



Enhanced cell-surface display of a heterologous protein using *SED1* anchoring system in *SED1*-disrupted *Saccharomyces cerevisiae* strain

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Yeast displaying enzymes on the cell surface are used for developing whole-cell biocatalysts. High enzyme activity on the cell surface is required in certain applications such as direct ethanol production from lignocellulosic materials. However, the cell surface enzyme activity is limited by several factors, one of which is the protein amount of the yeast cell wall. In this study, we attempted to improve the incorporation capacity of a displayed heterologous enzyme by disrupting a native cell-wall protein. β -Glucosidase (BGL1) from *Aspergillus aculeatus* was fused with *Saccharomyces cerevisiae* Sed1 and displayed on the cell surface of *S. cerevisiae* BY4741 strain and its *SED1* disruptant. Sed1 is one of the most abundant stationary phase yeast cell wall protein. A time course analysis revealed that BGL1 activity of the control strain reached saturation after 48 h of cultivation. In contrast, the BGL1 activity of the *SED1* disruptant increased until 72 h of cultivation and was 22% higher than that of the control strain. We also performed relative quantification of cell wall proteins of these strains by nanoscale ultra pressure liquid chromatography electrospray ionization quadrupole time-of-flight tandem mass spectrometry (nano-UPLC-MS^E). The amount of the cell wall-associated BGL1 per unit dry cell-weight of the *SED1* disruptant was 19% higher than that of the control strain. These results suggested that the incorporation capacity of the cell wall for BGL1 was increased by disruption of *SED1*. Disruption of *SED1* would be a promising approach for improving display efficiency of heterologous protein fused with Sed1.

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The yeast strains displaying cellulolytic enzymes have been developed as whole-cell biocatalysts for efficient ethanol production from pretreated lignocellulosic materials (1–6). In these strains, cellulolytic enzymes were fused with the anchoring domains of glycosylphosphatidylinositol (GPI) proteins and immobilized in the cell wall. The enzyme activity of the immobilized proteins is retained as long as the yeasts continue to grow (7). The biocatalyst is easily separated from the products, and the cell surface-displayed active enzymes can be reutilized without cell division (8). This approach simultaneously reduces the cost of yeast propagation and enzyme addition (9).

The high cellulolytic activity on yeast cell surface is required for the direct conversion of pretreated lignocellulosic materials using cellulase-displaying yeast. However, the cell surface-displayed enzyme activity is limited by certain factors, one of which is the protein incorporation capacity of the yeast cell wall. *Saccharomyces cerevisiae* contains approximately 60 native GPI proteins, most of which can be integrated into the cell wall via covalent attachment to β -1,6-glucan chains in the glucan layer (10). Therefore, heterologous displayed enzymes must compete with other GPI proteins for

the limited protein incorporation capacity of the cell wall (11). Thus, increasing the protein incorporation capacity of the yeast cell wall for further improvement of cell surface-displayed enzyme activity is important. In a previous study, the promoter and anchoring region of *S. cerevisiae* *SED1* were used for the efficient cell surface display of heterologous enzymes (12,13). The cell surface-associated activity of *Aspergillus aculeatus* β -glucosidase (BGL1) using the *SED1* anchoring system was 8.4-fold higher than that observed using the conventional *TDH3* promoter and α -agglutinin (*SAG1*) anchoring cell surface display system. However, the cell surface-displayed activity of BGL1 displayed using the *SED1* anchoring system reached saturation after 48 h of cultivation (12). This also underscored the restricted protein incorporation capacity of the yeast cell wall.

In this study, the *SED1* disruptant was successfully used as a host strain for increasing the amount of heterologous displayed enzymes on yeast cell surface using the *SED1* anchoring system. Sed1 is a stress-induced structural GPI protein and one of the most abundant cell-wall proteins found in the stationary phase (14). Therefore, it was expected that the deletion of *SED1* would enable cell wall-displayed enzymes to utilize the space previously occupied by Sed1. First, we evaluated the effect of the *SED1* disruption on the cell surface-associated BGL1 activity. In addition, relative quantification of the amount of cell wall-displayed BGL1 was performed to verify the improvement in the display efficiency in the *SED1* disruptant. Finally, ethanol was directly produced from

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cellobiose to evaluate the fermentation ability of the *SED1* disruptant.

