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Display of active beta-glucosidase on the surface of *Schizosaccharomyces pombe* cells using novel anchor proteins

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Abstract Here, we demonstrate display of beta-glucosidase (BGL) on the surface of *Schizosaccharomyces pombe* cells using novel anchor proteins. A total of four candidate anchor proteins (SPBC21D10.06c, SPBC947.04, SPBC19C7.05, and SPBC359.04c) were selected from among almost all of *S. pombe* membrane proteins. The C-terminus of each anchor protein was genetically fused to the N-terminus of BGL, and the fusion protein was expressed using *S. pombe* as a host. The highest cell surface-associated BGL activity (107 U/10^5 cells was achieved with SPBC359.04c serving as the anchor, followed by SPBC947.04 (44 U/10^5 cells) and SPBC21D10.06c (38 U/10^5 cells). *S. pombe* displaying BGL with SPBC359.04c as an anchor showed the highest growth on 2 % cellobiose (10.7×10^7 cells/mL after 41 h of cultivation from an initial density of 0.1×10^7 cells/mL). Additionally, culturing BGL-displaying *S. pombe* in medium containing cellobiose as the sole carbon source did not affect protein expression, and ethanol fermentation from cellobiose was successfully demonstrated using BGL-displaying *S. pombe*. This is the first report describing a cell surface display system for the functionalization of *S. pombe*.

Keywords *Schizosaccharomyces pombe* · Cell surface display · Beta-glucosidase · Cellobiose · Anchor protein

Introduction

Cell surface display is a versatile research technique, with applications in vaccine development, gene therapy, cell-based diagnostics, high-throughput polypeptide library screening, whole-cell biocatalysis, bioremediation, biosensor development, and even biofuels production (Kondo and Ueda 2004; Chen and Georgiou 2002; Wu et al. 2008). The immobilization of enzymes on surfaces is a crucial step in the development of tailor-made biocatalysts (Bornscheuer 2003). Thus, the construction of novel, recyclable, and regenerative whole-cell biocatalysts by arming the surface of yeast cells with enzymes is of particular interest. Moreover, it has been shown that cell wall immobilization can significantly enhance the stability and regeneration of particular enzymes (Schreuder et al. 1996). The cell surface display technique also has applications in directed evolution, as it facilitates both access of a substrate to an enzyme and selection of cells harboring the genetic information for a corresponding enzyme variant displayed on the surface, thus rendering tedious enzyme preparation and purification steps largely dispensable.

Cell surface display systems are typically based on the fusion of a protein of interest to a natural cell wall protein (Lee et al. 2003; Kondo and Ueda 2004). Baker's yeast (*Saccharomyces cerevisiae*) is an attractive host for expressing biotechnologically, pharmaceutically, and medicinally relevant proteins because the organism is safe to handle and tolerates a variety of cell wall modifications. Several *S. cerevisiae* display systems have been developed and applied in recent years with varying degrees of success

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(Kondo and Ueda 2004; Matsumoto et al. 2002; Wang et al. 2007; Yue et al. 2008). Most of the cell surface display methods developed for *S. cerevisiae* to date are based on the agglutinin and flocculin model systems (Saleem et al. 2008). These cell wall proteins, including alpha-agglutinin, Aga1, Cwp1, Cwp2, Tip1p, Srp1 (Van der Vaart et al. 1997), Flo1p (Tanino et al. 2007; Theunissen et al. 1993), Sed1p (Hardwick et al. 1992), and Tir1p (Marguet et al. 1988) all contain the glycosyl phosphatidylinositol (GPI) signal motif and are covalently crosslinked to beta-1,6-glucan in the cell wall. Other yeast strains have recently been equipped with a variety of functional displayed proteins, including antibodies, enzymes, and even combinatorial protein libraries (Breinig et al. 2006; Lee et al. 2006; Parthasarathy et al. 2006).

The fission yeast *Schizosaccharomyces pombe* is the second most frequently used yeast host, after *S. cerevisiae*. *S. pombe*, which is taxonomically and evolutionarily distant from the budding yeast (Takegawa et al. 2009), has been extensively characterized both genetically and physiologically. In contrast to budding yeast, *S. pombe* has not been widely used in the manufacture of fermented beverages and foods such as wine, beer, and bread. In terms of mRNA splicing, cell division control, and post-translational modifications, *S. pombe* appears to resemble a multicellular organism more than budding yeast (Zhao and Lieberman 1995). *S. pombe* is also being used extensively for high-level heterologous protein production, primarily because it shares many genetic and biochemical characteristics with higher eukaryotes (Russel 1989; Giga-Hama 1997). Recently, many types of recombinant human proteins have been successfully expressed in fission yeast, including transferrin (Mukaiyama et al. 2010), papillomavirus E7 protein, D2S dopamine receptor (Sander et al. 1994), human growth hormone (Idiris et al. 2006), and ScFv-GFP fusion protein (Naumann et al. 2011), in addition to recombinant proteins from other organisms (Giga-Hama and Kumagai 1999). However, to date, no cell surface display system suitable for *S. pombe* has been developed.

In the present study, we developed a novel and useful cell surface display system involving *S. pombe* by screening novel anchor proteins. A cellobiose-assimilating *S. pombe* strain was created by displaying beta-glucosidase (BGL) on the cell surface. To our knowledge, this is the first report describing a cell surface display system utilizing *S. pombe*.

