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Production of alkyl glucoside from cellooligosaccharides using yeast strains displaying *Aspergillus aculeatus* β-glucosidase 1

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Abstract

For efficient alkyl glucoside production from cellooligosaccharides, we constructed a yeast strain for alkyl glucoside synthesis by genetically inducing the display of β-glucosidase 1 (BGL1) from the filamentous fungus *Aspergillus aculeatus* No. F-50 on the cell surface. The localization of BGL1 on the cell surface was confirmed by immunofluorescence microscopy. The yeast strain displaying BGL1 catalyzed alkyl glucoside synthesis from *p*-nitrophenyl β-D-glucoside and primary alcohols. The highest yield of alkyl glucoside was 27.3% of the total sugar. The substrate specificities of the BGL1-displaying yeast strain and almond β-glucosidase were compared using different-chain-length cellooligosaccharides. The BGL1-displaying yeast showed efficient alkyl glucoside production from not only glucose but also cellohexaose. This yeast is applicable as a whole-cell biocatalyst for alkyl glucoside production from cellulose hydrolysates.
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Keywords: β-Glucosidase; Alkyl glucoside; Cell surface engineering; Whole-cell biocatalyst

1. Introduction

Cellulose is one of the most attractive resources in the world because of its renewability and abundance. The concept of a biorefinery, the production of useful materials from waste and unused biomass has been developed. Alkyl glucosides are useful materials and have applications in many areas such as the pharmaceutical, cosmetic, food and detergent industries. For example, lauryl glucoside is used as a synthetic detergent in the kitchen. Additionally, they are nonionic, biodegradable and nontoxic surfactants [1]. The chemical synthesis of glycoside derivatives, such as alkyl glucosides, requires numerous steps of protection and deprotection, and produces byproducts. On the other hand, glycosidase simplifies these reactions because of its regioselectivity and stereoselectivity [2]. Alkyl glycoside syntheses by glycosidase, carried out by transglycosylation between a reactive sugar donor and an alcohol acceptor or by the reverse hydrolysis of a glycoside donor and an alcohol, have been reported by many researchers [3–7].

β-Glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) is a member of a widespread group of enzymes that catalyze the hydrolysis of disaccharides, oligosaccharides, aryl glucosides and alkyl glucosides from their nonreducing end [8,9]. β-Glucosidases play an important role in bioconversion processes, and the characterization of these enzymes from various plants, bacteria and fungi has been reported by many researchers [10–12]. β-Glucosidases from different sources have different substrate specificities [8,9,13]. Using both the hydrolytic and transferase activities of these enzymes, various biotechnological applications have been developed. For example, the hydrolytic activity of the enzyme has been used in the degradation of cellulosic biomass and the synthesis of oligosaccharides and glycoconjugates such as alkyl glucosides [4,5,14,15]. To produce alkyl glucosides from cellulose, it is necessary to hydrolyze cellulose to glucose or cellooligosaccharides. Treatment of cellulosic material with acid results in a low yield of saccharides due to the formation of byproducts, e.g., 5-hydroxymethylfurfural [16]. Alternatively, the hydrolysis of cellulose using hot-compressed water produces cellooligosaccharides that consist of various-chain-length oligomers [17]. Thus, for efficient alkyl glucoside production with a high yield, it is beneficial to use a broad-substrate-specificity and highly active

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β-glucosidase with cellooligosaccharides. *Aspergillus aculeatus* β-glucosidase 1 (BGL1) is known to hydrolyze not only soluble cellooligosaccharides, but also insoluble cellooligosaccharides (when the degree of polymerization is at least 20) [13]. For the efficient use of β-glucosidase in an aqueous–organic biphasic medium, it is reported that the immobilization of β-glucosidase is effective for enhancing the accessibility and stability of enzyme [18]. However, the application of an alkyl glucoside synthesis reaction using *A. aculeatus* BGL1 in an aqueous–organic biphasic system has not been reported yet.

