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Efficient co-displaying and artificial ratio control of α-amylase and glucoamylase on the yeast cell surface by using combinations of different anchoring domains

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Abstract Recombinant yeast strains that display heterologous amylolytic enzymes on their cell surface via the glycosylphosphatidylinositol (GPI)-anchoring system are considered as promising biocatalysts for direct ethanol production from starchy materials. For the effective hydrolysis of these materials, the ratio optimization of multienzyme activity displayed on the cell surface is important. In this study, we have presented a ratio control system of multienzymes displayed on the yeast cell surface by using different GPI-anchoring domains. The novel gene cassettes for the cell-surface display of *Streptococcus bovis* α-amylase and *Rhizopus oryzae* glucoamylase were constructed using the *Saccharomyces cerevisiae SED1* promoter and two different GPI-anchoring regions derived from *Saccharomyces cerevisiae SED1* or *SAG1*. These gene cassettes were integrated into the *Saccharomyces cerevisiae* genome in different combinations. Then, the cell-surface α-amylase and glucoamylase activities and ethanol productivity of these recombinant strains were evaluated. The combinations of the gene cassettes of these enzymes affected the ratio of cell-surface α-amylase and glucoamylase activities and ethanol productivity of the recombinant strains. The highest ethanol productivity from raw starch was achieved by the strain harboring one α-amylase gene cassette carrying the *SED1*-anchoring region and two glucoamylase gene cassettes carrying the *SED1*-anchoring region (BY-AASS/GASS/GASS). This strain yielded 22.5±0.6 g/L of ethanol from 100 g/L of raw starch in 120 h of fermentation.

Keywords *Saccharomyces cerevisiae* · Cell-surface display · α-Amylase · Glucoamylase · Ratio control

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Introduction

The search for practical petroleum substitutes from renewable resources has become a global priority to combat the rapid rise in atmospheric carbon dioxide levels. Starchy materials are important feedstock for bioethanol production, as they contain a large amount of polysaccharides such as amylose and amylopectin (Fukuda et al. 2009). The most commonly used microorganism for industrial ethanol fermentation is *Saccharomyces cerevisiae*. Compared to bacteria, it demonstrates faster sugar consumption rate, provides a higher ethanol yield from glucose, and has greater resistance to ethanol and other compounds present in various biomass resources (Lau et al. 2010; Olsson and Hahn-Hägerdal 1993; Palmqvist et al. 1999). However, native *Saccharomyces cerevisiae* cannot utilize starchy materials without liquefaction and saccharification processes, which are both expensive and time-consuming. Therefore, it is important to develop an efficient and cost-effective process for ethanol production from starchy materials.

A promising strategy for the construction of efficient whole-cell biocatalysts for consolidated bioprocessing (CBP) involves displaying hydrolytic enzymes on the yeast cell

surface via the glycosylphosphatidylinositol (GPI)-anchoring system (Fujita et al. 2002, 2004; Kondo and Ueda 2004; Kotaka et al. 2008; Yamada et al. 2011; Yanase et al. 2010). In this system, a yeast cell is transformed by introducing fusion genes coding for hydrolytic enzymes and the anchoring domain of GPI protein. The fused proteins self-immobilize into the yeast cell wall to enable retention of enzyme activities during the growth of the yeast (Ueda and Tanaka 2000). Because the recovery of the biocatalyst from the products is easy, reutilization of the yeast cells allows reuse of the active enzymes on its cell surface, without the need for the cells to reproduce. Previously, a yeast strain displaying α-amylase (EC 3.2.1.1) and glucoamylase (1,4-α-D-glucan glucohydrolase; EC 3.2.1.3) on its cell surface was constructed via random integration of the multiple α-amylase and glucoamylase genes into the cell genome (δ-integration) (Yamakawa et al. 2012). The displayed enzyme activities of the strain were stably maintained even after 23 cycles of repeated batch fermentations.

The proportion of multienzyme activities and their synergetic effects are important for the effective degradation of polysaccharides. It was reported that the ratio optimization of α-amylase and glucoamylase activities by the response surface method enabled efficient starch hydrolysis (Bao et al. 2011).

The ratio of multienzymes displayed on the yeast cell surface is also important. Because the protein incorporation capacity of yeast cell wall is limited, heterologous GPI-anchoring fusion proteins compete for the limited capacity with the other heterologous and native GPI proteins in the recombinant yeast cell wall (Van der Vaart et al. 1997). Therefore, the ratio optimization process of heterologous hydrolytic enzyme activity on the yeast cell surface is necessary to construct efficient whole-cell biocatalysts for CBP.

In previous studies, the C-terminal domain of α-agglutinin (Sag1), which is a GPI protein in *Saccharomyces cerevisiae*, has been mainly used as an anchoring domain in fusion proteins (Murai et al. 1999; Yamada et al. 2010b; Yamakawa et al. 2012). The genes encoding these fusion proteins were expressed by constitutive promoters such as *TDH3* and *PGK1* promoters. However, the hydrolytic activities on the cell surface by these conventional gene cassettes are insufficient for efficient CBP of starchy substrates. Although the overexpression of target genes by using multicopy genome integration or 2 μ-based multicopy plasmid recombination allows for sufficient enzyme activities, it is difficult to control and optimize the expression levels of each gene by these methods.

