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Enhancement of β-glucosidase activity on the cell-surface of sake yeast by disruption of *SED1*

Atsushi Kotaka,[1,*] Hiroshi Sahara,[1] Kouichi Kuroda,[2] Akihiko Kondo,[3] Mitsuyoshi Ueda,[2] and Yoji Hata[1]

Research Institute, Gekkeikan Sake Co. Ltd., 101 Shimotoba-koyanagi-cho, Fushimi-ku, Kyoto 612-8385, Japan[1] *Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan*[2] *and Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan*[3]

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We determined the genetic background that would result in a more optimal display of heterologously expressed β-glucosidase (BGL) on the cell surface of yeast *Saccharomyces cerevisiae*. Amongst a collection of 28 strains carrying deletions in genes for glycosylphosphatidyl inositol (GPI)-anchored proteins, the Δ*sed1* and Δ*tos6* strains had significantly higher BGL-activity whilst maintaining wild type growth. Absence of Sed1p, which might facilitate incorporation of anchored BGL on the cell-surface, could also influence the activity of BGL on the cell surface with the heterologous gene being placed under the control of the *SED1* promoter. For the evaluation of its industrial applicability we tested this system in heterologous and homogenous *SED1*-disruptants of sake yeast, a diploid *S. cerevisiae* strain, in which either the *SED1* ORF or the complete gene including the promoter was deleted by use of the high-efficiency loss of heterozygosity method. Evaluation of disruptants displaying BGL showed that deletion of the *SED1* ORF enhanced BGL activity on the cell surface, while additional deletion of the *SED1* promoter increased further BGL activity on the cell surface. Compared to heterozygous disruption, homozygous disruption resulted generally in a higher BGL activity. Thus, homozygous deletion of both *SED1* gene and promoter resulted in the most efficient display of BGL reaching a 1.6-fold increase of BGL-activity compared to wild type.

[**Key words:** *SED1*; Cell-surface engineering; Gene disruption; Diploid sake yeast; β-glucosidase]

Novel biocatalysts can be engineered from *Saccharomyces cerevisiae* by displaying enzymes on the yeast cell surface. The yeast cell wall mainly consists of glucan and mannoprotein to which glycosylphosphatidyl inositol (GPI)-anchored proteins and Pir proteins are bound (1). By fusing enzymes of interest to GPI-anchored protein and a secretion signal they will become immobilized on the cell wall and displayed on the surface. Such cells are utilized as whole-cell biocatalysts, and the enzymes are simply harvested by centrifugation and easily recycled (2).

Previously we constructed sake yeast (*S. cerevisiae*) strains displaying *Aspergillus oryzae* β-glucosidases (BGLs) that catalyzed the production of isoflavone aglycones from isoflavone glycosides (3) or ethanol from barley β-glucan (4). For the production on an industrial scale, the activity of these whole-cell catalysts needs to be raised. The expression and stability of heterologous proteins is often enhanced by particular gene disruptions. For example, protease deficiency improves the production of heterologous proteins (5). In the case of heterologous proteins displayed on the cell surface, changing the architecture of the cell wall might have a comparable advantageous effect.

Sake yeast is used for brewing Japanese sake. It produces a high concentration of ethanol, proliferates rapidly, and is generally recognized as safe. These characteristics make this yeast an attractive organism for industrial application, but because sake yeast hardly sporulates it is difficult to genetically engineer by conventional means. Using our recently developed high-efficiency loss of heterozygosity (HELOH) method, we created diploid sake yeast with recessive traits (6) or that overproduced isoamyl acetate during fermentation in sake mash (7).

In this study, we aimed to obtain sake yeast strains with increased concentration of BGL on the cell surface. By screening *S. cerevisiae* BY4743 strains with deletions of GPI-anchored protein genes we found that *SED1* disruption enhanced BGL activity. By use of the HELOH method, *SED1* deletion mutants of diploid sake yeast were constructed, in which either the *SED1* ORF or the complete gene including the promoter were removed. The latter disruption in homozygous sake yeast resulted in the most effective BGL display, presumably because both heterologous gene expression, being driven by the *SED1* promoter, and incorporation of BGL in the cell wall were improved.

MATERIALS AND METHODS

Strains and media *Escherichia coli* DH5α [F′*endA1 hsdR17* (r_K^-/m_K^-) *supE44 thi-1* λ⁻*recA1 gyrA96* Δ*lacU169* (ϕ80*lacZ*ΔM15)] was used as the host strain for plasmid construction. Yeast deletion collection derived from BY4743 (*MAT***a**/*MAT*α *his3*Δ1/*his3*Δ1 *leu2*Δ0/*leu2*Δ0 *lys2*Δ0/*LYS2 MET15*/*met15*Δ0 *ura3*Δ0/*ura3*Δ0) was obtained from Open Biosystems (Huntsville, AL, USA). The sake yeast strain GRI-117-UK (*MAT***a**/*MAT*α *lys2*/*lys2 ura3*/*ura3*) was obtained before (4). *E. coli* was grown in Luria–Bertani medium containing 10 g/l Polypepton, 5 g/l yeast extract, and 10 g/l sodium chloride

* Corresponding author. Tel.: +81 75 623 2130; fax: +81 75 623 2132.
E-mail address: a_kotaka@gekkeikan.co.jp (A. Kotaka).