MATERIALS AND METHODS

Strains and media *Escherichia coli* NovaBlue (Merck Millipore, Darmstadt, Germany) was used as the host strain for recombinant DNA manipulation and was grown in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) containing 100 µg/mL ampicillin. The genetic properties of all strains used in this study are summarized in Table 1. The control strain BY-BG-SS was constructed in a previous study (12). BY4741*sed1Δ* was obtained from the yeast deletion *MAT-A* complete set (Thermo Fisher Scientific, Waltham, MA, USA). Yeast strains were screened and pre-cultured in synthetic dextrose (SD) medium [6.7 g/L of yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA) and 20 g/L of glucose] supplemented with appropriate amino acids and nucleic acids in a shaker incubator at 180 rpm for 24 h at 30°C. The precultured cells were then inoculated into Yeast extract peptone dextrose (YPD) medium [10 g/L yeast extract, 20 g/L Bactopeptone (Difco Laboratories), and 20 g/L glucose] and used for enzyme assay, growth assay, and ethanol fermentation as described below.

Yeast cells were harvested by centrifugation at 1000 ×g for 5 min, washed twice with distilled water, followed by centrifugation at 1000 ×g for 5 min. The wet cell weight was determined by weighing the cell pellet. The cell pellets were freeze-dried using the FreeZone 2.5 Plus system (Labconco, Kansas City, MO, USA) to determine the dry cell weight.

Growth assay The growth assay was conducted as described previously (13). The $\mu_{\max}$ values were calculated from the growth rates from 12 to 14 h by using the following equation: $\mu = 2.303 (\log_{10} OD_2 - \log_{10} OD_1) / (t_2 - t_1)$. The optical densities of cells at times t_1 (12 h) and t_2 (14 h) were termed as OD1 and OD2, respectively.

Yeast transformation The integrative plasmid pIBG-SS (12) was used for displaying BGL1. The plasmid was digested using *NdeI* (New England Biolabs, Ipswich, MA, USA) within *HIS3*, and the linearized plasmid was transformed into BY4741*sed1Δ* using the lithium acetate method (15) and integrated into the chromosomal *HIS3* locus by homologous recombination.

BGL1 activity assay Precultured cells were inoculated in 50 mL of YPD medium to an initial OD₆₀₀ of 0.05 and cultivated aerobically in a shaker incubator (150 rpm at 30°C) for 96 h. The culture broth was sampled every 24 h and cell-surface associated BGL1 activity was assayed using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as the substrate as described previously (12). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of *p*-nitrophenol per minute. Cell-surface BGL activities were normalized by the dry cell weight of each strain.

Extraction of cell wall proteins Precultured cells were inoculated in 50 mL of YPD medium to an initial OD₆₀₀ of 0.05 and cultivated aerobically in a shaker incubator (150 rpm at 30°C) for 72 h. The yeast cells were collected by centrifugation at 1000 ×g for 10 min at 20°C and washed twice with distilled water. The washed cells were treated with a cell-wall degrading enzyme (lyticase, Cellulytic Y Plus kit, Sigma-Aldrich, St. Louis, MO, USA) as described by Kutty et al. (16), followed by centrifugation at 9300 ×g for 5 min at 20°C to remove spheroplasts. The supernatant was used as the cell wall protein sample. Each protein sample extracted from 10 mg wet cells was mixed with 10 µL 1 mg/mL bovine serum albumin (BSA) and dried in a vacuum evaporator (CVE-3100, Tokyo Rikakikai, Osaka, Japan) overnight. The dried extracts were stored at -80°C until use.