We recently developed a novel gene cassette for the efficient cell-surface display of hydrolytic enzymes with the *Saccharomyces cerevisiae SED1* promoter and its anchoring region (Inokuma et al. 2014). The β-glucosidase and endoglucanase activities of recombinant yeast cells transduced with the novel gene cassette were 8.4- and 106-fold higher than those of the cells transduced with the conventional gene cassette carrying the *TDH3* promoter and the *SAG1*-anchoring region. In addition, the novel gene cassette could vary the enzyme-displaying efficiency, depending on its C-terminal anchoring domain. These results indicate the feasibility of the development of a novel ratio control system of the cell-surface multienzyme activities without multicopy genome integration.

The objectives of this study are the construction of efficient α-amylase and glucoamylase co-displaying yeast strains and artificial ratio control of these enzymes on the cell surface. First, we constructed the novel gene cassettes for the cell-surface display of *Streptococcus bovis* α-amylase and *Rhizopus oryzae* glucoamylase by using *Saccharomyces cerevisiae SED1* promoter and its anchoring region. These cassettes were integrated into the genome of *Saccharomyces cerevisiae* BY4741 strain by single-copy integration. Then, the cell-surface α-amylase and glucoamylase activities of the constructed strains were evaluated. Subsequently, direct ethanol production from raw starch was performed by using the recombinant strain displaying both α-amylase and glucoamylase. Finally, we demonstrated the artificial ratio control of the cell-surface α-amylase and glucoamylase activities by using combinations of gene cassettes with different anchoring domains.

Materials and methods

Strains and media

Escherichia coli strain DH5α (Toyobo, Osaka, Japan) was used as the host for recombinant DNA manipulation. *E. coli* transformants were grown at 37 °C in Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride) supplemented with 100 μg/mL of ampicillin. The genetic properties of all yeast strains used in this study are summarized in Table 1. The cell-surface display cassettes of amylolytic enzymes were expressed in the haploid yeast strain *Saccharomyces cerevisiae* BY4741 (Life Technologies, Carlsbad, CA, USA). The yeast transformants were screened and precultured in synthetic dextrose (SD) medium [6.7 g/L yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA) and 20 g/L glucose] supplemented with appropriate amino acids and nucleic acids in a shaker incubator (180 rpm at 30 °C; BR-43FL; Taitec, Saitama, Japan), followed by aerobic culturing in YPD medium [10 g/L yeast extract, 20 g/L Bacto-peptone (Difco Laboratories) and 20 g/L glucose] in a shaker incubator (150 rpm at 30 °C; GBR-200; Taitec). The yeast cells were harvested by centrifugation at 1000×*g* for 5 min, washed twice with distilled water, and recentrifuged at 1000×*g* for 5 min. Wet cell weight of the washed yeast cells was determined by weighing the cell pellet.

Table 1 Characteristics of integrative plasmids used in this study

Plasmids	Relevant genotype	Source/references
pRS403	*HIS3* Integrative vector without display cassette	Agilent Technologies
pRS405	*LEU2* Integrative vector without display cassette	Agilent Technologies
pRS406	*URA3* Integrative vector without display cassette	Agilent Technologies
pIUPGSBAAG	*URA3* Integrative vector with cell-surface display cassette of *Streptococcus bovis* α-*amylase*	Yamakawa et al. (2012)
pδU-PGGlucRAG	*URA3* δ-Integrative vector with cell-surface display cassette of *R. oryzae glucoamylase*	Yamada et al. (2010a)
pIEG-SS	*HIS3* $SED1_P$-*T. reesei EGII-*$SED1_A$-$SAG1_T$	Inokuma et al. (2014)
pIEG-SA	*HIS3* $SED1_P$-*T. reesei EGII-*$SAG1_A$-$SAG1_T$	Inokuma et al. (2014)
pIAA-SS	*HIS3* $SED1_P$-*Streptococcus bovis* α-*amylase-*$SED1_A$-$SAG1_T$	This study
pIGA-SS	*HIS3* $SED1_P$-*R. oryzae glucoamylase-*$SED1_A$-$SAG1_T$	This study
pIGA-SA	*HIS3* $SED1_P$-*R. oryzae glucoamylase-*$SAG1_A$-$SAG1_T$	This study
pIL2GA-SS	*LEU2* $SED1_P$-*R. oryzae glucoamylase-*$SED1_A$-$SAG1_T$	This study
pIL2GA-SA	*LEU2* $SED1_P$-*R. oryzae glucoamylase-*$SAG1_A$-$SAG1_T$	This study
pIU5GA-SS	*URA3* $SED1_P$-*R. oryzae glucoamylase-*$SED1_A$-$SAG1_T$	This study
pIU5GA-SA	*URA3* $SED1_P$-*R. oryzae glucoamylase-*$SAG1_A$-$SAG1_T$	This study

S. bovis Streptococcus bovis, *R. oryzae Rhizopus oryzae*, *T. reesei Trichoderma reesei*

The estimated dry cell weight of a yeast cell is approximately 0.15-fold its wet cell weight (Inokuma et al. 2014). Cell pellets were used for enzyme assays and ethanol fermentation.

