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Direct ethanol fermentation of the algal storage polysaccharide laminarin with an optimized combination of engineered yeasts

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Abstract

Laminarin is the algal storage glucan and represents up to 35% of the dry weight of brown macroalgae. In this study, a novel laminarinase, Gly5M, was first found using focused proteome analysis of a laminarin-assimilating marine bacterium, *Saccharophagus degradans*, and the encoding gene was isolated. A Gly5M-displaying yeast strain was prepared with the cell surface display system using *Saccharomyces cerevisiae*. It showed a laminarin-degrading activity on the cell surface and caused the dominant accumulation of gentiobiose. The obtained gentiobiose was converted into glucose and could be assimilated by an *Aspergillus aculeatus* β -glucosidase (BG)-displaying yeast strain. When Gly5M- and BG-displaying yeasts were anaerobically cultivated together in fermentation medium containing 20 g/L laminarin as a sole carbon source, the coculture system with the combination of optimized ratios of the 2 yeast strains directly produced 5.2 g/L ethanol. This coculture system of the 2 engineered yeast strains would be a platform for the use of laminarin and contribute to the complete utilization of brown macroalgae.

Keywords: Macroalgae, Laminarin, *Saccharomyces cerevisiae*, Cell surface engineering, Coculture, Ethanol

1. Introduction

Brown macroalgae are a promising resource for the production of biofuels and renewable chemicals (Enquist-Newman et al., 2014; Horn et al., 2000a; Takagi et al., 2016b; Wargacki et al., 2012; Wei et al., 2013). They can inhabit diverse environments and grow at rates that far exceed those of terrestrial biomass without the requirement of arable land, fresh water, or fertilizer (Ross et al., 2008). Since they do not contain lignin, which is a persistent material and essential for structural support in most terrestrial plants (John et al., 2011), simple biorefinery processes can efficiently produce sugars from brown macroalgae (Wargacki et al., 2012). Moreover, commercial-scale cultivation of edible brown macroalgae is already being practiced in many countries, and the process from harvest to fermentation of this biomass has been established (Enquist-Newman et al., 2014; Roesijadi et al., 2010). Considering these aspects, the utilization of brown macroalgae for biofuel production is desirable to prevail against negative impacts on the environment and food supplies.

Laminarin is a β -1,3-glucan with occasional β -1,6-branches, with a degree of polymerization of 20–40 (Pang et al., 2005; Sova et al., 2013). It is a storage polysaccharide produced in brown macroalgae and represents 0–35% of the dry weight, depending on the algal species, harvesting season, habitat, and method of extraction (Kadam et al., 2015). Moreover, laminarin has structural variations with different ratios of β -1,3/1,6-bonds among algal species. For instance, laminarin from *Eisenia bicyclis* is characterized by a high content of 1,6-bound glucose residues (the ratio of β -1,3/1,6-bonds is 3:2) present in both the branches and the main chain (Menshova et al., 2014). Meanwhile, laminarin from *Laminaria digitata* contains β -1,6-bound glucose residues as singular branches from the major chain of β -1,3-glucan, and the ratio of β -1,3/1,6- bonds is about 7:1 (Pang et al., 2005).

70 Recently, degradation of alginate, which is one of the major components of brown
71 macroalgae, and bioethanol production from its monosaccharide 4-deoxy-L-erythro-5-
72 hexoseulose uronic acid were demonstrated (Enquist-Newman et al., 2014; Takagi et al.,
73 2016b; Wargacki et al., 2012). Moreover, many studies have developed the ways to utilize
74 other carbohydrates composing brown macroalgae such as mannitol, cellulose, and
75 hemicellulose (Chujo et al., 2015; Lin and Tanaka, 2006). However, the complete utilization
76 of macroalgae cannot be realized without the exploitation of the algal storage glucan,
77 laminarin. Although several studies have reported degradation of laminarin by laminarinase
78 (β -1,3-endoglucanase or β -1,3-exoglucanase), laminarin was broken down incompletely and
79 resulted in the accumulation of oligosaccharides due to the lack of harnessing a β -glucosidase
80 (Kawai et al., 2006; Kumagai and Ojima, 2010). Furthermore, while ethanol fermentation of
81 laminarin has been attempted with several microbes (Adams et al., 2009; Adams et al., 2011;
82 Horn et al., 2000a, b; Lee and Lee, 2011; Mountfort and Rhodes, 1991), the yeast
83 *Saccharomyces cerevisiae*, which is one of the most beneficial hosts for bioethanol
84 production, has not been engineered for direct ethanol fermentation of laminarin.

