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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.

[**Key words:** *Saccharomyces cerevisiae*; Cell surface display; β-Glucosidase; *SED1*; Mass spectrometry; *Aspergillus aculeatus*]

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 μ_{max} values were calculated from the growth rates from 12 to 14 h by using the following equation: $\mu = 2.303\ (\log_{10}OD2 - \log_{10}OD1)/(t2 - t1)$. The optical densities of cells at times *t*1 (12 h) and *t*2 (14 h) were termed asOD1 and OD2, respectively.

Yeast transformation The integrative plasmid pIBG-SS (12) was used for displaying BGL1. The plasmid was digested using *Nde*I (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 OD_{600} 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 (*p*NPG) 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 OD_{600} 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, CelLytic 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% *Rapi*Gest 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. *Rapi*Gest 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 [Glu^1]-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 OD_{600} 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 CO_2 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).

TABLE 1. Characteristics of strains used in this study.

Strain	Relevant genotype	Source
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Thermo Fisher Scientific
BY-BG-SS	BY4741/pIBG-SS	12
BY4741*sed1Δ*	BY4741, *sed1Δ*::*kanMX4*	Thermo Fisher Scientific
BY*sed1Δ*-BG	BY4741*sed1Δ*/pIBG-SS	This study

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

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