In recent years, microorganisms have been used to display enzymes on the cell surface by fusion with cell-wall-anchored proteins and used as an immobilized enzyme [19–23]. For a cost-effective bioconversion process, the utilization of such microorganisms is attractive because the enzymes are immobilized on the cell surface during cultivation. Previously, we reported direct and efficient ethanol production from cellulosic materials using a yeast strain co-displaying cellulase and *A. aculeatus* BGL1 [22,23]. In this study, we have constructed several BGL1-displaying strains of the yeast *Saccharomyces cerevisiae* to investigate a suitable host strain. The optimum water content, temperature and alcohol were investigated using BGL1-displaying GRI-117-UK, which showed the highest β-glucosidase activity. It was shown that alkyl glucoside can be produced from glucose or cellooligosaccharides using this cell surface-engineered strain.

2. Materials and methods

2.1. Strains and media

Escherichia coli NovaBlue [*endA1 hsdR17*(r_{K12}^- m_{K12}^+) *supE44 thi-1 gyrA96 relA1 lac recA1/F'*{*proAB*$^+$ *lacI*^{q}*ZΔM15::Tn10*(*tet*^{r})}] (Novagen Inc., Madison, WI, USA) was used as the host strain for the recombinant DNA manipulations. *S. cerevisiae* YPH499, YPH500, YPH501, W303-1A, BY4742 and GRI-117-UK were used for cell surface display (Table 1). *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 100 μg/ml ampicillin. Yeast cells were cultivated in SD medium [2.0% glucose, 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA) with appropriate supplements, 2.0% agarose] for use in transformation experiments. Following precultivation, yeast cells were cultivated aerobically at 30 °C in YPD medium [1% yeast extract (Nacalai Tesque Inc., Kyoto, Japan), 2% glucose and 2% peptone (Difco Laboratories, Detroit, MI, USA)].

2.2. Construction of plasmid and transformation of yeast cells

The plasmid pK113-AaBGL1 used for the cell surface display of *A. aculeatus* BGL1, was constructed as follows: A DNA sequence containing the gene encoding mature BGL1 fused with the gene encoding the FLAG peptide tag at the C-terminus was amplified by PCR using forward and reverse primers 5′-atgactcgagcggatgaactggcgttctctcctcctttctacccctctccgtgggccaatgg-3′ and 5′-gcatctcgagtccttgtcatcgtcatccttgtagtcttgcaccttcgggagcgccgcgtgaaggggcag-3′, respectively, and plasmid pBG211 as a template [24]. The resulting DNA product was digested using *Xho*I and introduced into the *Xho*I site of the cell surface-expression plasmid pK113 (donated by Gekkeikan Corporation) containing genes with the *SED1* promoter, the secretion signal sequence of the glucoamylase gene from *Rhizopus oryzae* and the 3′-half region of the α-agglutinin gene.

The introduction of the constructed plasmid (Fig. 1) into *S. cerevisiae* was performed using the YEASTMAKER^{TM} yeast transformation system (Clontech Laboratories Inc., Palo Alto, CA, USA). The plasmids pK113-AaBGL1 and pK113 were digested within the *URA3* gene using *Nco*I, and these linearized DNA products were used to transform the *S. cerevisiae* strains listed in Table 1.

2.3. Immunofluorescence detection of surface-displayed BGL1

The immunofluorescence labeling of cells was performed as follows: after cultivation in YPD medium for 72 h at 30 °C, yeast cells were collected, washed with phosphate-buffered saline (PBS: 50 mM phosphate, 150 mM NaCl, pH 7.4), and resuspended in PBS containing 1% bovine serum albumin (BSA) for 30 min at 4 °C (optical density at 600 nm; OD_{600} = 10). The yeast cells were then incubated for 1.5 h at 4 °C in PBS containing 1% BSA with the rabbit anti-*A. aculeatus* BGL1 IgG as the primary antibody at a dilution of 1:100. They were then incubated for 1 h at 4 °C with a secondary antibody, goat anti-rabbit IgG conjugated with Alexa Fluor^{TM} 488 (Molecular Probes Inc., Eugene, OR, USA). After washing with PBS, the green fluorescences of the cells were analyzed using a flow cytometer (FACS Calibur; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) at a low flow rate, correspond-