85 In this study, we focused on a marine bacterium *Saccharophagus degradans* 2–40 with
86 a powerful ability to degrade marine biomass (Andrykovitch and Marx, 1988; Weiner et al.,
87 2008). This bacterium has multi-component enzyme systems to degrade at least 10 different
88 kinds of carbon polysaccharides, including cellulose, hemicellulose, agar, pectin, alginate,
89 and laminarin (Hutcheson et al., 2011). Therefore, we screened laminarinases derived from
90 *S. degradans* to be displayed on the *S. cerevisiae* cell surface based on the result of qualitative
91 proteome analysis. The technology of yeast cell surface display has several advantages
92 (Kuroda and Ueda, 2013): (i) yeast cells can be used as whole-cell biocatalysts without

purification of enzymes, (ii) enzymes can be readily recycled by the recovery of yeast cells, and (iii) degradation products can be efficiently uptaken by yeast cells because the displayed enzymes are localized on the yeast cell surface. After the confirmation of the display of a laminarinase and its activity toward laminarin, the further degradation of laminarin by β -glucosidase-displaying yeast was examined. Finally, we demonstrated direct ethanol production from laminarin by the coculture system composed of the 2 yeast strains displaying laminarinase or β -glucosidase.

2. Materials and Methods

2.1. Strains, media, and culture conditions

S. degradans 2-40 (ATCC 43961) was precultured in minimal media containing 2.3% (w/v) Instant Ocean salt mixture (Aquarium Systems, Mentor, OH, USA), 0.2% (w/v) glucose, 0.05% (w/v) ammonium chloride, 0.1% (w/v) yeast extract, and 50 mM Tris-HCl (pH 7.4) at 30°C for 12 h. Cultured cells were harvested by centrifugation at $6000 \times g$ for 10 min at 25°C and resuspended in fresh minimal media containing 0.2% (w/v) glucose (Nacalai Tesque, Kyoto, Japan) or laminarin from *E. bicyclis* (Nacalai Tesque), and the cell growth at 30°C was monitored by measuring the optical density at 600 nm (OD₆₀₀) in triplicate.

Escherichia coli strain DH5 α (F⁻, ϕ 80dlacZ Δ M15, Δ (lacZYA-argF) U169, *deoR*, *recA1*, *endA1*, *hsdR17* (r_K⁻, m_K⁺), *phoA*, *supE44*, λ ⁻, *thi-1*, *gyrA96*, *relA1*; Toyobo, Osaka, Japan) was used as the host for recombinant DNA manipulation and grown in Luria-Bertani medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) sodium chloride] containing 100 μ g/mL ampicillin.

S. cerevisiae strain BY4741/ Δ *sed1* (Kuroda et al., 2009) (*MATa*, *his3*, *leu2*, *met15* Δ 0,

ura3, *YDR077w::kanMX4*; EUROSCARF, Frankfurt, Germany) was used for the cell surface display of enzymes. Yeast host cells were grown in yeast extract peptone dextrose medium [1% (w/v) yeast extract, 2% (w/v) glucose, and 2% (w/v) peptone] for transformation. For the activity assay of laminarinase or β -glucosidase, cells displaying laminarinase or β -glucosidase were cultivated in synthetic dextrose (SDC) medium [0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, 2% (w/v) casamino acids, 0.003% (w/v) L-leucine, 0.002% (w/v) L-tryptophan, 0.002% (w/v) L-histidine, and 0.002% (w/v) adenine] buffered at pH 6.0 with 50 mM 2-morpholinoethanesulfonic acid (MES).

2.2. Sample preparation for proteome analysis

For discovering candidate enzymes for yeast cell surface display, samples were prepared from *S. degradans* as previously described with minor modifications (Takagi et al., 2016a). After cultivation, each cell culture was instantly cooled in ice-cold water and centrifuged at $6000 \times g$ for 10 min at 4°C. Harvested cells were resuspended in 700 μ L lysis buffer [2% (w/v) 3-(3-cholamidepropyl) dimethylammonio-1-propanesulfonate, 10 mM dithiothreitol, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), 7 M urea, and 2 M thiourea in 50 mM Tris-HCl (pH 7.5)]. The cells were then disrupted by sonication using a Bioruptor UCD-250T sonicator (Cosmo Bio, Tokyo, Japan) (250 W, 15 s on and 15 s off/15 cycles, on ice). The crude solution was centrifuged at $12,000 \times g$ for 10 min at 4°C, and the supernatant was obtained. After reduction, alkylation, and digestion by trypsin of each supernatant, the tryptic digests were subjected to nano liquid chromatography (LC)-mass spectrometry (MS)/MS analysis (Esaka et al., 2015).