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and 100 mg/l ampicillin. Yeast was cultivated aerobically at 30 °C in YPD medium containing 10 g/l yeast extract, 20 g/l Polypepton and 20 g/l glucose or SDC-Ura medium containing 6.7 g/l yeast nitrogen base without amino acids (Becton Dickinson and Company, Franklin Lakes, NJ, USA), 20 g/l glucose, 20 g/l Casamino acids (Becton Dickinson and Company), and 0.77 g/l complete supplement mixture-URA (MP Biomedicals, Solon, OH, USA). Uracil auxotrophic yeast strains were selected by 5-fluoroorotic acid (5-FOA) medium containing 6.7 g/l yeast nitrogen base without amino acids, 20 g/l glucose, 1 g/l 5-FOA, 20 mg/l uracil and 50 mg/l lysine hydrochloride (8).

Plasmid constructions Plasmid pRSU-SED1, used for integration of the *URA3* marker into the wild-type *SED1* gene, is pRS406 (Stratagene, La Jolla, CA, USA) with *SED1* cloned as an *Eco*RI-*Sal*I fragment and containing an engineered *Hpa*I restriction site after amplification by fusion PCR (9) using primers listed in Table 1 and *S. cerevisiae* chromosomal DNA as the template. The plasmid pYCUE-BGL1 (Fig. 1) for displaying *A. oryzae* β-glucosidase (BGL) on the cell surface by anchoring through the fused α-agglutinin contains the pRS416 (Stratagene) backbone consisting of ColE1 *ori*, Amp^r and *CEN6*/*ARS4* regions (amplified with the primers CAC-F and CAC-R) recombined with an *Aat*II–*Not*I fragment with the BGL-expression cassette that was isolated from plasmid pK113-BGL1 (3).

Yeast transformation The DNA fragments or plasmids were transformed into *S. cerevisiae* by the lithium acetate method (10).

Disruption of genes The *SED1* ORF (disruption *sed1-1*) or the complete *SED1* gene including the promoter (disruption *sed1-2*) were deleted from sake yeast (Fig 2) by the two-step gene disruption, high-efficiency loss of heterozygosity (HELOH) method (6, Fig 3). In the first step, heterozygous disruptants (DS1 and DS3) were constructed by PCR-mediated gene replacement and marker recycling (11), using the primers listed in Table 1. For marker recycling, the transformants whose deletion regions were replaced by the integration cassette containing *URA3* were cultivated aerobically at 30 °C in YPD for 72 h, and then plated on 5-FOA medium. Colonies appearing on 5-FOA plate were tested for the occurrence of loop out recombination (desirable genotype) or loss of heterozygosity (LOH, undesirable genotype) by colony PCR using primers 1F/(down)R, yielding heterozygous disruptants DS1 and DS3. The same PCR was used to identify homozygous disruptants (DS2 and DS4) which were obtained from DS1 and DS3 after integration of pRSU-SED1 digested with *Hpa*I into the wild-type *SED1* allele, followed by aerobic cultivation of the integrants at 30 °C in YPD for 72 h and selection on 5-FOA medium. In this case, occurrence of LOH resulted in the desirable genotype. *SED1* gene of GRI-117-UK is different from that of S288C. These sequence data have been submitted to the DDBJ database under accession no. AB530985.

Zymolyase treatment The phenotype of *SED1* disruptants was tested as described (12). The parental strain GRI-117-UK and its *SED1* disruptants were cultivated in YPD at 30 °C for 72 h, after which cells were collected and resuspended in 10 U/ml Zymolyase (Seikagaku Biobusiness, Tokyo) solution (pH 7.5) to an optical density of 1.0 at 600 nm. Cell-suspensions were incubated at 30 °C, and their cell densities were automatically measured with a Bio-photorecorder (Advantec Toyo Co. Ltd., Tokyo).

Measurement of BGL activity Cells were aerobically cultivated in SDC-Ura medium at 30 °C for 72 h, harvested by centrifugation, washed with distilled water, and resuspended in 50 mM sodium acetate (pH 5.0) with 1 mM *p*-nitrophenyl β-D-glucopyranoside as a substrate (4). After incubation at 37 °C for 5 min, cells were pelleted and the *p*-nitrophenol released in the supernatant was determined by measuring the absorbance at 415 nm.