Table 1
β-Glucosidase activity and fluorescence intensity of six BGL1-displaying cells

Strain (genotype)	Activities of transformant (U/g dry wt of cells)	Fluorescence intensity
YPH499 (*MATα ura3-52 lys2-801 ade2-11 trpl-Δ63 his3-Δ200 leu2-ΔI*)	309.0 ± 15.2	58.2 ± 3.1
YPH500 (*MATα ura3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-Δl*)	323.3 ± 30.1	65.2 ± 9.8
YPH501 (*MATα ura3-52 lys2-801 ade2-101 trpl-Δ63 his3-Δ200 leu2-I*)	328.2 ± 6.8	60.2 ± 6.0
W303-1A (*MATα ade2-1 ura3-1 his2-11 trp1-1 leu2-3 leu2-112 canl-100*)	327.6 ± 12.6	51.6 ± 4.1
BY4742 (*MATα ura3 Δ0 his3Δ0 lys2Δ0*)	316.3 ± 11.5	55.3 ± 4.8
GRI-117-UK (*MATa/α ura3/ura3 lys2/lys2*)	435.5 ± 18.1	78.2 ± 7.2

The values are averages of three independent experiments.

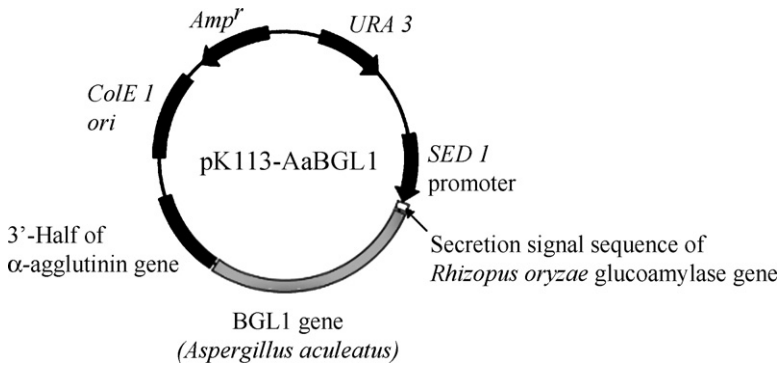

Fig. 1. Construction of plasmid pK113-AaBGL1 used for display of BGL1 on the yeast-cell surface.

ing to 150–300 cells/s. The average fluorescence intensity of 10,000 cells was calculated by subtracting the average fluorescence intensity of control cells (parent strains) from that of each sample. Fluorescently labeled cells were also observed using a fluorescence microscope (BZ-8000, Keyence Co., Osaka, Japan).

2.4. Enzyme assay

We modified the method of Fujita et al. to assay β-glucosidase [23]. β-Glucosidase activity was measured in a 50 mM sodium acetate buffer (pH 5.0) at 30 °C using 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) (Nacalai Tesque) as the substrate. The OD_{600} of the reaction mixture was adjusted to 0.05. The reaction was stopped by adding the equivalent of 1 M Na_2CO_3. Supernatants were separated by centrifugation at 20,000 × *g* for 5 min at 4 °C, and the amount of released *p*-nitrophenol was estimated by measuring the absorbance at 400 nm. One unit of β-glucosidase activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from the substrate/min.

2.5. Synthesis of alkyl glucosides

Alkyl glucoside synthesis was performed as follows: BGL1-displaying yeast cells were grown in YPD medium at 30 °C, and yeast cells were collected by centrifugation at 3000 × *g* for 5 min at 4 °C and washed with distilled water twice. 1.6 U of BGL1-displaying yeast strains or almond β-glucosidase (Sigma, St. Louis, MO) was added to the reaction mixture, which consisted of 7–9.5 m1 of primary alcohol and 3–0.5 ml of 50 mM sodium acetate buffer (pH 5.0) containing either 36 mg of cellooligosaccharide (Seikagaku Co., Tokyo, Japan) or glucose (Nacalai Tesque) or 60 mg of *p*NPG. The reaction was constantly shaken at 300 rpm at 30 or 50 °C. After the reaction, the organic phase was separated by centrifugation for 5 min at 15,000 × *g* at room temperature and the amount of alkyl glucoside was measured.