Materials and methods

Bacterial strains and media

The *S. pombe* strains used in this study are listed in Table 1. All strains were grown in complete YM medium (BD Biosciences, San Jose, CA, USA) or EMM broth (ForMedium, Norfolk, UK) with the addition of appropriate auxotrophic supplements (225 mg/L leucine, uracil; Nacalai Tesque, Inc., Kyoto, Japan) when necessary. In some experiments, yeast were grown on EMM broth without dextrose (ForMedium) containing 20 % cellobiose (Sigma-Aldrich Corp., St. Louis, MO, USA) as the sole carbon source. *Escherichia coli* NovaBlue (Novagen, San Diego, CA, USA) was grown in LB medium and served as the host strain for all plasmid manipulations.

Plasmids construction and yeast transformation

Plasmids for BGL expression on the cell surface using various anchor proteins were constructed as follows. To remove the *Sal*I site of BGL gene, the *Aspergillus aculeatus* gene encoding BGL was amplified by PCR using pδW-PGAGBGL (Yamada et al. 2010) as a template with the primer pairs *Nhe*I_*Sal*I_F/BGL_del*Sal*I_R and BGL_del*Sal*I_F/BGL_*Nco*I_R. A second round of PCR was carried out using both amplified fragments as templates with the primers *Nhe*I_*Sal*I_F and BGL_*Nco*I_R. The amplified gene fragment encoding BGL was digested with *Nhe*I/*Nco*I and ligated into plasmid pDUAL-FFH51 (Matsuyama et al. 2006). The resulting plasmid was designated pDUAL51-BGL.

The gene fragment encoding a candidate anchor protein was amplified by PCR using *S. pombe* FY7132 genomic DNA as a template with the primers SPBC21D10.06c_F and SPBC21D10.06c_R. The amplified fragment was digested with *Nhe*I/*Sal*I and ligated into plasmid pDUAL51-BGL. The resulting plasmid was designated pDUAL51-SPBC21D10.06c_BGL. The genes encoding the candidate anchor proteins SPBC947.04, SPBC19C7.05, and SPBC359.04c were amplified using the following primer pairs: SPBC947.04_F/SPBC947.04_R, SPBC19C7.05_F/SPBC19C7.05_R, and SPBC359.04c_R/SPBC359.04c_R, respectively. Each amplified fragment was digested with *Nhe*I/*Sal*I and ligated into plasmid pDUAL51-BGL. The resulting plasmids were designated pDUAL51-SPBC947.04_BGL, pDUAL51-SPBC19C7.05_BGL, and pDUAL51-SPBC359.04c_BGL. In these constructs, the C-terminus of the anchor protein was fused to the N-terminus of BGL.

All plasmids were introduced into *S. pombe* FY7132 (YGRC/NBRP) or *S. pombe* OB1 (kindly provided by Prof. Kawamukai at Shimane University) after *Not*I digestion as described in a previous report (Matsuyama et al. 2006). The resulting strains were designated SPBC21D10.06c-BGL-7132, SPBC947.04-BGL-7132, SPBC19C7.05-BGL-7132, SPBC359.04c-BGL-7132, SPBC21D10.06c-BGL-OB1, SPBC947.04-BGL-OB1, SPBC19C7.05-BGL-OB1, and SPBC359.04c-BGL-OB1 (Table 1).

Table 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant phenotype, description, or sequence (5′-3′)	Source
S. pombe strains		
FY7132	*h90 leu1-32*	YGRC/NBRP
OB1	*h90 leu1-32 ura4-D18*	Kindly provided Prof. Kawamukai
SPBC21D10.06c-BGL-7132	FY7132 expressing SPBC21D10.06c-BGL fusion protein under tif51 promoter	This study
SPBC947.04-BGL-7132	FY7132 expressing SPBC947.04-BGL fusion protein under tif51 promoter	This study
SPBC19C7.05-BGL-7132	FY7132 expressing SPBC19C7.05-BGL fusion protein under tif51 promoter	This study
SPBC359.04c-BGL-7132	FY7132 expressing SPBC359.04c-BGL fusion protein under tif51 promoter	This study
SPBC21D10.06c-BGL-OB1	OB1 expressing SPBC21D10.06c-BGL fusion protein under tif51 promoter	This study
SPBC947.04-BGL-OB1	OB1 expressing SPBC947.04-BGL fusion protein under tif51 promoter	This study
SPBC19C7.05-BGL-OB1	OB1 expressing SPBC19C7.05-BGL fusion protein under tif51 promoter	This study
SPBC359.04c-BGL-OB1	OB1 expressing SPBC359.04c-BGL fusion protein under tif51 promoter	This study
Plasmids		
pDUAL-FFH51	Vector under tif51 promoter control	RIKEN BRC
pDUAL2-FFG1	Vector for EGFP expression	RIKEN BRC
pDUAL51-BGL	Vector for BGL expression, from *Aspergillus aculeatus*	This study
pDUAL51-SPBC21D10.06c_BGL	Vector for BGL expression using SPBC21D10.06c anchor protein; the C-terminus of SPBC21D10.06c was fused to the N-terminus of BGL	This study
pDUAL51-SPBC947.04_BGL	Vector for BGL expression using SPBC947.04 anchor protein; the C-terminus of SPBC947.04 was fused to the N-terminus of BGL	This study
pDUAL51-SPBC19C7.05_BGL	Vector for BGL expression using SPBC19C7.05 anchor protein; the C-terminus of SPBC19C7.05 was fused to the N-terminus of BGL	This study
pDUAL51-SPBC359.04c_BGL	Vector for BGL expression using SPBC359.04c anchor protein; the C-terminus of SPBC359.04c was fused to the N-terminus of BGL	This study
Oligonucleotide primers		
*Nhe*I_*Sal*I_F	TACGCTAGCGTCGACGATGAACTGGCGTTCTCTCCTCCTTTCTACCCC	
BGL_del*Sal*I_R	CCCTCGGGCACGTGCTCCGCGGTGTCCACTCCATAGTACGGGTCGCCAGATGAGGCCTTCAGG	
BGL_del*Sal*I_F	CCTGAAGGCCTCATCTGGCGACCCGTACTATGGAGTGGACACCGCGGAGCACGTGCCCGAGGG	
BGL_*Nco*I_R	GCATCCATGGTCATTGCACCTTCGGGAGCGCCGCGTGAAGGGGC	
SPBC21D10.06c_F	GCATGCTAGCATGAATTCATACGCAATTTTATTGTCTCTTTTCTTTTCATTCGAAAGG	
SPBC21D10.06c_R	GCATGTCGACATATTCCAAAGCTCTAAGAACAACATTAGCCGC	
SPBC947.04_F	GCATGCTAGCATGAGCCTGTTTCCCCAAATTCTACTAAGGC	
SPBC947.04_R	GCATGTCGACTAAAGGCACAGCCTTAAAGTAAACATTTGATGCACG	
SPBC19C7.05_F	GCATGCTAGCATGAATGACGATAGCAGCAGCAGTAGCAGCGGCGATTCC	
SPBC19C7.05_R	GCATGTCGACCCCAAGCTTAGCATATCCATCGTATTTGCGAAAAGC	
SPBC359.04c_F	GCATGCTAGCATGAATTCGTTAAAGTCACTTTGCCTTAAATGTATTGTG	
SPBC359.04c_R	GCATGTCGACATAGGCCACGAGAATAACATCGTCCGCCC	