Sample preparation for mass spectrometry analysis Dried protein samples were resuspended in 38 µL 0.1% RapiGest SF (Waters Corporation, Milford, MA, USA), reduced with dithiothreitol, S-alkylated with iodoacetamide, and digested with 1:50 (w/w) sequencing grade modified trypsin for 18 h. RapiGest SF was hydrolyzed by the addition of 2.5 µL 10% trifluoroacetic acid, and the insoluble fraction (dodeca-2-one) was removed by centrifugation at 13,000 ×g for 5 min at 4°C. The digested peptides were stored at -80°C until use.

Analysis by nano-UPLC-MS^E Protein identification with nanoscale ultra pressure liquid chromatography electrospray ionization quadrupole time-of-flight tandem mass spectrometry (nano-UPLC-MS^E) were performed according to the procedure described by Pacheco et al. (17) with modifications as follows. A nanoACQUITY system (Waters Corporation) was equipped with a Symmetry C18 trapping column (180 µm × 20 mm; particle size, 5 µm) and an HSS T3 analytical column (75 µm × 150 mm; particle size, 1.8 µm). The temperature of the analytical column was set at 35°C. The composition of solvent A was 0.1% formic

acid in water, and solvent B consisted of 0.1% formic acid in acetonitrile. The digested peptide samples obtained from three independent cultures of each strain were used for subsequent data analysis. Each sample (1 µL) was transferred to the trapping column and flushed with 0.5% solvent B for 1 min at a flow rate of 15 µL/min. The sample was then eluted from the trapping column to the analytical column by increasing the organic solvent concentration from 3% to 60% B over 60 min at a flow rate of 0.5 µL/min. The precursor ion masses and associated fragment ion spectra of the digested peptides were measured using a Synapt HDMS mass spectrometer (Waters Corporation). Human [Glu¹]-fibrinopeptide B (Sigma Aldrich) was used as the lock mass compound for the external calibration of the time-of-flight analyzer of the mass spectrometer. The lock mass compound was delivered to the NanoLockSpray interface by the auxiliary pump of the nanoACQUITY system at a concentration of 500 fmol/µL and a flow rate of 1 µL/min, and sampled every 60 s. The mass spectrometer was programmed to switch between low (4 eV) and high (20–40 eV) energies in the collision cell, with a scan time of 0.5 s per function over a mass range of 50–1990. The ions exceeding 100 counts were selected for MS^E fragmentation in the collision cell.

Data processing The LC-MS^E data were processed using ProteinLynx Global SERVER (PLGS) v2.5.2 (Waters Corporation) and searched in the associated *S. cerevisiae* S288c database (<http://www.uniprot.org/uniprot/?query=taxonomy:559292>), to which the *A. aculeatus* BGL1 and BSA sequences had been appended. The protein identification for the processed data files was performed using the Identity^E algorithm within PLGS. At least three fragment ions were required for peptide identification, and seven fragment ions and one peptide were required for identification of protein. The false positive rate of protein identification was set at 4% with a randomized database. The precursor and fragment ion tolerances were determined automatically. Carbamidomethyl C and oxidation M were set as the fixed and variable modification, respectively. Three missed trypsin cleavage sites were allowed.

Relative quantitative analysis of cell wall-associated BGL1 Relative quantitative analysis between samples was performed using Expression software (version 2) within PLGS as described previously (17). Automatic normalization was applied to the data set using BSA of each sample as the internal standard protein to normalize comparative proteomic data.

Ethanol fermentation from cellobiose and glucose Precultured cells were inoculated in 50 mL of YPD medium to an initial OD₆₀₀ of 0.05 and cultivated aerobically in a shaker incubator (150 rpm at 30°C) for 72 h. The yeast cells were collected by centrifugation at 1000 ×g for 10 min at 20°C and washed twice with distilled water. The ethanol fermentation was conducted following the procedures described by Inokuma et al. (18) with minor modification. Briefly, the fermentation was performed in 10 mL fermentation medium [10 g/L yeast extract, 20 g/L bactopeptone (Difco Laboratories), and 100 g/L cellobiose or glucose] in closed 100 mL bottles equipped with a CO₂ outlet for 10 h at 30°C. The initial cell concentration was 50 g wet cells/L. The concentrations of cellobiose, glucose, and ethanol in the fermentation medium were determined using high performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) as described previously (18).