2.3. LC-MS/MS analysis

Focused proteome analysis was performed as previously described (Morisaka et al., 2012). Tryptic digests were separated by reversed-phase chromatography using a long liquid chromatographer (Ultimate 3000; Thermo Fisher Scientific, Waltham, MA, USA) equipped with a monolithic silica capillary column (200 cm × 0.1 mm ID) coupled to a MS/MS (LTQ Velos Mass Spectrometer; Thermo Fisher Scientific) system at a flow rate of 500 nL/min. Each sample analysis was performed with 3 biological replicates in the same way as previously described (Takagi et al., 2016a).

2.4. Data analysis

The MS data of each sample were used for protein identification using the MASCOT algorithm (Matrix Science, London, UK), working on Proteome Discoverer 1.4 (Thermo Fisher Scientific) against the amino acid sequence data of *S. degradans* 2-40, including the 4007 sequences from NCBI (<http://www.ncbi.nlm.nih.gov/>) with a peptide tolerance of 1.2 Da, MS/MS tolerance of 0.8 Da, and maximum number of missed cleavages of 1. For trypsin digestion, cysteine carbamidomethylation (+57.021 Da) was set as a variable modification. The data were then filtered at a q value ≤ 0.01 corresponding to a 1% false discovery rate on a spectral level. Proteins identified by at least 2 peptides per protein from any data of 3 biological replicates were accepted as “identified proteins.”

2.5. Construction of plasmids

All of the primers used for constructing plasmids are listed in Table 1, and KOD-Plus-Neo DNA polymerase (Toyobo) was used for PCR. The gene encoding Gly5M (Sde_3023) was PCR-amplified from *S. degradans* 2-40 genomic DNA using infusion Gly5M-F and

infusion Gly5M-R primers. The gene encoding *Aspergillus aculeatus* BG I (BG) was PCR-amplified from pBG (Nakanishi et al., 2012) using infusion BG-F and infusion BG-R primers. Each of the amplified fragments was inserted into the vector pULD1 (Kuroda et al., 2009) using the In-Fusion HD cloning kit (Takara Bio Inc., Shiga, Japan) after digestion by *Bgl*II and *Xho*I. The resulting plasmids were named pGLY5M and pBGI, respectively.

2.6. Yeast transformation

Constructed plasmids were introduced into yeast using the lithium acetate method (Ito et al., 1983), with the Frozen-EZ Yeast Transformation Kit (Zymo Research, Irvine, CA, USA). Transformants were selected on SDC plate medium containing the appropriate amino acids.

2.7. Immunofluorescence microscopy

To confirm the display of enzymes on the yeast cell surface, cells were immunofluorescently labeled using a FLAG epitope tag as follows: Cells were incubated in phosphate-buffered saline (PBS; pH 7.4) containing 1% (w/v) bovine serum albumin for 30 min prior to immunostaining. A mouse monoclonal anti-FLAG M2 antibody (1:300 dilution; Sigma-Aldrich) was used as the primary antibody. A mixture of cells and antibody were incubated for 1.5 h at room temperature on a rotator, and the cells were washed with PBS (pH 7.4). As the secondary antibody, Alexa Fluor 488- conjugated goat anti-mouse IgG antibody (1:300 dilution; Invitrogen, Carlsbad, CA, USA) was incubated with cells for 1.5 h at room temperature on a rotator. After washing with PBS (pH 7.4), the cells were suspended in 30 μ L of PBS (pH 7.4) and observed by fluorescence microscopy. Fluorescence was

detected using an inverted microscope IX71 (Olympus, Tokyo, Japan) through a UMNIBA2 mirror unit with a BP470-490 excitation filter, DM505 dichroic mirror, and BA510-550 emission filter (Olympus). Live images were obtained using the Aqua Cosmos 2.0 software program (Hamamatsu Photonics, Shizuoka, Japan) to control a digital charge-coupled device camera (Hamamatsu Photonics).