Evaluation of BGL activity

Beta-glucosidase activity was screened using a simple plate assay as follows. *S. pombe* FY7132 carrying one of the BGL expression plasmids was seeded on an EMM agar plate. After overnight incubation at 30 °C, 0.75 % soft agar containing 0.034 % 4-methylumbelliferyl-β-D-glucoside (Nacalai Tesque, Inc., Kyoto, Japan) in 50 mM acetate buffer (pH 5.0) was overspread on the plate. The plate was incubated for an additional 16 h at 30 °C, after which the fluorescence of the colonies was observed using a UV Transilluminator FAS-III (Toyobo, Osaka, Japan).

Beta-glucosidase activity was quantitatively assessed in 50 mM sodium acetate buffer (pH 5.0) at 30 °C with 2 mM *p*-nitrophenyl-β-D-glucopyranoside (pNPG) (Nacalai Tesque) as the substrate. After 24-h cultivation in EMM medium, the cells were separated by centrifugation, washed three times with PBS, and resuspended in reaction buffer. The amount of *p*-nitrophenol released was determined by measuring the absorbance at 400 nm. One unit of β-glucosidase activity was defined as the amount of enzyme producing 1 μmol *p*-nitrophenol/min at 37 °C and pH 5.0.

Growth analysis

Growth on cellobiose was evaluated by incubating cells at 30 °C on EMM without glucose but containing 2 % cellobiose. The initial number of cells was adjusted to 0.1×10^7 cells/mL using hemocytometer and microscope.

Fluorescence microscopy analysis and flow cytometric analysis

The EGFP expression plasmid pDUAL2-HFG1 (Matsuyama et al. 2006) was introduced into BGL-expressing *S. pombe* OB1 cells using uracil auxotrophy. Cells were cultivated EMM without glucose but containing 2 % cellobiose for 24 h and observed with fluorescent microscopy. For flow cytometric analysis, the cells were collected and diluted into test tubes containing sheath solution, and green fluorescence was measured using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The green fluorescence signal from 10,000 cells was excited with a 488-nm argon laser and collected through a 530/30-nm band-pass (FL1) filter. The data were analyzed using BD CELLQuest software (BD Biosciences).

Ethanol fermentation from cellobiose or glucose

For ethanol fermentation, yeast cells were aerobically grown in 100 ml of YM medium at 30 °C for 48 h. Cells were centrifuged at 3,000×*g* for 5 min and inoculated into fermentation medium (YM medium containing additional 20 g/l glucose or cellobiose as sole carbon source). The initial cell density was adjusted to 10^7 cells/mL. Ethanol fermentation was carried out at 30 °C with mild agitation in 50 ml bottles.

Ethanol, cellobiose, and glucose concentrations were simultaneously measured using HPLC (Shimadzu, Kyoto, Japan) with a Shim-pack SPR-Pb column (Shimadzu, Kyoto, Japan). The operating conditions were carried out at 80 °C, with water as the mobile phase at a flow rate of 0.6 ml/min, and detection was performed with a refractive index detector (Shimadzu RID10A). HPLC analysis was applied to a sample of culture supernatant after separating the culture broth by centrifugation at 14,000×*g* for 10 min.

