

The effects of pH on the reverse phosphorolytic activity of 1.5 μM Cphy1019 was measured at 30 °C in 100 mM of the following buffers: sodium citrate (pH 3.0–5.5), 2-(*N*-morpholino)ethanesulfonic acid–NaOH (pH 5.5–7.0), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid–NaOH (pH 7.0–8.5), and glycine–NaOH (pH 8.5–10.5). The temperature dependence of 764 nM Cphy1019 was measured in 25 mM MOPS–NaOH (pH 6.5) at various temperatures for 10 min. The thermal and pH stabilities were defined as the

reverse phosphorolytic activity remaining after incubation of Cphy1019 (2.5 μM) at various temperatures for 10 min in 25 mM MOPS–NaOH (pH 6.5) and at various pH values at 30 °C for 30 min, respectively.

1.5. Kinetic analysis

The reverse phosphorolytic reactions were carried out at 30 °C with 548 nM Cphy1019 and various concentrations of L-rhamnose (1–40 mM) as the acceptor or β-D-glucose 1-phosphate (0.5–10 mM) as the donor with 10 mM of each opposite substrate in 25 mM MOPS–NaOH (pH 6.5). The kinetic parameters were calculated by curve-fitting the experimental data with the Michaelis–Menten equation $\{v = k_{cat}[E]_0 \cdot [S]/(K_m + [S])\}$ using Grafit version 7.0.2 (Erithacus Software Ltd, London, UK).

1.6. Structural analysis

The product for structural determination was generated in a reaction mixture containing 636 nM Cphy1019, 50 mM β-D-glucose 1-phosphate, and 50 mM L-rhamnose in a final volume of 500 μL of 100 mM MOPS–NaOH (pH 6.5) incubated at 30 °C for 20 h. The reaction mixture was desalted using Amberlite MB-3 (Organo, Tokyo, Japan) and separated on a Toyopearl HW-40F column (26 mm ϕ × 320 mm; Tosoh, Tokyo, Japan) equilibrated with distilled water at a flow rate of 0.5 mL/min. Fractions containing the product were collected and desalted again using Amberlite MB-3, followed by lyophilization to obtain 5.2 mg of the purified disaccharide (16 μmol, yield 64%).

The one-dimensional (^{1}H and ^{13}C) and two-dimensional [double-quantum-filtered correlation spectroscopy (DQF-COSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC)] nuclear magnetic resonance (NMR) spectra of the product were taken in D_2O with 2-methyl-2-propanol as an internal standard using a Bruker Avance 500 spectrometer (Bruker Biospin, Rheinstetten, Germany). Proton signals were assigned based on the DQF-COSY spectrum. ^{13}C signals were assigned with the HSQC spectrum, based on the assignment of proton signals. The linkage position in each disaccharide was determined by detecting inter-ring cross peaks in the HMBC spectrum.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2011.12.019.

References

1. Luley-Goedl, C.; Nidetzky, B. *Biotechnol. J.* **2010**, *5*, 1324–1338.
2. Kitaoka, M.; Hayashi, K. *Trends Glycosci. Glycotechnol.* **2002**, *14*, 35–50.
3. Ohdan, K.; Fujii, K.; Yanase, M.; Takaha, T.; Kuriki, T. *Biocatal. Biotransform.* **2006**, *24*, 77–81.
4. Suzuki, M.; Kaneda, K.; Nakai, Y.; Kitaoka, M.; Taniguchi, H. *New Biotechnol.* **2009**, *26*, 137–142.
5. Nishimoto, M.; Kitaoka, M. *Biosci. Biotechnol. Biochem.* **2007**, *71*, 2101–2104.
6. Nishimoto, M.; Kitaoka, M. *Carbohydr. Res.* **2009**, *344*, 2573–2576.
7. Nakajima, M.; Nishimoto, M.; Kitaoka, M. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 1652–1655.
8. Nakajima, M.; Nishimoto, M.; Kitaoka, M. *J. Biol. Chem.* **2009**, *284*, 19220–19227.
9. Senoura, T.; Ito, S.; Taguchi, H.; Higa, M.; Hamada, S.; Matsui, H.; Ozawa, T.; Jin, S.; Watanabe, J.; Wasaki, J.; Ito, S. *Biochem. Biophys. Res. Commun.* **2011**, *408*, 701–706.
10. Nihira, T.; Nakai, H.; Chiku, K.; Kitaoka, M. *Appl. Microbiol. Biotechnol.*, **2012**. doi:10.1007/s00253-011-3515-9.
11. De Groeve, M. R.; De Baere, M.; Hoflack, L.; Desmet, T.; Vandamme, E. J.; Soetaert, W. *Protein Eng. Des. Sel.* **2009**, *22*, 393–399.
12. Nakai, H.; Petersen, B. O.; Westphal, Y.; Dilokpimol, A.; Abou Hachem, M.; Duus, J. Ø.; Schols, H. A.; Svensson, B. *Protein Eng. Des. Sel.* **2010**, *23*, 781–787.
13. Desmet, T.; Soetaert, W. *Process Biochem.* **2012**, *47*, 11–17.
14. Cantarel, B. L.; Coutinho, P. M.; Rancurel, C.; Bernard, T.; Lombard, V.; Henrissat, B. *Nucleic Acids Res.* **2009**, *37*, D233–D238.
15. Bendtsen, J. D.; Nielsen, H.; von Heijne, G.; Brunak, S. *J. Mol. Biol.* **2004**, *340*, 783–795.
16. Nielsen, H.; Engelbrecht, J.; Brunak, S.; von Heijne, G. *Protein Eng.* **1997**, *10*, 1–6.
17. Inoue, Y.; Yasutake, N.; Oshima, Y.; Yamamoto, Y.; Tomita, T.; Miyoshi, S.; Yatake, T. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2594–2599.
18. Chaen, H.; Yamamoto, T.; Nishimoto, T.; Nakada, T.; Fukuda, S.; Sugimoto, T.; Kurimto, M.; Tsujisaka, Y. *J. Appl. Glycosci.* **1999**, *46*, 423–429.
19. Ehrmann, M. A.; Vogel, R. F. *FEMS Microbiol. Lett.* **1998**, *169*, 81–86.
20. Andersson, U.; Levander, F.; Rådström, P. *J. Biol. Chem.* **2001**, *276*, 42707–42713.
21. Warnick, T. A.; Methé, B. A.; Leschine, S. B. *Int. J. Syst. Evol. Microbiol.* **2002**, *52*, 1155–1160.
22. Nakajima, M.; Nihira, T.; Nishimoto, M.; Kitaoka, M. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 465–471.
23. Honda, Y.; Kitaoka, M.; Hayashi, K. *Biochem. J.* **2004**, *377*, 225–232.
24. Toukach, P. V. *J. Chem. Inf. Model.* **2011**, *51*, 159–170.
25. Lowry, O. H.; Lopez, J. A. *J. Biol. Chem.* **1946**, *162*, 421–428.