2.8. Thin layer chromatography analysis

After precultivation in SDC medium buffered at pH 6.0 for 24 h at 30°C, the Gly5M- or BG-displaying yeast cells were cultivated in SDC medium buffered at pH 6.0 for 24 h at 30°C and collected by centrifugation at $3000 \times g$ for 5 min at 4°C. After the cells with an OD₆₀₀ of 20 were washed with PBS (pH 7.4), 0.5 mL of the cell suspension was mixed with 0.5 mL of 0.5% (w/v) laminarin or gentiobiose solution dissolved in PBS (pH 7.4). The mixture was incubated at 30°C for 12 h for Gly5M-displaying yeast or 30 min for BG-displaying yeast, on a rotator. Then, the mixture was centrifuged at $3000 \times g$ for 5 min at 4°C, and the supernatant was collected. The changes in the carbohydrate profile after reaction by displayed enzymes were examined using thin layer chromatography (TLC). Degradation products were developed with ethyl acetate/acetic acid/water (3:2:1, by volume) and visualized using 20% (v/v) sulfuric acid solution in ethanol containing 0.2% (w/v) 1,3-naphthalenediol (Nacalai Tesque), followed by heating the TLC plate at 80°C for 5 min.

2.9. Ethanol production

After Gly5M- and BG-displaying yeast cells were individually cultivated in SDC medium buffered at pH 6.0 for 48 h at 30°C, the yeast cells were centrifuged at $3000 \times g$ for

5 min at 4°C. The harvested cells were inoculated into the fermentation medium containing 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) laminarin, 2% (w/v) casamino acids, 0.003% (w/v) L-leucine, 0.002% (w/v) L-tryptophan, 0.002% (w/v) L-histidine, and 0.002% (w/v) adenine, buffered at pH 7.0 with 50 mM MES. The total initial OD₆₀₀ in the fermentation medium was adjusted to 50, varying the mixture ratio of Gly5M- and BG-displaying yeast cells. All fermentations were performed in closed tubes at 30°C on a rotator.

2.10. Measurement of sugars and ethanol

After filtering by an Ultrafree-MC 0.45-mm centrifugal filter device (Millipore, MA, USA), gentiobiose in degradation products from laminarin and ethanol in the fermentation medium were analyzed by high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) together with a RID-10A refractive index detector (Shimadzu) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). The HPLC system was operated at 60°C with 5 mM H₂SO₄ (flow rate, 0.75 mL/min) as the mobile phase. The concentration of total sugars was determined using the phenol-sulfuric acid method (Dubois et al., 1956).

3. Results

3.1. Cell growth and qualitative proteome analysis of *S. degradans* cultivated in the presence of laminarin or glucose

To confirm whether *S. degradans* can assimilate laminarin, we cultivated the cells in minimal medium containing laminarin or glucose, respectively (Fig. 1). In both media, the cell density continued to increase until 12 h and then decreased at 15 h. From this result, we confirmed the growth of *S. degradans* in the presence of laminarin or glucose as a sole carbon

source and determined a culture time of 12 h for the following proteome analysis.

To identify necessary enzymes for laminarin degradation, qualitative proteome analysis was performed when *S. degradans* was cultured in the presence of laminarin or glucose as a sole carbon source. The obtained mass spectrometry data were used for protein identification by comparison with the sequence data of *S. degradans* from NCBI (<http://www.ncbi.nlm.nih.gov/>). Consequently, with the cut-off criteria, we successfully identified 92 laminarin-specifically produced proteins, including 6 carbohydrases, in the samples from 3 biological replicates (Table S1). Among the carbohydrases, Gly16G (94.2 kDa), Lam16B (158.6 kDa), and Gly5M (94.9 kDa) belonged to glycoside hydrolases family 5 or 16, which suggested that these carbohydrases might be laminarinases according to the Carbohydrate-Active Enzymes (CAZy) database (<http://www.cazy.org/>). Although Gly16G and Lam16B had already been predicted as laminarinases (Hutcheson et al., 2011), the catalytic machinery of Gly16G was missing according to NCBI. In addition, the molecular weight of Lam16B was quite large and may cause an insufficient display efficiency (Kondo and Ueda, 2004). On the other hand, the function of Gly5M remained unknown (Taylor et al., 2006; Zhang and Hutcheson, 2011). With these considerations, we supposed that Gly5M might be a novel laminarinase and hence selected this enzyme for the yeast cell surface display.