2.6. High-performance liquid chromatography (HPLC)

The quantity of alkyl glucosides was measured by HPLC, using a refractive-index detector (model RID-10A; Shimadzu, Kyoto, Japan) and an ODS column. HPLC was carried out at 30 °C using an acetonitrile–water mixture (60:40, v/v) as the mobile phase at a flow rate of 1.0 ml/min.

2.7. Thin-layer chromatography (TLC)

The end products were analyzed by TLC. Aliquots (10 μl) of the reaction mixture were spotted on a silica gel 60 F_{254} thin-layer chromatography plate (Merck, Darmstadt, Germany), which was developed using an ethyl acetate–2-propanol–water mixture (4:3:1, v/v/v). After spraying sulfuric acid and ethanol (5:95, v/v) on the plates, sugars were detected by heating at 120 °C for 10 min.

3. Results

3.1. Display of BGL1 on yeast-cell surface

To synthesize the alkyl glucosides from a primary alcohol and glucosides, we constructed yeast strains displaying BGL1 on their cell surface. The gene encoding BGL1 fused with the gene encoding the secretion signal sequence of the *R. oryzae* glucoamylase gene and the 3′-half region of the α-agglutinin gene were expressed under the *SED1* promoter (Fig. 1). Transformants with the linearized plasmid pK113-AaBGL1 underwent auxotroph screening. To select β-glucosidase-positive transformants, β-glucosidase activity was determined using *p*NPG as the substrate. The hydrolysis of the substrate was detected in cells harboring the β-glucosidase expression cassette. On the other hand, no hydrolysis was detected in the control strain (GRI-117-UK).

The BGL1 activities on the cell surface of various yeast strains (Table 1) were compared. BGL1-displaying GRI-117-UK showed the highest β-glucosidase activity, and the activity reached 435.5 U/g dry wt of cell. To compare the productivity of BGL1 on the cell surface of various yeast strains and to confirm the display of BGL1 on the yeast cells, immunofluorescence labeling of the cells was carried out using the rabbit anti-*A. aculeatus* BGL1 antibody as the primary antibody and Alexa Fluor™ 488-conjugated goat anti-rabbit IgG as the secondary antibody. As shown in Table 1, BGL1 activity and fluorescence intensity had the correlation. The intensity of BGL1-displaying GRI-117-UK was the highest. This result indicated that the largest amount of BGL1 was produced on the GRI-117-UK

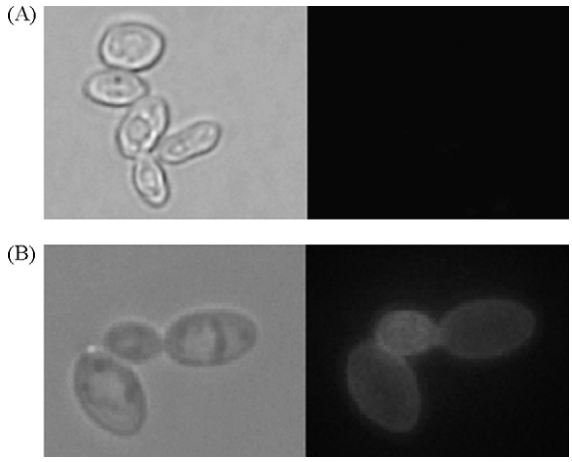

Fig. 2. Immunofluorescence labeling of the transformed yeast cells using rabbit anti-*Aspergillus aculeatus* BGL1 IgG and Alexa Fluor™ 488-conjugated goat anti-rabbit IgG. Left and right columns show phase and immunofluorescence micrographs, respectively. (A) GRI-117-UK (control) and (B) BGL1-displaying yeast strain GUK:BGL1.