Plasmid construction and yeast transformation

The plasmids and primers used in this study are listed in Tables 2 and 3, respectively. The 3′ half of the *SAG1*-coding region (963 bp) and the full length of the *SED1*-coding region, except for the start codon, (1014 bp) were used as the *SAG1*- and *SED1*-anchoring regions, respectively. The integrative plasmid for cell-surface display of *Streptococcus bovis* α-amylase was constructed as follows: The DNA fragments encoding prepro-α-factor and *Streptococcus bovis* α-amylase were amplified from plasmid pIUPGSBAAG (Yamakawa et al. 2012) by PCR with the AA-F and AA-R primers. Similarly, the vector fragment containing *SED1* promoter, the sequence for *SED1*-anchoring region, and *SAG1* terminator was amplified from pIEG-SS (Inokuma et al. 2014) by PCR with the SSvector-F and SSvector-R primers. These fragments were connected by the isothermal assembly method (Gibson et al. 2009), and the resulting plasmid was named as pIAA-SS.

The integrative plasmids for cell-surface display of *R. oryzae* glucoamylase with *SED1*-anchoring region were constructed as follows: The DNA fragment encoding *R. oryzae* glucoamylase was amplified from plasmid pδU-PGGlucRAG (Yamada et al. 2010a) by PCR with the GA-F and GA-R primers. Similarly, the vector fragment containing *SED1* promoter, the sequence for *SED1*-anchoring region, and *SAG1* terminator was amplified from pIEG-SS by PCR with the SSvector-F2 and SSvector-R2 primers. These fragments were connected by the isothermal assembly method (Gibson et al. 2009), and the resulting plasmid was named pIGA-SS. The DNA fragment encoding a part of the 3′ noncoding region of *YFL021W* and *YFL020C* genes (I2 region) was amplified from *Saccharomyces cerevisiae* BY4741 genomic DNA by PCR with the I2-F and I2-R primers. The DNA fragment

Table 2 Characteristics of yeast strains used in this study

Strains	Relevant genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Invitrogen
BY-403	BY4741/pRS403	Inokuma et al. (2014)
BY-AASS	BY4741/pIAA-SS	This study
BY-GASS	BY4741/pIGA-SS	This study
BY-AASS/GASS	BY-AASS/pIL2GA-SS	This study
BY-AASS/GASA	BY-AASS/pIL2GA-SA	This study
BY-AASS/GASS/GASS	BY-AASS/GASS/pIU5GA-SS	This study
BY-AASS/GASS/GASS	BY-AASS/GASS/ pIU5GA-SA	This study
BY-AASS/GASA/GASA	BY-AASS/GASA/pIU5GA-SA	This study

Table 3 PCR primers used in this study

Primers	Sequence
AA-F	aatacgttcgctctattaagatgagatttccttcaatttttactgc
AA-R	aataggacagttgataatttcttgtcatcgtcatccttgtagtc
SSvector-F	acaaggatgacgatgacaagaaattatcaactgtcctattatctgcc
SSvector-R	aaaattgaaggaaatctcatcttaatagagcgaacgtattttattttg
GA-F	aatacgttcgctctattaagatgcaactgttcaatttgcc
GA-R	gttgataatttactcgagccagcggcaggtgcaccagccttag
SSvector-F2	aggctggtgcacctgccgctggctcgagtaaattatcaactgtcc
SSvector-R2	ggcaaattgaacagttgcatcttaatagagcgaacgtatttt
I2-F	tgtactgagagtgcaccatagggaatacctcgtcaaaacaagac
I2-R	acctgagtattcccacagttgatgtggaaatataagttatgcaagag
LEU2-F	ataacttatatttccacatcaactgtgggaatactcaggtatcg
LEU2-R	tttcacaccgcatagatccgctacgtcgtaaggccgtttct
GASSvector-F	gaaacggccttacgacgtagcggatctatgcggtgtgaaatac
GASSvector-R	tgttttgacgaggtattccctatggtgcactctcagtacaatctg
I5-F	tgtactgagagtgcaccatattgttgtgtaaatgttctatctgacact
I5-R	attgaattgaaaagctgtggcaggttgtgctcactgtatatagtctc
URA3-F	tatacagtgagcacaacctgccacagcttttcaattcaattcatc
URA3-R	tttcacaccgcatagatccggggtaataactgatataattaaattgaag
GASSvector-F2	aattatatcagttattaccccggatctatgcggtgtgaaatac
GASSvector-R2	atagaacatttacacaacaatatggtgcactctcagtacaatctg
GA-R2	gagcttttggcgctcgagccagcggcaggtgcaccagccttag
SAvector-F	aggctggtgcacctgccgctggctcgagcgccaaaagc