Results

Expression of active BGL on the surface of *S. pombe* cells

We cloned 31 *S. pombe* membrane protein genes to identify candidate anchor proteins (Table S1). These proteins seems to be membrane-localized, because their function (including predicted function) contain "cellular protein" and/or "membrane", and/or "surface". The cloned genes were genetically fused to the N-terminus of *A. aculeatus* BGL (Yamada et al. 2010) and integrated into *S. pombe* FY7132 genome using leucine auxotrophic maker. The anchor-BGL fusion protein was expressed using *S. pombe* FY7132 as a host under constitutive promoter tif51 promoter. The BGL activity of each fusion protein was evaluated using a plate assay. Four *S. pombe* colonies carrying the following anchor protein genes, SPBC21D10.06c, SPBC947.04, SPBC19C7.05, and SPBC359.04c showed bright fluorescence when 4-methylumbelliferyl-β-D-glucoside was used as a substrate (data not shown). The activity of BGL on the cell surface and in the culture medium was quantitatively evaluated for each of these colonies using pNPG as a substrate. After 24 h of cultivation in EMM, the cell numbers were 6.0×10^7 cells/mL (SPBC21D10.06c), 6.5×10^7 cells/mL (SPBC947.04), 5.5×10^7 cells/mL (SPBC19C7.05), and 5.5×10^7 cells/mL (SPBC359.04c), which suggest almost as same levels among these strains. When *S. pombe* FY7132 served as the host (Fig. 1a, b), the highest BGL activity was associated with SPBC359.04c (85 U/10^5 cells), followed by SPBC947.04 (8.5 U/10^5 cells), SPBC21D10.06c (8.0 U/10^5 cells), and SPBC19C7.05 (0.71 U/10^5 cells). In the case of *S. pombe* OB1 serving as the host, the highest BGL activity was associated with SPBC359.04c (107 U/10^5 cells), followed by SPBC947.04 (45 U/10^5 cells), SPBC21D10.06c (38 U/10^5 cells), and SPBC19C7.05 (2.5 U/10^5 cells). With both strains, the BGL activity associated with the cell surface was greater than that in the culture supernatant (under 10 U/mL), demonstrating that BGL was successfully expressed on the surface of