RESULTS

Construction of a novel BGL1-displaying yeast strain In this study, the *SED1* disruptant of *S. cerevisiae* BY4741 (BY4741*sed1Δ*) was used as the host strain for efficient display of BGL1. The plasmid pIBG-SS, which harbors the BGL1 display cassette with the *SED1* promoter, glucoamylase secretion signal peptide from *Rhizopus oryzae*, and the *SED1* anchoring region, was integrated into the chromosomal *HIS3* locus of BY4741*sed1Δ* by homologous recombination, and the constructed strain was referred to as BY*sed1Δ*-BG. BY*sed1Δ*-BG and BY-BG-SS (control strain constructed in our previous study (12)) were used in subsequent experiments.

Cell surface activity of BGL1 and growth of recombinant yeast strains The cell surface-associated BGL activity of BY-BG-SS and BY*sed1Δ*-BG was evaluated as described in Materials and methods. BGL activities per dry cell-weight of these strains are shown in Fig. 1. In the control strain (BY-BG-SS), BGL1 activity increased rapidly during the first 48 h of cultivation, after which the activity level attained saturation. This result was in good agreement with our previous report (12). In contrast, the BGL1 activity of BY*sed1Δ*-BG surpassed the saturation stage, and increased until 72 h of cultivation. BY*sed1Δ*-BG exhibited 22% higher activity than the control strain after 72 h of cultivation. To verify effects of *SED1* disruption for yeast cell growth, growth

TABLE 1. Characteristics of strains used in this study.

Strain	Relevant genotype	Source
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Thermo Fisher Scientific
BY-BG-SS	BY4741/pIBG-SS	12
BY4741 <i>sed1Δ</i>	BY4741, <i>sed1Δ::kanMX4</i>	Thermo Fisher Scientific
BY <i>sed1Δ</i> -BG	BY4741 <i>sed1Δ</i> /pIBG-SS	This study

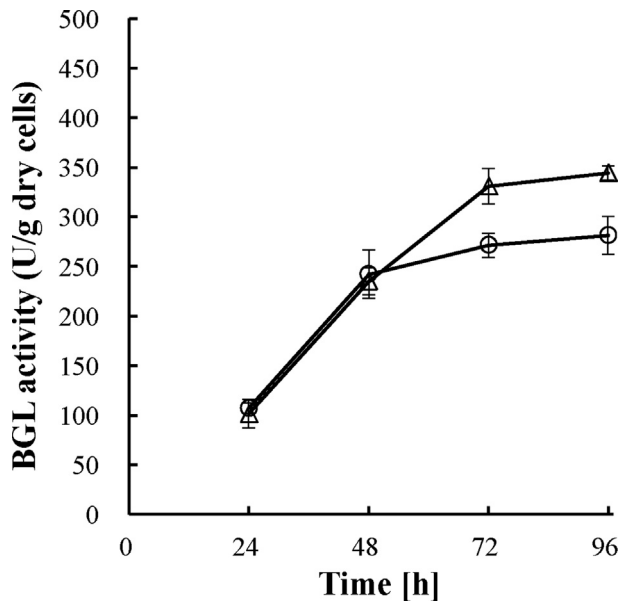


FIG. 1. Time course of cell surface-associated BGL1 activity. BY-BG-SS (circles) and BYsed1Δ-BG (triangles). Error bars indicate the standard deviations of three independent experiments.

assay of the recombinant strains was also performed. As shown in Table 2, the disruption of *SED1* did not substantially affect cell growth in YPD medium.