encoding *LEU2* was amplified from pRS405 (Agilent Technologies, Santa Clara, CA, USA) by PCR with the LEU2-F and LEU2-R primers. Similarly, the vector fragment containing the gene cassette for the cell-surface display of glucoamylase was amplified from pIGA-SS by PCR with the GASSvector-F and GASSvector-R primers. These three fragments were connected by the isothermal assembly method (Gibson et al. 2009), and the resulting plasmid was named pIL2GA-SS. The DNA fragment encoding a part of the 3′ noncoding region of *YLL055W* and *YLL054C* genes (I5 region) was amplified from *Saccharomyces cerevisiae* BY4741 genomic DNA by PCR with the I5-F and I5-R primers. The DNA fragment encoding *URA3* was amplified from pRS406 (Agilent Technologies) by PCR with the URA3-F and URA3-R primers. Similarly, the vector fragment containing the gene cassette for cell-surface display of glucoamylase was amplified from pIGA-SS by PCR with the GASSvector-F2 and GASSvector-R2 primers. These three fragments were connected by the isothermal assembly method (Gibson et al. 2009), and the resulting plasmid was named pIU5GA-SS.

The integrative plasmids for the cell-surface display of *R. oryzae* glucoamylase with *SAG1*-anchoring region were constructed as follows: The DNA fragment encoding *R. oryzae* glucoamylase was amplified from plasmid pδU-PGGlucRAG by PCR with the GA-F and GA-R2 primers. Similarly, the vector fragment containing *SED1* promoter, the sequence for *SAG1*-anchoring region, and *SAG1* terminator was amplified from pIEG-SA (Inokuma et al. 2014) by PCR with the SAvector-F and SSvector-R2 primers. These fragments were connected by the isothermal assembly method (Gibson et al. 2009), and the resulting plasmid was named pIGA-SA. The vector fragment containing the gene cassette for the cell-surface display of glucoamylase was amplified from pIGA-SA by PCR with the GASSvector-F and GASSvector-R primers. This vector fragment was connected with the DNA fragments encoding I2 region and *LEU2* by the isothermal assembly method (Gibson et al. 2009), and the resulting plasmid was named pIL2GA-SA.

The vector fragment containing the gene cassette for the cell-surface display of glucoamylase was amplified from pIGA-SA by PCR with the GASSvector-F2 and GASSvector-R2 primers. This vector fragment was connected with the DNA fragment encoding I5 region and *URA3* by the isothermal assembly method (Gibson et al. 2009), and the resulting plasmid was named pIU5GA-SA.

The pIAA-SS was digested with *Msc*I within the *HIS3*. Other plasmids were digested with *Nde*I within the *HIS3*, the I2 region, or the I5 region. Then, the linearized plasmids were transformed into *Saccharomyces cerevisiae* BY4741 by the lithium acetate method (Chen et al. 1992) and integrated into each of the homologous regions of the chromosomal DNA by homologous recombination.

Enzyme assay

α-Amylase and glucoamylase activities of the yeast cells were evaluated by using α-amylase and saccharification assay kits (Kikkoman Corp., Chiba, Japan), as described previously (Yamakawa et al. 2012). For both the enzymes, 1 unit of the enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per minute.

Ethanol fermentation from raw starch

Ethanol fermentation of the raw corn starch was performed in a 50-mL polypropylene tube (Corning Inc., Corning, NY, USA) by using a heat block (Thermo Block Rotator SN-06BN; Nissin, Tokyo, Japan) as described previously (Matano et al. 2012). *Saccharomyces cerevisiae* strains were cultivated in 500 mL of YPD medium for 48 h at 30 °C under aerobic conditions. The yeast cells were collected by centrifugation at 1000×*g* for 10 min at 20 °C and washed twice with distilled water. The cells were then resuspended in 10 mL of the fermentation medium [10 g/L yeast extract, 20 g/L Bactopeptone, and 100 g/L raw corn starch (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 0.5 g/L potassium pyrosulfite (Nacalai Tesque, Inc., Kyoto, Japan) to prevent contamination by anaerobic bacteria] at an initial cell

concentration of 50 g wet cell/L. Fermentation was initiated by the addition of yeast cells into the tube, followed by axial rotation at 25 rpm for 120 h at 35 °C. The ethanol concentration in the fermentation medium was determined by high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan), as described previously (Hasunuma et al. 2011).

Growth assay

The yeast transformants were precultured in 5 mL of YPD medium in a shaker incubator at 180 rpm for 18 h at 30 °C. The preculture medium was inoculated into 5 mL of YPD medium in an L-shaped test tube at an initial optical density of 0.05 at 660 nm (OD_{660}). The yeast cells were then cultured at 30 °C, and the OD_{660} of the cell suspension was measured every 30 min by using the TVS062CA Bio-photorecorder (Advantec Toyo, Tokyo, Japan). The μmax values were calculated from the growth rates from 4 to 6 h by using the following equation: $\mu = 2.303\ (\log_{10}OD2 - \log_{10}OD1)/(t2 - t1)$. The optical densities of cells at times $t1$ (4 h) and $t2$ (6 h) were termed as OD1 and OD2, respectively.