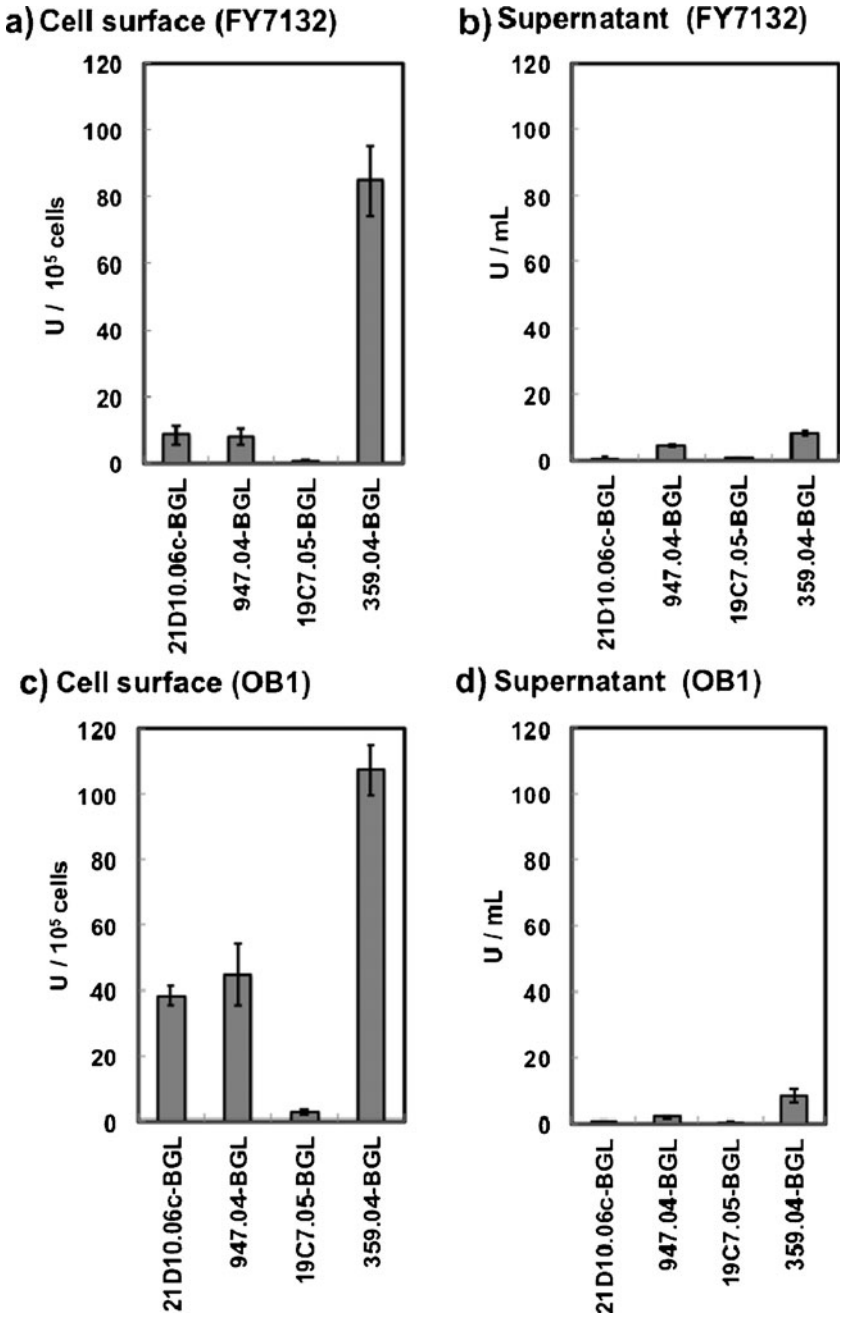

Fig. 1 Activity of BGL on the surface of *S. pombe* cells (**a** and **c**) or in the culture supernatant (**b** and **d**) after 24 h of cultivation in EMM medium with the addition of appropriate auxotrophic supplements. *S. pombe* FY7132 (**a** and **b**); *S. pombe* OB1 (**c** and **d**). SPBC21D10.06c, SPBC947.04, SPBC19C7.05, and SPBC359.04c represent the anchor proteins used. The N-terminus of BGL was fused to the C-terminus of each anchor protein. All data are averages from three independent experiments, and *error bars* represent the standard deviation

S. pombe cells and that fusion with an anchor protein did not affect the enzyme's function.

Growth of BGL-displaying *S. pombe* using cellobiose as the sole carbon source

We also evaluated the ability of BGL-displaying *S. pombe* strains FY7132 and OB1 to grow on 2 % cellobiose or glucose as the sole carbon source (Fig. 2). The initial cell density was 0.1×10^7cells/mL. From Fig. 2a, after 21 h of cultivation, the density of FY7132 using SPBC359.04c as the anchor was approximately 7.5×10^7 cells/mL and 6.2×10^7 cells/mL for FY7132 with SPBC947.04 as the anchor, both of which were slightly lower than the densities achieved after 24 h of growth on 2 % glucose (11.7×10^7cells/mL for SPBC359.04c and 11.1×10^7 cells/mL for SPBC947.04, respectively). After 41 h, the cell densities grown on cellobiose reached approximately 9.2×10^7 cells/mL (SPBC359.04c) and 7.7×10^7 cells/mL (SPBC947.04).

Strain OB1 using SPBC359.04c as the anchor reached 5.7×10^7 cells/mL after 17 h of cultivation when cellobiose was used as carbon source, which was higher than other OB1

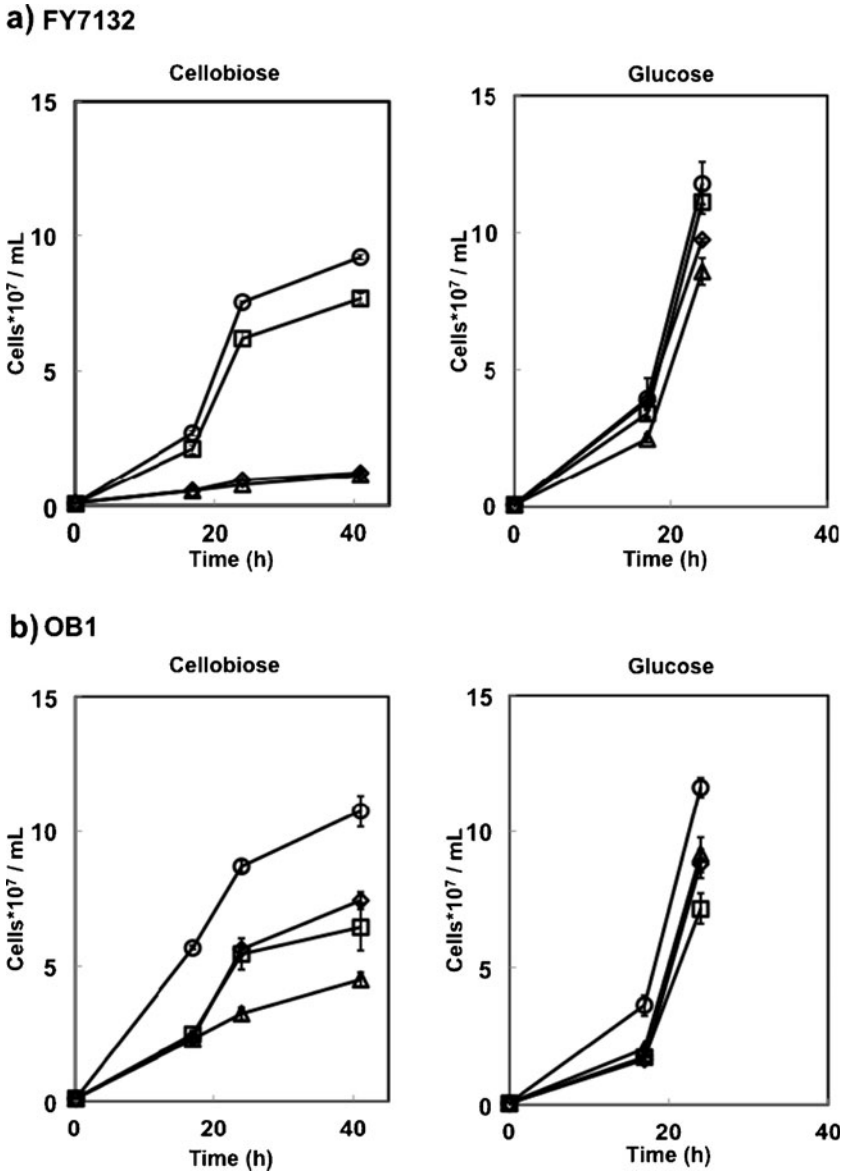

Fig. 2 Growth of BGL-displaying *S. pombe* in medium containing cellobiose (*left panels*) or glucose (*right panels*) as the sole carbon source. **a** *S. pombe* FY7132; **b** *S. pombe* OB1. *Symbols* shown in panels **a** and **b** are as follows: SPBC21D10.06c-BGL (*diamonds*), SPBC947.04-BGL (*squares*), SPBC19C7.05-BGL (*triangles*), and SPBC359.04c-BGL (*circles*). All data are averages from three independent experiments, and *error bars* represent the standard deviation