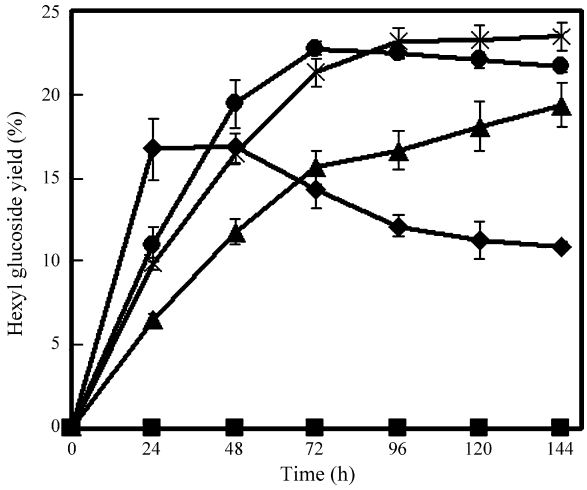

Fig. 3. Effect of water content on hexyl glucoside production using BGL1-displaying strain in 1-hexanol/water two-phase system. Symbols for water contents (v/v): squares, 5%; triangles, 10%; crosses, 15%; circles, 20%; diamonds, 30%. The data points represent the averages of the results of four independent experiments.

cell surface. In further experiments, we used this strain and named it GUK:BGL1. As shown in Fig. 2, the green fluorescence was observed for GUK:BGL1 around yeast-cell surface (Fig. 2B), whereas no fluorescence was observed on the cell surface of the control strain (Fig. 2A). These results suggest that BGL1 was successfully displayed on the surface of the yeast cells.

3.2. Synthesis of alkyl glucosides using BGL1-displaying yeast strain

The influence of water content on alkyl glucoside synthesis via transglycosylation was examined using GUK:BGL1. The reaction mixture containing *p*NPG as a sugar donor and 1-hexanol as an alcohol acceptor was adjusted to pH 5.0 and 30 °C was shaken at 300 rpm. As shown in Fig. 3, hexyl glucoside was not synthesized at a water content of 5% (v/v). For the maximum hexyl glucoside yield, the optimum water content was approximately 20%. The effect of temperature on hexyl glucoside synthesis was examined by transglycosylation performed at 30 °C and 50 °C (Fig. 4). The reaction rate at 50 °C was faster than that at 30 °C during the first 12 h. When the synthesis was carried out at 50 °C, the reaction was completed after 12 h with a 27.3% yield. Upon further reaction, after 12 h, the yield of hexyl glucoside decreased. To perform an efficient synthesis, the optimum chain length of the alcohol in the reaction mixture was investigated using 1-hexanol and 1-octanol as the organic phase. The reaction was completed in 12 h, and the yield of hexyl glucoside decreased after 12 h (Fig. 5). The reaction rate and reaction yield obtained using 1-hexanol were higher than those obtained using 1-octanol.

3.3. Substrate specificity

To examine the possibility of alkyl glucoside production from cellulose, hexyl glucoside production from glucose or cellooligosaccharides was compared between GUK:BGL1 and commercial almond β-glucosidase. As shown in Fig. 6, almond β-glucosidase and GUK:BGL1 were able to synthesize hexyl glucoside from glucose to cellohexaose in 72 h. Almond β-glucosidase was less active for compounds with long chain lengths. On the other hand, the BGL1-displaying yeast GUK:BGL1 showed activity for glucose as well as cellohexaose. Thin-layer chromatography of the hexanol layer was performed to examine the reaction products released from cellooligosaccharides using GUK:BGL1 or almond β-glucosidase. As shown in Fig. 7, hexyl glucosides produced by β-glucosidase were identified by comparison with the standard R_f in the hexanol layer. The amounts of hexyl glucoside show a tendency similar to Fig. 6. We confirmed the other spots were glucose, which was produced by the hydrolysis of cellooligosaccharides, by TLC (data not shown). In addition, cellooligosaccharides and