3.2. Construction of Gly5M-displaying yeast and its characterization

In order to display Gly5M on the yeast cell surface, a multi-copy expression plasmid pGLY5M (Fig. 2a) was constructed and transformed into the *S. cerevisiae* BY4741/ Δ *sed1* strain. Immunofluorescence staining was performed to confirm the display of each enzyme

on the yeast cell surface with mouse monoclonal anti-FLAG M2 antibody. Yeast cells transformed with pULD1-s (Kuroda et al., 2009), which contains a strep-tag instead of a FLAG-tag, were used as a negative control. Consequently, Gly5M was successfully displayed (Fig. 2b). After Gly5M-displaying yeast cells were reacted with laminarin for 12 h, the degradation products of laminarin were detected by TLC analysis (Fig. 3). The results showed that Gly5M-displaying yeast had a degradation activity toward laminarin and generated oligosaccharides. They were confirmatively analyzed by HPLC, and the main degradation product was identified as gentiobiose, which is a disaccharide of glucose with a β -1,6-bond (Fig. 4).

3.3. Hydrolysis of gentiobiose by β -glucosidase-displaying yeast

For the further degradation of laminarin, we attempted the hydrolysis of gentiobiose by BG-displaying yeast (Nakanishi et al., 2012). After pBGI (Fig. 2a) was transformed into the *S. cerevisiae* BY4741/ Δ sed1 strain, we confirmed the display of BG on the yeast cell surface (Fig. 2b). Subsequently, TLC analysis was performed to analyze the degradation products from gentiobiose by BG-displaying yeast. The result suggested that gentiobiose was converted into glucose and completely assimilated by BG-displaying yeast cells (Fig. 5).

3.4. Ethanol fermentation of laminarin by yeast coculture system

As Gly5M-displaying yeast degraded laminarin and generated gentiobiose, which was further converted into glucose by BG-displaying yeast, we constructed a coculture system of Gly5M- and BG-displaying yeasts to directly produce ethanol from fermentation medium including laminarin as a sole carbon source. When Gly5M- and BG-displaying yeasts were

anaerobically cultivated together in the fermentation medium with different initial inoculation ratios of the 2 yeast strains, the coculture system of Gly5M- and BG-displaying yeasts successfully produced ethanol (Fig. 6a). Especially, the highest ethanol production at 24 h was achieved when the initial OD₆₀₀ values of the Gly5M- and BG-displaying yeasts were adjusted to 35 and 15, respectively. Subsequently, the ethanol production by the coculture of Gly5M- and BG-displaying yeasts (35:15) reached 5.2 g/L (a conversion efficiency of 46%) at 96 h, which was superior to those of single cultures of Gly5M- and BG-displaying yeasts (Fig. 6b). The total sugar concentration in the fermentation medium of 35:15 at 24 h was 3.5 g/L and much lower than those of 50:0 (18.4 g/L) and 0:50 (11.2 g/L) (Fig. 7). These results indicated that the coculture system synergistically degraded and assimilated laminarin to produce ethanol.

4. Discussion

In this study, a novel laminarinase Gly5M was identified from the results of proteome analysis, and its degradation activity toward laminarin was detected using the yeast cell surface display method. The main degradation products from laminarin by Gly5M-displaying yeast was identified as gentiobiose and subsequently converted into glucose by BG-displaying yeast. Furthermore, the coculture system of Gly5M- and BG-displaying yeasts was designed for the direct ethanol production from laminarin.

Previous studies showed that laminarinase incompletely broke down laminarin and generated oligosaccharides (Kawai et al., 2006; Menshova et al., 2014). Nonetheless, the further degradation of laminarin has not been investigated. In order to solve this problem and produce ethanol from laminarin, we designed a coculture system of Gly5M- and BG-

displaying yeasts to convert laminarin into glucose through generation and hydrolysis of gentiobiose. In fact, the assimilated percentage of laminarin by 35:15 was 82.5%, whereas those by 50:0 and 0:50 were 8.0% and 44.0%, respectively (Fig. 7). Moreover, the ethanol concentration by 35:15 resulted in a 9.5- and a 1.7-fold increase at 24 h, compared to 50:0 and 0:50, respectively (Fig. 6a). Interestingly, without Gly5M-displaying yeast, BG-displaying yeast slowly but substantially produced ethanol from laminarin. From this result, BG from *A. aculeatus* is assumed to have laminarin-degrading activity as well as the β -glucosidase described previously (Wang et al., 2015). Because degradation of laminarin by Gly5M-displaying yeast would be rate-limiting, ethanol production reached a maximum when the initial OD₆₀₀ values of the Gly5M- and BG-displaying yeasts were adjusted to 35 and 15, respectively, where Gly5M-displaying yeast was the majority. The total OD₆₀₀ value of yeast cells did not change during fermentation (data not shown), so that Gly5M- and BG-displaying yeast cells could stay in the same composition, which would enable the successive culture without adjusting the OD₆₀₀ ratio.