strains. After 41 h of cultivation, OB1 using SPBC359.04c as the anchor reached 10.7×10^7 cells/mL, which was almost the same as that from glucose (11.6×10^7 cells/mL; Fig. 2b). Cells carrying only the pDUAL control plasmid did not increase in number (data not shown), demonstrating that BGL-displaying *S. pombe* can directly assimilate cellobiose.

Protein expression in BGL-displaying *S. pombe* with cellobiose serving as the sole carbon source

Protein expression by BGL-displaying *S. pombe* strains growing on a medium containing cellobiose as the sole carbon source was evaluated using enhanced green fluorescent protein (EGFP) as a model protein. The EGFP expression multicopy plasmid pDUAL2-FFG1 (Matsuyama et al. 2006) was introduced into BGL-expressing *S. pombe* OB1 cells using uracil auxotrophy. Bright fluorescence was observed after 24 h of cultivation (Fig. 3a), demonstrating that protein expression is unaffected in BGL-expressing cells using cellobiose as the sole carbon source. Figure 3b shows the fluorescence intensity of each BGL-expressing strains. BGL-expressing *S. pombe* strains carrying control vector showed no fluorescence (Fig. 3b, upper panels); however, BGL-expressing *S. pombe* strains carrying EGFP expression vector showed blight fluorescence and almost as same levels among these strains (Fig. 3b, lower panels). Although cell growth was dependent on the anchor protein (Fig. 2a and b), the EGFP fluorescence intensity from a single cell was almost same levels among these *S. pombe* strains.