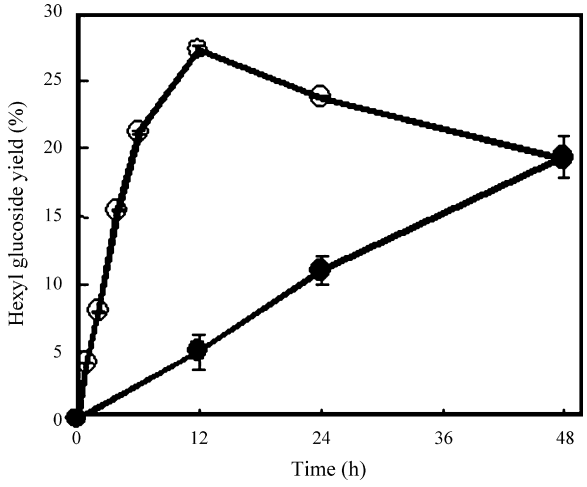

Fig. 4. Effect of reaction temperature on hexyl glucoside production. Symbols for temperatures: closed circles, 30 °C; open circles, 50 °C. The data points represent the averages of the results of three independent experiments.

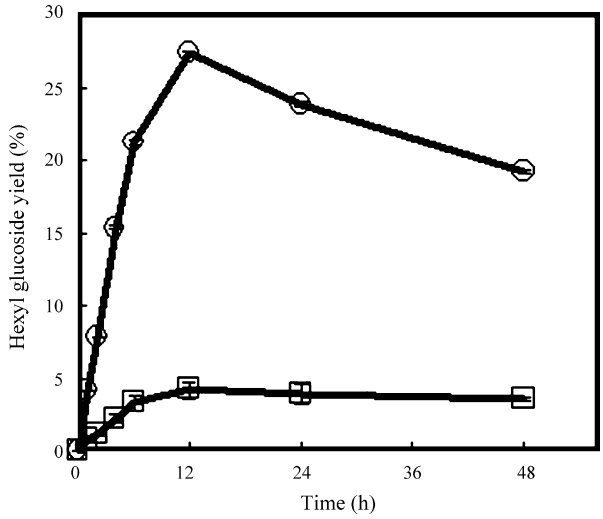

Fig. 5. Effect of chain length of primary alcohol on the yield of alkyl glucoside. Symbols for alcohols: open circles, 1-hexanol; open squares, 1-octanol. The data points represent the averages of the results of three independent experiments.

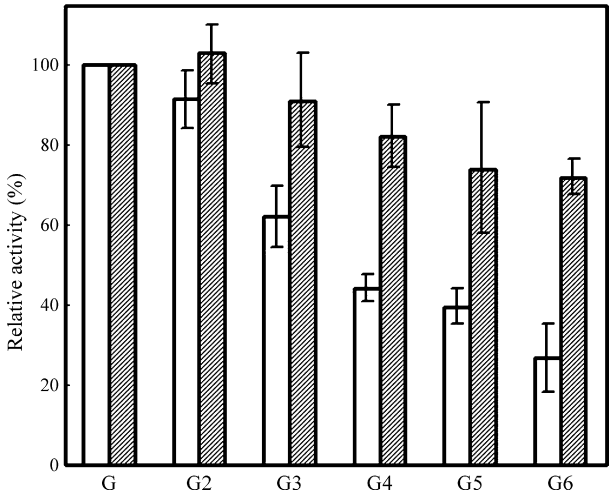

Fig. 6. Hexyl glucoside production from various cellooligosaccharides using BGL1-displaying yeast strain or almond β-glucosidase. The unshaded bars represent almond β-glucosidase and the shaded bars represent the BGL1-displaying yeast strain. G, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose; G6, cellohexaose. The data points represent the averages of the results of three independent experiments.

alkyl cellooligosaccharides were not detected in the organic layer.

4. Discussion

We have constructed six yeast strains displaying the *A. aculeatus* β-glucosidase 1, known to be highly reactive to cellooligosaccharides, on the yeast-cell surface and investigated the difference among the strains for β-glucosidase activity and productivity (Table 1). Every yeast strain displaying BGL1 under the control of the *SED1* promoter, showed an approximately 20-fold higher activity than the previous *GAPDH*-promoter-regulated display [23]. In particular the industrial strain GRI-117-UK showed the highest β-glucosidase