Results

Construction of yeast strains

A haploid yeast strain, *Saccharomyces cerevisiae* BY4741, was used as the host strain in this study. Figure 1 shows the gene cassettes constructed for the cell-surface display of α-amylase and glucoamylase. The AASS cassette encodes the prepro-α-factor, the *Streptococcus bovis* α-amylase, and the full length of *Saccharomyces cerevisiae SED1* as a GPI-anchoring region. The GASS and GASA cassettes encode *R. oryzae* glucoamylase with its original secretion signal peptide, fused with the full-length *SED1*, and the 3′-half of *Saccharomyces cerevisiae SAG1* as the GPI-anchoring regions, respectively. All cassettes have the *SED1* promoter and the *SAG1* terminator. The plasmids containing these cassettes are listed in Table 2. The plasmid with the AASS cassette was integrated into the *HIS3* locus of the chromosomal DNA by homologous recombination. The plasmids with the GASS and GASA cassettes were integrated into the 3′ noncoding region of *YFL021W* and *YFL020C* genes and/or into the 3′ noncoding region of *YLL055W* and *YLL054C* genes by homologous recombination. The constructed strains listed in Table 1 were used for further experiments.

α-Amylase and glucoamylase activities of recombinant yeast strains

Recombinant yeast strains displaying *Streptococcus bovis* α-amylase (BY-AASS) and *R. oryzae* glucoamylase (BY-GASS) were cultivated aerobically for 96 h. The culture broth was sampled every 24 h, and the enzyme activities in these cells were determined as described in the "Materials and methods" section. The α-amylase activity of BY-AASS reached 14.3±1.4 U/g dry cells at 24 h and then gradually decreased, whereas no significant glucoamylase activity was detected (Fig. 2a). On the other hand, the glucoamylase activity of BY-GASS increased gradually to reach 18.7±0.1 U/g dry cells at 48 h, whereas no α-amylase activity was detected (Fig. 2b).

The recombinant strain displaying both α-amylase and glucoamylase (BY-AASS/GASS) showed high α-amylase and glucoamylase activities (26.5±1.3 U/g dry cells at 24 h and 19.0±0.4 U/g dry cells at 48 h, respectively; Fig. 2c), which were the same or higher than those of the strains displaying either one of the two enzymes (Fig. 2a, b). These results suggest that the gene cassettes carrying the *Saccharomyces cerevisiae SED1* promoter and its anchoring region can be used for the efficient co-displaying of multienzymes on the yeast cell surface.

Direct ethanol fermentation from raw starch

To evaluate the effects of cell-surface display of α-amylase and glucoamylase on the hydrolysis of starchy materials, direct ethanol production from raw starch was performed using the cells of the strain displaying both α-amylase and glucoamylase (BY-AASS/GASS) after 48 h of aerobic

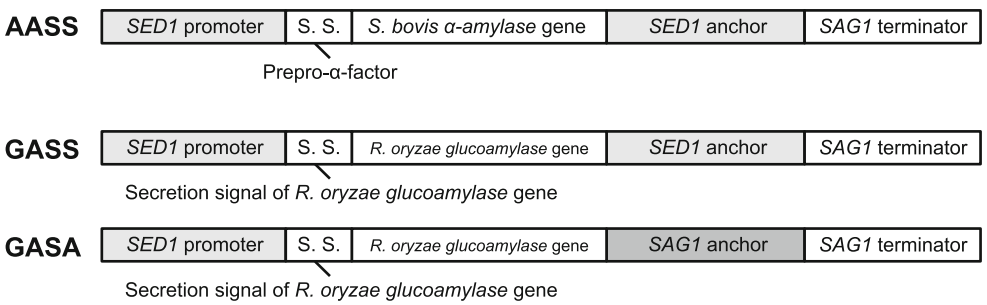

Fig. 1 Construction of novel gene cassettes for the yeast cell-surface display of α-amylase and glucoamylase