Relative quantitative analysis of cell wall-associated BGL1 The cell-wall proteins of BY-BG-SS and BYsed1Δ-BG were extracted after 72 h of cultivation to compare the amounts of BGL1 in their cell walls. The amount of cell wall proteins extracted from 10 mg wet cells of BY-BG-SS and BYsed1Δ-BG were 58.1 ± 3.3 and 51.2 ± 2.7 μ g, respectively. The identification and relative quantitative analysis of the extracted proteins were performed using nano-UPLC-MS^E as described in Materials and methods. The identified cell wall proteins and detected peptides are listed in Table 3 and Supplementary Table S1, respectively. Thirty-five peptides derived from *A. aculeatus* BGL1 were detected in both strains. The result of relative quantitative analysis of cell wall-associated BGL1 are shown in Fig. 2. The amount of BGL1 per unit dry cell-weight of BYsed1Δ-BG was 19% higher than that of BY-BG-SS at 72 h. Although 4 native *S. cerevisiae* cell wall proteins (Cwp1, Ecm33, Pir1, and Bgl2) were

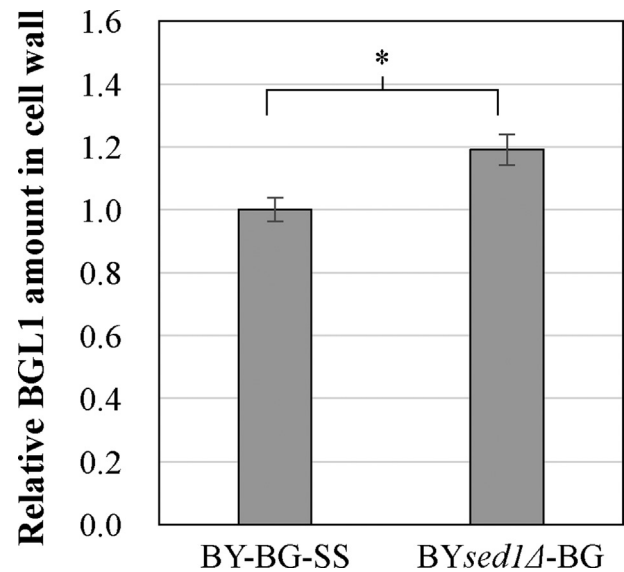


FIG. 2. The relative quantification of BGL1 in the cell-walls of BY-BG-SS and BYsed1Δ-BG by nano-UPLC-MS^E. Three independent experiments of protein profile analysis were performed for each sample. Asterisk represent a statistically significant ($p < 0.05$) difference in BGL amount as evaluated by paired comparisons using Student's *t* test.

also identified in the analysis (Table 3), there was no significant difference in the abundance of these proteins (data not shown).

Ethanol fermentation from cellobiose using BGL1-displaying yeast strain Direct ethanol production from 100 g/L cellobiose was performed using yeast cells cultivated for 72 h to evaluate the effect of *SED1* disruption on fermentative ability. Both BY-BG-SS and BYsed1Δ-BG almost completely consumed 100 g/L cellobiose after 10 h of fermentation and produced 41.8 ± 0.8 and 42.3 ± 1.5 g/L ethanol, respectively (Fig. 3A and B). Unexpectedly, the cellobiose consumption rate and ethanol production rate were similar in both the strains. In contrast, glucose accumulation in the supernatant was higher in BYsed1Δ-BG than in BY-BG-SS during the fermentation (Fig. 3C).

DISCUSSION

One of the factors limiting the cell surface display of heterologous enzyme activity is that these enzymes are in competition with other GPI proteins for the limited protein incorporation capacity of the cell wall (11,12). In this study, we demonstrated an improvement in the display efficiency of a BGL1-Sed1 fusion protein by disruption of the endogenous *SED1* in *S. cerevisiae*. Sed1 is an abundant stationary phase cell wall protein (14). Time course analysis of BGL1 activity revealed that BY-BG-SS and BYsed1Δ-BG exhibited highly similar activities until 48 h of cultivation (Fig. 1). However, the BGL1 activity of BY-BG-SS appeared to saturate after 48 h of cultivation. In contrast, BGL1 activity of BYsed1Δ-BG continued to increase until 72 h and was approximately 22% higher than that of BY-BG-SS. In addition, no significant effect on cell growth was observed in the *SED1* disruptant (Table 2).