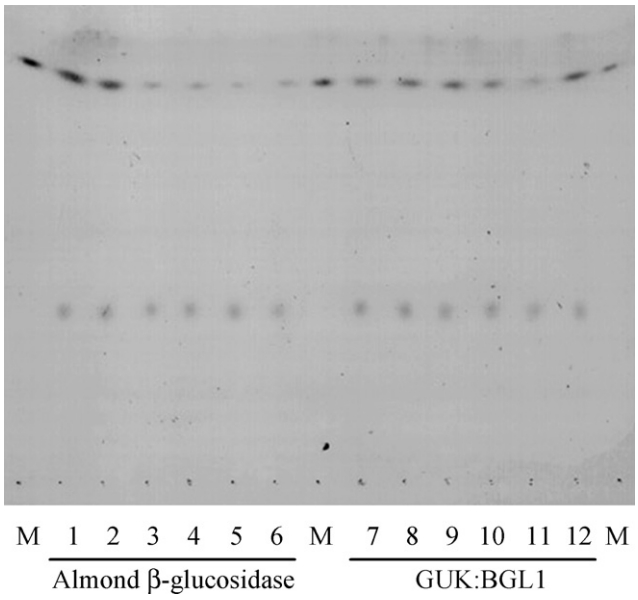

Fig. 7. TLC after reaction using glucose or cellooligosaccharides as a substrate. M, hexyl glucoside; 1, 7, glucose; 2, 8, cellobiose; 3, 9, cellotriose; 4, 10, cellotetraose; 5, 11, cellopentaose; 6, 12 cellohexaose.

activity due to its high productivity of BGL1. Therefore, alkyl glucoside synthesis in a water–organic two-phase system was examined using BGL1-displaying GRI-117-UK that is, the GUK:BGL1 strain.

To perform high-yield alkyl glucoside production, water content or water activity is considered to be an important factor [25]. The optimum water content for almond β-glucosidase, which is known to produce alkyl glucoside efficiently, is nearly 8% [18]. On the other hand, we have investigated the optimum water content for hexyl glucoside production using GUK:BGL1, and it was determined to be 20.0% (v/v) (Fig. 3). This is advantageous because, in the reaction mixture, a large amount of substrate is prepared since the glycosyl donor, for example, cellooligosaccharide is primarily hydrophilic and most of the glycoside exists in the water phase. Furthermore, to obtain higher reaction rates, the temperature dependence of cell surface BGL1 activity was studied. As shown in Fig. 4, the reaction rate of GUK:BGL1 was faster at 50 °C than at 30 °C. The thermal stability of β-glucosidase on the cell surface is similar to that of native BGL1, which has been reported to be stable at 50 °C [13]. The activities of GUK:BGL1 for hexanol and octanol substrates are shown in Fig. 5. The enzyme catalyzed hexanol more efficiently than it catalyzed octanol, which is similar to the result reported using other glucosidase [26]. The substrate specificities of GUK:BGL1 and almond β-glucosidase were compared using cellooligosaccharides with different chain lengths. GUK:BGL1 catalyzed long-chain-length oligosaccharides more efficiently than almond β-glucosidase (Fig. 6). Hexyl glucoside and other spots were detected by TLC of the organic layer (Fig. 7). By performing TLC, we confirmed that the other spots were glucose (data not shown). Therefore, this BGL1-displaying yeast strain can be used to produce alkyl glucoside efficiently from cellooligosaccharides, which are present in the hydrolysates of cellulose prepared using hot-compressed water or by cellulase

treatment. BGL1-displaying yeast should produce alkyl glucoside efficiently from such a hydrolysate.

As discussed above, the efficient synthesis of alkyl glucoside from cellooligosaccharides and alcohol was achieved by developing a yeast strain displaying *A. aculeatus* BGL1. Further studies, for example, an examination on the effect of additives such as DMSO, are required to enhance the interface of such a yeast strain for the high-yield synthesis of alkyl glucoside from cellulooligosaccharides. To produce alkyl glucosides from cellulose, it is necessary to hydrolyze cellulose to cellooligosaccharides and transglycosylate cellooligosaccharides to alkyl glucoside in an aqueous–organic biphasic medium. Direct alkyl glucoside production from cellulose is expected by using the yeast strains co-displaying cellulase and BGL1 on the surface.

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