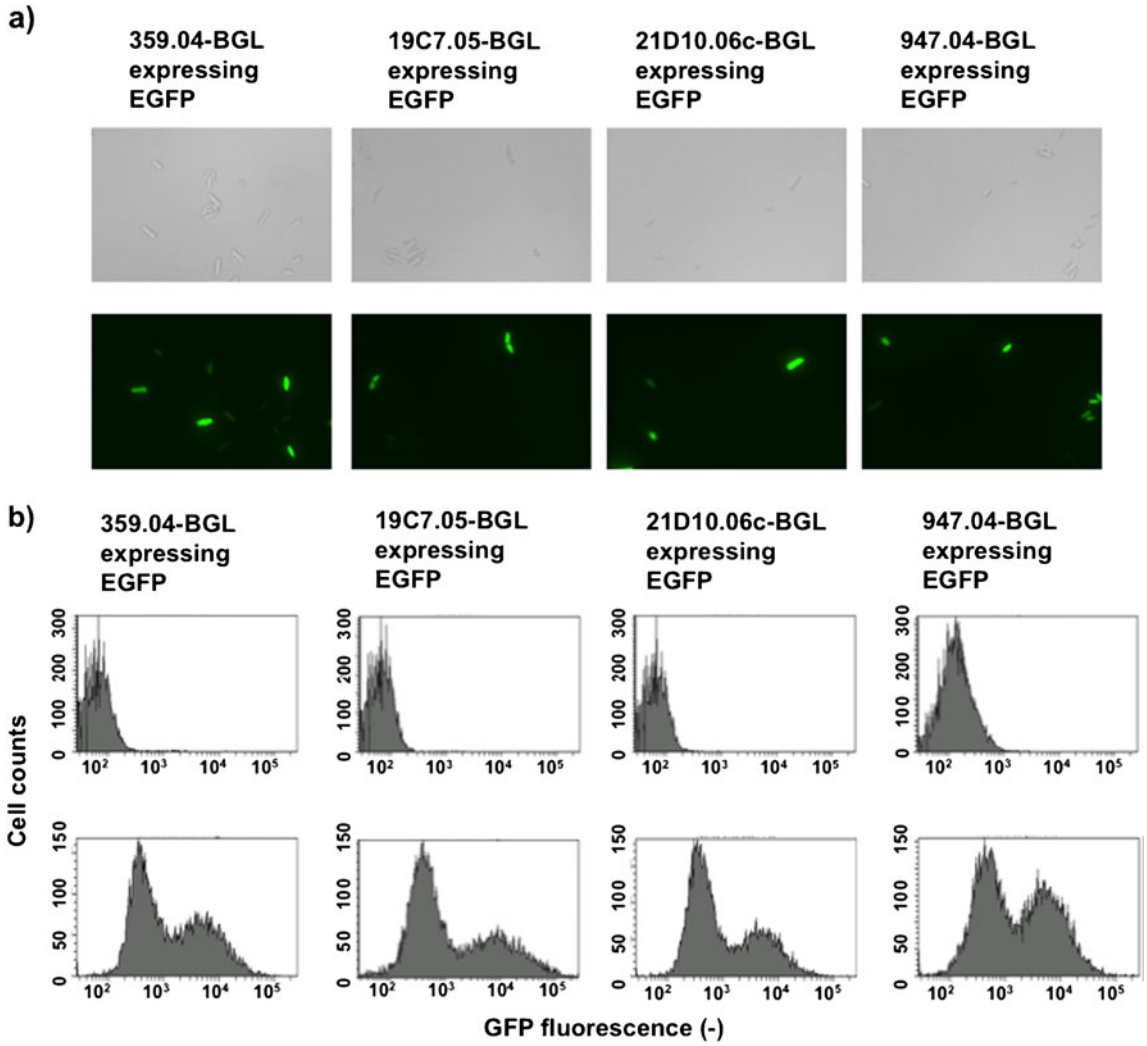

Fig. 3 Fluorescence analysis of *S. pombe* carrying the EGFP expression plasmid and displaying BGL fused with various anchor proteins. (**a**) Images of fluorescence microscopy. Upper panels show transmission images and lower panels show fluorescence images; (**b**) Flow cytometer analysis. Upper panels show BGL-expressing *S. pombe* carrying empty vectors as controls, and lower panels show BGL-expressing *S. pombe* carrying EGFP-expression vectors. Cells were examined after 24 h of cultivation in medium containing cellobiose as the sole carbon source

Ethanol fermentation from cellobiose using BGL-displaying *S. pombe*

To evaluate ethanol productivity of BGL-displaying *S. pombe*, ethanol fermentations from cellobiose or glucose were performed (Fig. 4a, b). After 24 h of fermentation at 30 °C, SPBC359.04c and SPBC947.04 consumed cellobiose and produced 13.2 g/L ethanol (Fig. 4a). The ethanol yield from the initial sugars (including 10 g/L glucose contained YM medium) reached 70 % of the theoretical yield. SPBC21D10.06c and SPBC19C7.05 consumed only a little cellobiose (3.6 and 7.5 g/L of consumed cellobiose, respectively) and the ethanol produced was only 4.0 and 4.3 g/L, respectively, which were produced from glucose contained in YM medium. *S. pombe* FY7132 carrying empty vector, as a control strain, did not consume cellobiose (Fig. 4a). In the case of glucose, all strains consumed glucose completely and produced about 11 g/L of ethanol with 72 % of the theoretical yield in 24 h (Fig. 4b).

Discussion

The aim of this study was to develop a cell surface display system using *S. pombe* as a host. Using BGL as a model protein, we identified several suitable novel anchor proteins and demonstrated that *S. pombe* cells displaying BGL on the surface are capable of growth on media containing

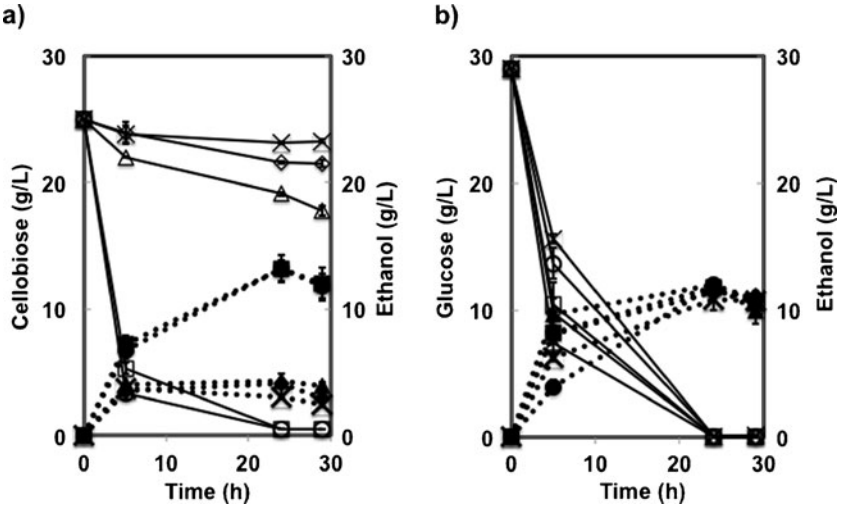

Fig. 4 Time course of ethanol production from **a** cellobiose and **b** glucose using BGL-displaying *S. pombe* strains. *Open symbols* and *solid lines* show sugar concentration and *closed symbols* and *dotted lines* show ethanol concentration. *Symbols* shown in panels **a** and **b** are as follows: SPBC21D10.06c-BGL (*diamonds*), SPBC947.04-BGL (*squares*), SPBC19C7.05-BGL (*triangles*), SPBC359.04c-BGL (*circles*), and FY7132 carrying empty vector (*crosses*) as a control. All data are averages from three independent experiments, and *error bars* represent the standard deviation

cellobiose as the sole carbon source. Finally, ethanol fermentation from cellobiose was successfully demonstrated using these BGL-displaying *S. pombe* strains.

The entire *S. pombe* genome has been sequenced and all of the proteins encoded have been annotated. We tested 31 proteins as candidates to anchor BGL for display on the surface of *S. pombe* cells. These proteins seems to be membrane-localized, because their function (including predicted function) contain "cellular protein" and/or "membrane", and/or "surface" in the *S. pombe* genome database (http://old.genedb.org/genedb/pombe/). A total of four proteins were selected as suitable anchor candidates: SPBC21D10.06c, SPBC947.04, SPBC19C7.05, and SPBC359.04c, because other candidates showed no BGL activities when they were used as an anchor protein. Cells expressing BGL fused with SPBC359.04c showed high BGL activity on the cell surface (Fig. 1a, c) and grew on media containing cellobiose as the sole carbon source (Fig. 2a, b).