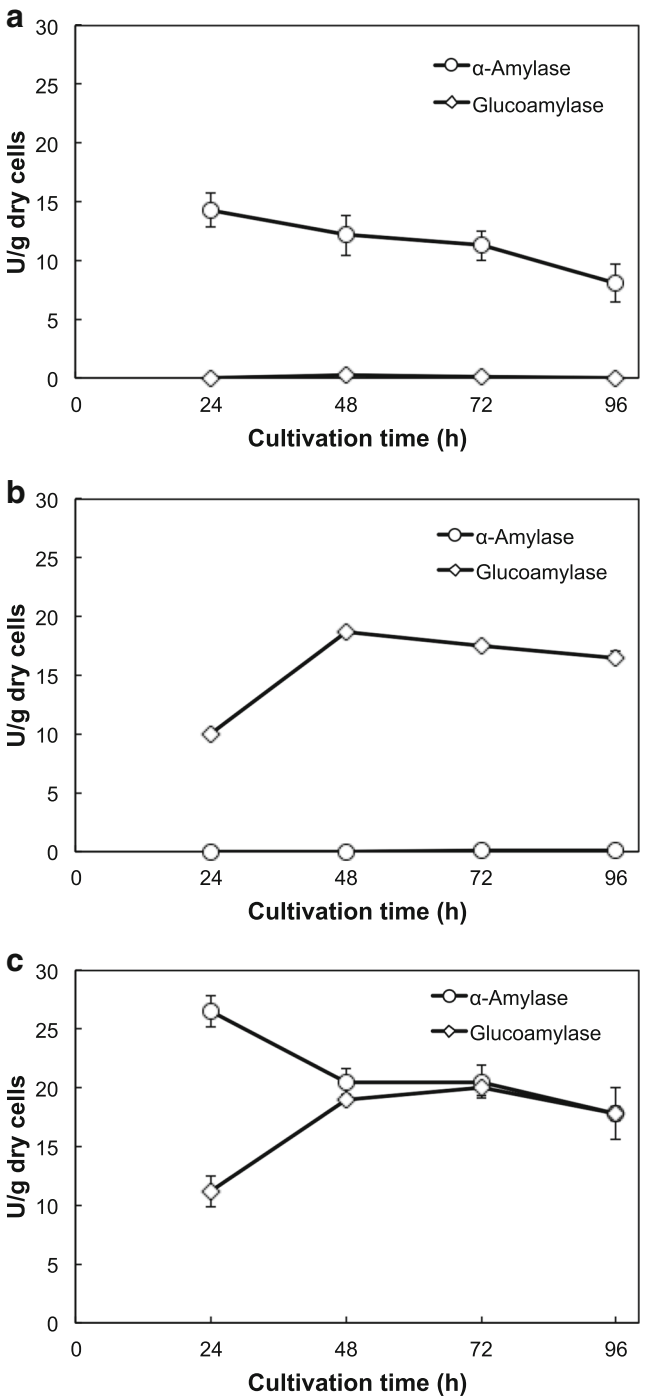

Fig. 2 Time course of α-amylase and glucoamylase activities displayed on yeast cell surface of BY-AASS (**a**), BY-GASS (**b**), and BY-AASS/GASS (**c**) strains. *Error bars* indicate the standard deviations of three independent experiments

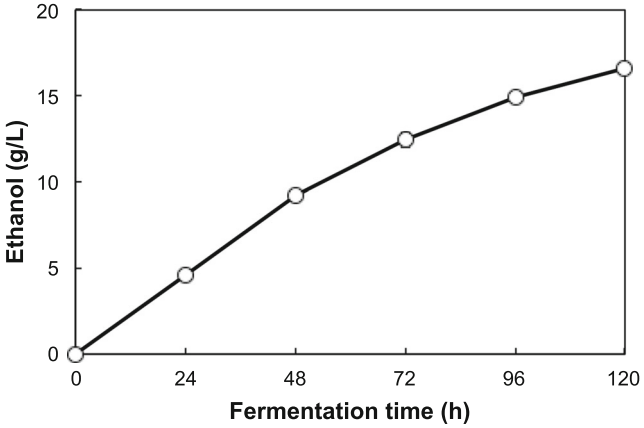

Fig. 3 Time course of direct ethanol production from 100 g/L of raw starch using BY-AASS/GASS strain. *Error bars* indicate the standard deviations of three independent experiments

cultivation. As shown in Fig. 3, the BY-AASS/GASS strain produced 16.6±0.3 g/L of ethanol from raw starch after 120 h of fermentation. Theoretically, the final ethanol concentration is approximately 50.5 g/L (based on 100 g starch/L×0.90 g starch/g raw starch×1.1 g glucose/g starch×0.51 g ethanol/g glucose) (Ohta et al. 1991). Thus, the potential ethanol yield by the BY-AASS/GASS strain in 120 h is approximately 33 %.

Ratio control of cell-surface α-amylase and glucoamylase activities

To control the ratios of α-amylase and glucoamylase displayed on the cell surface, recombinant strains displaying both α-amylase and glucoamylase were constructed using the two types of glucoamylase-displaying cassettes carrying different GPI-anchoring regions (Table 2). The BY-AASS/GASA and BY-AASS/GASA/GASA strains possessed one or two copies of gene cassettes carrying the *SAG1*-anchoring region (GASA cassette in Fig. 1), respectively. The BY-AASS/GASS and BY-AASS/GASS/GASS strains possessed one or two copies of gene cassettes carrying the *SED1*-anchoring region (GASS cassette in Fig. 1), respectively. The BY-AASS/GASS/GASA strain possessed one copy each of GASS and GASA cassettes. The enzyme activities and the ethanol productivity from raw starch of these strains were evaluated. As shown in Fig. 4, the types of the GPI-anchoring regions and the copy number of the glucoamylase-displaying cassettes influenced the ratio of enzyme activity and the ethanol productivity. Among these strains, the BY-AASS/GASS/GASS strain demonstrated the highest ratio of glucoamylase activity to α-amylase activity (1.17 at 48 h cultivation) and ethanol production (22.5±0.6 g/L after 120 h fermentation). This result suggests that the glucoamylase activity is an important bottleneck of direct ethanol production from raw starch using these strains.