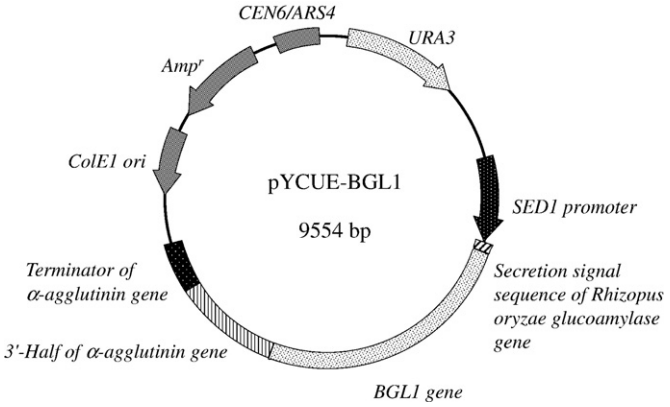

FIG. 1. Expression plasmid for cell surface display of β-glucosidase (BGL).

RESULTS

Identification of BY4743 mutants with optimal BGL activity A limiting factor for the activity of whole-cell biocatalysts engineered to display an enzyme of interest on the cell surface could be the presence of other cell-wall anchored proteins. We investigated this possibility by screening a collection of 28 strains with a disrupted GPI-anchored protein gene for BGL activity after transformation with plasmid pYCUE-BGL1, which expresses a fusion protein of *A. oryzae* β-glucosidase (BGL) and α-agglutinin from the *SED1* promoter (Fig. 1). Normalized against the BGL activity of the parental strain (BY4743) harboring pYCUE-BGL1 deletion of *ECM33* (Δ*ecm33*) caused the highest increase in BGL activity but the growth of this strain, like that of Δ*gas1* and Δ*sag1*, was too slow to be suitable for industrial application (data not shown). Among the deletion strains that grew as wild type, Δ*sed1* and Δ*tos6* displayed the highest BGL activity (Fig. 4).

TABLE 1. PCR primers used in this study.

Product name purpose	Primer name	Primer sequence[a] (5′–3′)
DNA fragment contains ColE1 *ori*, Amp^r and *CEN6*/*ARS4*	CAC-F	CGCGCGGCCGCACTGACTCGCTGCGCTCGG (*Not*I)
	CAC-R	GCGGGACGTCGGACGGATCGCTTGCCTGTA (*Aat*II)
sed1-URA3-1		
Deletion of *SED1*	sed1-URA3-1-1F	GCCCTCTTTTGAACTGTCATATAAATACCT
	sed1-URA3-1-1R	ACACCACCGTCTTAATAGAGCGAACGTATT
	sed1-URA3-1-2F	CTCTATTAAGACGGTGGTGTTTGACACATC
	sed1-URA3-1-2R	CAGCCACATTATCTTCCATAAATGTGCTGA
	sed1-URA3-1-3F	TATGGAAGATAATGTGGCTGTGGTTTCAGG
	sed1-URA3-1-3R	TTAGATTCGGGGGTAATAACTGATATAATT
	sed1-URA3-1-4F	GTTATTACCCCCGAATCTAAGGGCACTACC
	sed1-URA3-1-4R	TTATAAGAATAACATAGCAACACCAGCCAA
sed1-URA3-2		
Deletion of *SED1* and its promoter region	sed1-URA3-2-1F	GAAGCAGCGACGATCGTAACTATATTGTCA
	sed1-URA3-2-1R	ACACCACCGTTTAACCTTCACTATCATAAA
	sed1-URA3-2-2F	TGAAGGTTAAACGGTGGTGTTTGACACATC
	sed1-URA3-2-2R	=sed1-URA3-1-2R
	sed1-URA3-2-3F	=sed1-URA3-1-3F
	sed1-URA3-2-3R	=sed1-URA3-1-3R
	sed1-URA3-2-4F	=sed1-URA3-1-4F
	sed1-URA3-2-4R	=sed1-URA3-1-4R
SED1 (full length)	SED1(LOH)1F	CGAGGTCGACATGAAATTATCAACTGTCCT (*Sal*I)
Amplification of the fragment cloned into the integrative plasmid used in the second stage	SED1(LOH)1R	TTAGATTCGTTAACTGGGACAGAAGACTCA (*Hpa*I)
	SED1(LOH)2F	TCCCAGTTAACGAATCTAAGGGCACTACCA (*Hpa*I)
	SED1(LOH)2R	GCAGGAATTCTTATAAGAATAACATAGCAA (*Eco*RI)
Confirmation of disruption	SED1(down)R	TCGGATGATAGGTTTGCACAGCTGGATCCT

[a] Restriction sites for the enzymes shown in parentheses are underlined.