Although it cannot be compared directly because the GPI-anchoring domain was different, some previous studies have indicated the effects of the *SED1* disruption for the yeast cell surface display of proteins fused with the α -agglutinin anchoring domain. Kuroda et al. (19) reported that the immunostaining efficiency of the FLAG-tagged red fluorescence protein displayed on the yeast cell surface using the α -agglutinin anchoring domain was improved by *SED1* disruption and in the presence of a high copy number plasmid harboring the FLAG-tagged protein display cassette. Kotaka et al.

TABLE 2. Growth profiles of BY-BG-SS and BYsed1Δ-BG.

Strain	$\mu_{\max}$ (h ⁻¹)	Final cell density (g dry cells/L at 96 h)
BY-BG-SS	0.397 ± 0.016	3.52 ± 0.10
BYsed1Δ-BG	0.383 ± 0.011	3.35 ± 0.03

TABLE 3. Identified proteins of cell wall.

Protein name	Description
<i>A. aculeatus</i> Bgl1	<i>A. aculeatus</i> β -glucosidase
Cwp1	GPI-anchored protein that localizes to birth scars of daughter cells
Ecm33	GPI-anchored protein of unknown function, possible role in apical bud growth
Pir1	O-glycosylated protein required for cell wall stability
Bgl2	Endo- β -1,3-glucanase

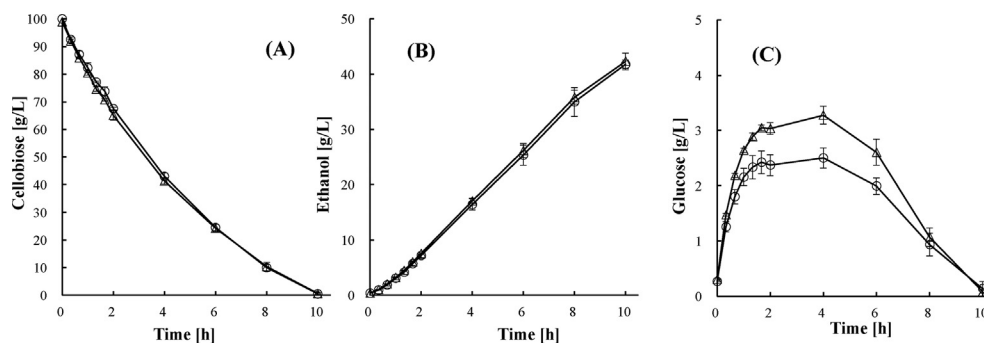


FIG. 3. Concentration of (A) cellobiose, (B) ethanol, and (C) glucose during direct ethanol production from cellobiose of BY-BG-SS (circles) and BYsed1Δ-BG (triangles) strains. Error bars indicate the standard deviations of three independent experiments.

(20) expressed a fusion protein of BGL1 and the α -agglutinin anchoring domain in a diploid sake yeast, and the cell surface-associated BGL1 activity was improved by the disruption of *SED1*. However, they did not perform a further investigation about the causes of the activity improvement, such as the time course analysis of BGL1 activity and quantitative analysis of the displayed proteins.

In this study, relative quantification of the actual amount of the cell wall-associated BGL1 was performed by tandem mass spectrometry. BYsed1Δ-BG showed 19% higher amount of BGL1 per unit dry cell weight than BY-BG-SS at 72 h of cultivation (Fig. 2). This result was in agreement with the ratio of the cell surface BGL1 activity of these strains at 72 h (Fig. 1). It was suggested that the deletion of *SED1* enable other GPI proteins inclusive of the BGL1-Sed1 to utilize the space previously occupied by original Sed1. Overall, these results suggest that the improvement in the cell surface-associated BGL1 activity by *SED1* disruption was because of the increase in the amount of the cell wall-associated BGL1. To our knowledge, this is the first report of relative quantification of displayed heterologous enzymes on yeast cell surface by tandem mass spectrometry.