Effects of cell-surface display of enzymes on yeast cell growth

To examine the effect of cell-surface display of α-amylase and glucoamylase on yeast cell growth, the growth rate and the final cell density of BY-AASS, AASS/GASS, and

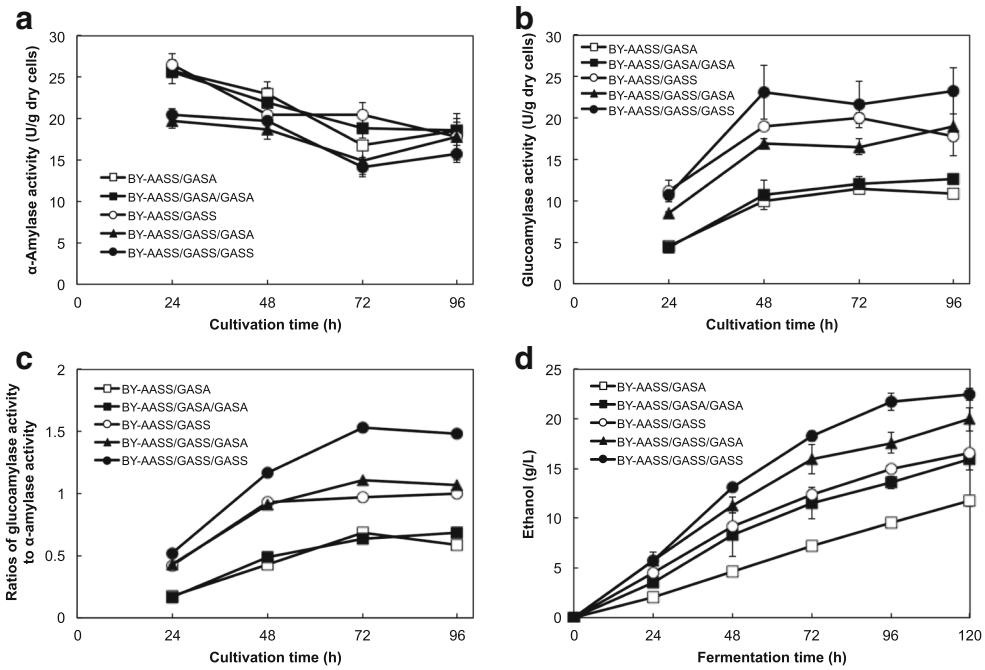

Fig. 4 Time course of α-amylase (**a**) and glucoamylase (**b**) activities displayed on yeast cell surface, ratio of glucoamylase activity to α-amylase activity (**c**), and direct ethanol production from 100 g/L of raw starch (**d**). *Error bars* indicate the standard deviations of three independent experiments

AASS/GASS/GASS strains were evaluated. A similar growth assay for BY-403 strain transformed with an empty vector (pRS403) was performed, as a control for BY-AASS strain. The time courses of cell growth and the growth profiles of these strains are shown in Fig. 5 and Table 4, respectively. The cell-surface display of these enzymes had no significant effect on the growth profiles of the tested strains.

Discussion

In this study, we demonstrated the construction of efficient α-amylase and glucoamylase co-displaying yeast strains and artificial ratio control of these enzymes on the cell surface.

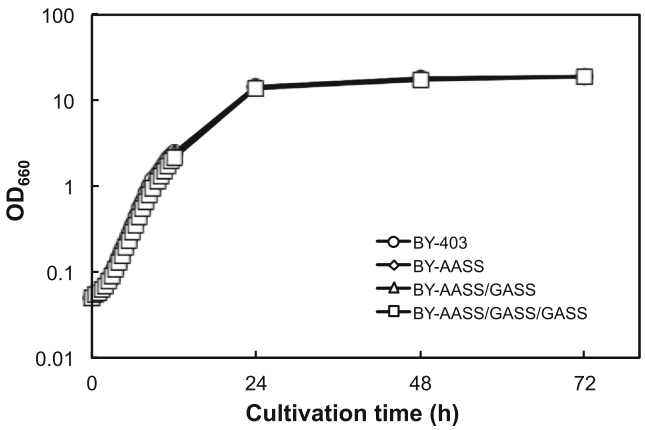

Fig. 5 Cell growth of recombinant yeast strains. *c* indicates the standard deviations of three independent cultivations

The novel recombinant strains displaying both α-amylase and glucoamylase were constructed by single-copy integration method. The cell-surface glucoamylase activities of the recombinant yeast strains were successfully controlled using combinations of glucoamylase-displaying cassettes carrying different GPI-anchoring regions (GASS and GASA cassettes in Fig. 1). To the best of our knowledge, this is the first report on the artificial ratio control of multienzyme activities displayed on the yeast cell surface by using different GPI-anchoring domains.

SED1 encodes a major stress-induced structural GPI protein in *Saccharomyces cerevisiae* (Shimoi et al. 1998). It has previously been reported that the *SED1* promoter indicates high expression level during the stationary phase, and simultaneous utilization of the *SED1* promoter and the sequence for the Sed1 anchoring domain in a gene cassette enabled highly efficient enzyme integration into the cell wall (Inokuma et al. 2014). The cell-surface α-amylase and glucoamylase activity of BY-AASS and BY-GASS strains were 14.3±1.4 U/g dry cells at 24 h and 18.7±0.1 U/g dry cells at 48 h, respectively (Fig. 2a, b). The α-amylase activity of BY-AASS was 8.9-fold

Table 4 Growth profiles of recombinant yeast strains

Strains	μmax (h^{-1})	Final cell density (OD_{660} at 72 h)
BY-403	0.438±0.015	19.2±0.2
BY-AASS	0.426±0.019	19.2±0.5
BY-AASS/GASS	0.433±0.006	19.3±0.2
BY-AASS/GASS/GASS	0.417±0.022	18.8±0.8

higher than that of MT8-1/pIUPGSBAAG (Yamakawa et al. 2012) harboring a single gene cassette carrying the *PGK1* promoter and the *SAG1*-anchoring region (0.24±0.02 U/g wet cells, which is comparable to approximately 1.6 U/g dry cells at 24 h). These results are in good agreement with our previous report (Inokuma et al. 2014).