The SPBC21D10.06c anchor protein was the largest, at 949 a.a., followed by SPBC947.04 (973 a.a.), SPBC359.04c (358 a.a.), and SPBC19C7.05 (150 a.a.). With the exception of SPBC19C7.05, all of these anchor proteins are larger than *S. cerevisiae* Aga1 (320 a.a.). The four anchor proteins we evaluated have a predicted signal sequence at the N-terminus but do not have a GPI anchor motif. The sequence of the most suitable anchor protein we identified, SPBC359.04c, is similar to that of both SPBC947.04 and SPAC186.01; however, no cell surface BGL activity was observed when SPAC186.01 was used as the anchor protein (data not shown). Sharifmoghadam and Valdivieso (2008) designated SPBC21D10.06c as MAP4. The N-terminal serine- and threonine-rich domain of MAP4 is required for cell wall localization (Sharifmoghadam and Valdivieso 2008). Several similar proteins were identified by a BLAST search, primarily from *Schizosaccharomyces japonicus* (Sipiczki et al. 1998). Although we also tried to use all of the membrane proteins on the surface from *S. japonicus* as anchors, we found that none were suitable as anchors for BGL (data not shown).

The highest cell surface-associated BGL activity (85 U/10^5 cells of *S. pombe* FY7132 and 107U/10^5 cells of *S. pombe* OB1) was achieved using SPBC359.04c as the anchor. The slight difference in BGL activities when using a same anchor protein in different host strains might be might be due to the specific character of each *S. pombe* strain. This activity was somewhat lower than that reported for BGL-displaying *S. cerevisiae* (Yamada et al. 2010). However, there are several ways in which the BGL activity of our cell surface display system can be improved, such as through optimization of the linker between BGL and the anchor protein (Washida et al. 2001), and optimization of the promoter and expression system (Kuroda et al. 2009; Idiris et al. 2010). Development of a more suitable anchor protein might also improve the activity of the displayed protein (Tanaka et al. 2011). As shown in Figs. 1 and 2, BGL activities were not necessary correlated the cell growth rate on cellobiose, which corresponds to the previous report (Tanaka et al. 2011). One possible explanation is that the difference of the substrate, pNPG (BGL activity evaluation) and cellobiose (cell growth), might cause some effects such as accessibility to the cell surface. However, these results suggest that the evaluation of growth on cellobiose as well

as the evaluation of BGL activity is important for enhancing direct cellobiose assimilation.

Using EGFP as a model protein, we also demonstrated protein expression in BGL-displaying *S. pombe* cells grown on medium containing cellobiose as the sole carbon source. Although growth of *S. pombe* displaying BGL on the cell surface depends on BGL activity, the amount of EGFP expressed did not depend upon the anchor protein used (Fig. 3). These results suggest that protein expression is not affected by cellobiose serving as the sole source of carbon, and that our *S. pombe* BGL display system is suitable for protein production from cellulosic materials used as a carbon source.

There are numerous reports of cell surface display systems using the yeast *S. cerevisiae* as a host. Amylase-displaying *S. cerevisiae* is capable of direct fermentation of starch (Yamakawa et al. 2010), and direct fermentation of cellulose has been demonstrated in cellulase-displaying yeast (Lilly et al. 2009). In addition, lipase-displaying yeast cells have been used as whole-cell biocatalysts. Other yeasts, such as *Pichia pastoris*, *Yarrowia lipolytica*, and *Kluyveromyces lactis* can be used as hosts for cell surface display. In this study, we identified several novel *S. pombe* proteins suitable for use as anchors to display BGL on the cell surface. It is possible that these anchor proteins would be suitable for use with other yeast species as well.

For several decades, *S. pombe* has been used as a model organism to study biological ethanol fermentation and protein production (Choi et al. 2010). Although additional research is needed to improve the protein and ethanol production capabilities of *S. pombe*, this organism has a number of characteristics that make it attractive for environmentally friendly ethanol fermentation and protein production. For example, the genetic and physiological background of *S. pombe* has been well-characterized, and the organism is capable of faster fermentation rates and can grow under both aerobic and anaerobic conditions. In addition, *S. pombe* has an exceptional flocculation capability, high ethanol and osmotic tolerance, is easy to manipulate, and can be safely used in food products (Flores et al. 2000; Ge et al. 2005; Hu et al. 2003; Humberto de Queiroz et al. 1993). In this study, ethanol fermentation from cellobiose was successfully demonstrated using BGL-displaying *S. pombe* (Fig. 4). The cell surface display system described here will facilitate the use of *S. pombe* in biomass assimilation such as cellobiose and whole-cell biocatalysis, thereby expanding the potential uses for this organism. Our results suggest the possibility to produce useful compounds from cellobiose as well as glucose.

In summary, we developed a *S. pombe* cell surface display system involving expression of BGL anchored to one of four different cell surface proteins: SPBC21D10.06c, SPBC947.04, SPBC19C7.05, or SPBC359.04c. We also demonstrated that BGL-displaying *S. pombe* is capable of direct growth on cellobiose and that protein expression is unaffected when cellobiose serves as the sole carbon source. Finally, ethanol fermentation from cellobiose was successfully demonstrated using BGL-displaying *S. pombe*. The system we describe can be used for cell surface display of other functional proteins, such as cellulases and lipases. We are currently developing a whole-cell biocatalyst system using *S. pombe* as a host.

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Conflict of interest The authors declare that they have no conflict of interest.

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