In previous studies, the abundance of cell wall-associated proteins has been evaluated using fluorescence activated cell sorting (FACS) and fluorescence microscopy (6,19). However, these conventional techniques have some problems for specific quantification of cellulosic enzymes immobilized in yeast cell wall. First of all, FACS and fluorescence microscopy can only detect fluorescence proteins or fluorescent-tagged proteins, while our technique enables the direct detection of cellulosic enzymes without any tagging and labeling. In addition, FACS does not discriminate intracellular proteins and cell wall proteins. In contrast, our technique enables specific quantification of cellulosic enzymes immobilized in yeast cell wall because cell wall proteins are separated from yeast cells before the quantification. The yeast cell surface display system is used in a wide range of applications, for example, they are used as bioadsorbents, biosensors, and whole-cell biocatalysts (21–23). Therefore, the analytical method utilized in this study would be a promising approach to evaluate the display efficiency on yeast cell surface.

In this study, 4 native *S. cerevisiae* cell wall proteins (Cwp1, Ecm33, Pir1, and Bgl2) were also identified (Table 3), while most cell wall-associated GPI proteins, including Sed1, were not detected in both BY-BG-SS and BYsed1Δ-BG. Protein identification was conducted based on the precise mass measurement of tryptic peptides from each protein. However, GPI proteins are often enriched with O- and N-linked carbohydrate side chains (24). Especially, Sed1 contains enriched glycosylation sites throughout the molecule. Therefore, the masses of several tryptic peptides derived from GPI proteins are altered dramatically by glycosylation, which might render identification and quantification of GPI

proteins by tandem mass spectrometry difficult. A tandem mass spectrometry analysis demonstrated in this study is useful for specific quantification of cell-surface displayed heterologous proteins, but it would be necessary to develop a whole new method in order to understand more about the protein incorporation capacity of yeast cell wall.

In the direct ethanol production from cellobiose, no significant difference was observed in the cellobiose degradation rate and ethanol production rate of BY-BG-SS and BYsed1Δ-BG despite the higher cell surface-associated BGL1 activity of BYsed1Δ-BG (Fig. 3A and B). One possible reason for this observation could be product inhibition for BGL1. The cellobiose degradation rate of BY-BG-SS and BYsed1Δ-BG was higher than their glucose consumption rate (Supplementary Fig. S1) and redundant glucose accumulated in the fermentation medium (Fig. 3C). At the early phase of the fermentation, cellobiose degradation rate of BYsed1Δ-BG was slightly higher than that of BY-BG-SS (Fig. 3A), and higher concentration of glucose was accumulated in BYsed1Δ-BG than BY-BG-SS (Fig. 3C). Decker et al. (25) reported that *A. aculeatus* BGL1 was inhibited by glucose. Therefore, high glucose accumulation in BYsed1Δ-BG might stringently inhibit the cell surface-associated BGL1 activity, which may explain the similar cellobiose degradation rates of BY-BG-SS and BYsed1Δ-BG.

In this study, we displayed only BGL1 on the yeast cell surface. However, in direct ethanol production from lignocellulosic materials, synergistic reaction using different cellulolytic enzymes are required for the efficient degradation of these materials (23). In multi-enzyme displaying yeast strains, the displayed enzymes must compete not only with the native GPI proteins also among themselves for the limited protein incorporation capacity of the cell wall (13). Although further investigation is needed, disruption of *SED1* and other cell wall protein-encoding genes might be more effective in displaying multi enzymes on the cell surface. Recently, we reported another approach for improvement of enzyme displaying efficiency on yeast cell surface by utilization of *S. cerevisiae* Sed1 signal peptide. Although we used the secretion signal peptide derived from *R. oryzae* glucoamylase which is one of the most commonly used signal peptide sequences for the displaying heterologous enzymes on yeast cell surface, a combination of Sed1 signal peptide and *SED1* disruption demonstrated in this study would be a promising strategy for construction of an efficient whole-cell biocatalyst.

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jbiosc.2017.09.013>.

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