These gene cassettes were also useful for the efficient co-displaying of α-amylase and glucoamylase with high activities (Fig. 2c). In addition, the efficient cell-surface display of these enzymes showed no negative effect on the growth profiles of the recombinant strains (Fig. 5 and Table 4). These results suggest that recombinant yeast cells displaying multienzymes using efficient enzyme-display cassettes are highly promising biocatalysts for the efficient ethanol production from starch.

In this study, we revealed the relationship between ethanol productivity from raw starch and a ratio of the cell-surface α-amylase and glucoamylase activity of the recombinant strain. The result shown in Fig. 4 indicates that α-amylase activity is sufficient for the degradation of raw starch in all strains constructed in this study, and the glucoamylase activity is an important bottleneck of direct ethanol production from raw starch using these strains. Similar result was previously reported in the ratio optimization of commercial α-amylase and glucoamylase activity for the efficient simultaneous saccharification and fermentation of cassava starch (Bao et al. 2011). The BY-AASS/GASS/GASS strain showed the highest ethanol production from raw starch (22.5±0.6 g/L after 120 h fermentation). The ethanol yield of this strain (approximately 44 % in 120 h) is higher than that of the MT8-1/δGSBAAG strain (approximately 31 % in 120 h) constructed via random genome integration of the multiple α-amylase and glucoamylase gene cassettes (δ-integration) (Yamakawa et al. 2012), although the total copy number of gene cassettes integrated into the genome was only three. This suggests that highly efficient enzyme-display cassettes used in this study allow constructions of multienzyme-displaying strains and ratio control of these enzyme activities without a random genome integration such as the δ-integration method.

The BY-AASS/GASS/GASA and BY-AASS/GASS/GASS strains indicated higher ethanol productivity than the other recombinant strains (Fig. 4d). On the other hand, the α-amylase activity of these strains was slightly lower than that of the other strains (Fig. 4a). These results suggest that α-amylase and glucoamylase compete for the limited protein incorporation capacity of the cell wall of these strains. In this study, the ratios of the displayed enzymes were evaluated using the enzyme activities, but a quantification of each enzyme displayed on yeast cell surface by other experiments would be necessary for more understanding about the protein incorporation capacity and the ratio control system by using combinations of different anchoring domains.

Although the ethanol yield of the recombinant strains displaying both α-amylase and glucoamylase was improved by using the ratio control system, the ratio optimization process remains to be completed. Further ratio control of the cell-surface α-amylase and glucoamylase might be necessary for the construction of the strain optimized for efficient CBP of starchy materials. On the other hand, it was reported that the ethanol yield of α-amylase- and glucoamylase-displaying strains from raw starch could be improved by addition of the substrate-binding domain of *Streptococcus bovis* α-amylase at its N-terminus, diploidization of the yeast strains, and the co-expression of the *Saccharomyces cerevisiae* maltose transporter gene *AGT1* (Yamakawa et al. 2012). The ethanol yield of the recombinant strain from raw starch rose to 88 from 31 % after 120 h of fermentation by these modifications. Similarly, the ethanol yield of the BY-AASS/GASS/GASS strain may also be improved by incorporating these modifications.

A method to optimize the multicellulolytic enzyme activities displayed on the yeast cell surface by the δ-integration method was reported previously (Yamada et al. 2010b). They introduced three cellulase genes into cell genome simultaneously by using the δ-integration method (cocktail δ-integration) and then selected the recombinant strain by its cellulolytic activity. After reiteration of δ-integrations and selections, the recombinant strain with high cellulolytic activity was constructed. The cocktail δ-integration method is advantageous in that it allows ratio optimization of multienzyme expression levels for efficient degradation, even when the optimum cellulase expression ratio is unknown. However, their method is not suitable for the artificial ratio control of multienzyme activity because the copy number of each gene integrated into the genome is random. Even if the ratio-optimized strain can be constructed with this method, it is difficult to reconstruct the recombinant strain with the same ability. On the other hand, the ratio control system used in this study has an advantage in that it allows the artificial ratio control of multienzymes without δ-integration. In this method, we used highly efficient enzyme-display cassettes. The random integration of multiple gene cassettes is not required because these cassettes provide sufficient cell-surface enzyme activities with low-copy integration. Thus, the cell-surface enzyme activity can be easily and artificially controlled by using different combinations of GPI-anchoring domains with distinct cell-surface immobilizing efficiencies. Although both the methods have their respective advantages and disadvantages, a combination of the advantages of both these methods would facilitate the construction of stable multienzyme-displaying strains optimized for efficient CBP of various biomass resources.

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