Codisplay of Gly5M and BG on the single yeast cell surface would be an alternative method for the direct ethanol production from laminarin, judging from the examples of other carbon resources (Fujita et al., 2004; Katahira et al., 2004). However, we adopted the yeast coculture system because we assumed that it would have the following advantages. First, from practical aspects, the initial inoculation ratio of the 2 yeast strains can be readily optimized for efficient ethanol production, while, in the codisplay method, fine adjustment of the ratio of displayed enzymes is difficult and time-consuming. Second, the initial inoculation ratio of the 2 yeast strains can be tuned depending on the kinds of laminarins with different structures from various algal species, which would enable the individual

optimizations for ethanol production from each laminarin. Correspondingly, our results showed that ethanol productivity depended on the initial inoculation ratio of the 2 yeast strains (Fig. 6a), and hence the proportion of the 2 enzymes displayed on the yeast cell surface would be an important factor to increase ethanol production.

Previous efforts to utilize laminarin for ethanol fermentation have focused on using the native laminarin-assimilating yeast *Pichia angophorae* (Adams et al., 2011; Horn et al., 2000a). However, the host-vector systems of *P. angophorae* are not well-developed, and its genetics and physiologies are poorly understood. While ethanol fermentation of laminarin was demonstrated by the well-characterized standard industrial yeast *S. cerevisiae* with the addition of laminarinase (Adams et al., 2009), the use of additional enzymes is not encouraged in terms of cost. In contrast, we established an engineered *S. cerevisiae* coculture system with the yeast cell surface display method to directly produce ethanol from laminarin, which would be superior to previously-developed methods that employ poorly-characterized microbes or supplementary laminarinase.

In conclusion, we demonstrated that the yeast coculture system can directly produce ethanol from laminarin as a sole carbon source. Although laminarin is one of the major components of brown macroalgae, it has not been well-utilized so far. The coculture system developed here would promote the use of laminarin and contribute to the complete utilization of brown macroalgae.

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Author's contributions

K.M. - designed and carried out the experiments, and drafted the manuscript, T.T., Y.S. and K.K. - participated in the experimental design and revised the manuscript, M.U. - contributed to the experimental design and revised the manuscript critically.

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Table 1 Plasmids and primers used in this study

Plasmid or primer	Feature or sequence
<i>Plasmids</i>	
pGLY5M	Cell surface display of Gly5M with FLAG-tag, <i>URA3</i>
pBGI	Cell surface display of BG I with FLAG-tag, <i>URA3</i>
<i>Primers</i>	
Infusion Gly5M-F	5'-TTGCTCGTTTCTGCCTGTGGCGGAGGCTCCAGCGGCACTAAG-3'
Infusion Gly5M-R	5'-GTAATCAGATCCACCGTCAACTGCTTCAACACTCCACCAATTCATATTCCACTGG-3'
Infusion BG-F	5'-TTGCTCGTTTCTGCCGATGAACTGGCGTTCTCTCCTCCTTTCTACCCCTC-3'
Infusion BG-R	5'-GTAATCAGATCCACCTTGCACCTTCGGGAGCGCCGCGTGAAG-3'

Figure legends

Fig. 1. Cell growth of *S. degradans* cultured in the presence of laminarin or glucose as a sole carbon source. Growth of *S. degradans* with laminarin (closed triangle) or glucose (open circle) was monitored by measuring the OD₆₀₀. Results are the means of 3 independent experiments, and error bars represent the standard errors.

Fig. 2. (a) Constructed plasmids and (b) fluorescence observation of yeast cells after immunofluorescence labeling using an anti-FLAG antibody and an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody. Phase-contrast micrographs (column 1) and immunofluorescence micrographs (column 2). *S. cerevisiae* displaying Gly5M (A), BG (B), and control cells harboring pULD1-s (C). The scale bar is 5 μ m.

Fig. 3. TLC analysis of degradation products from laminarin. Standards are laminaribiose (L2), laminaritriose (L3), and gentiobiose (G2) (Lane 1-2). The reactions were performed by Gly5M-displaying yeast cells (Lane 3), control yeast cells harboring pULD1 (Lane 4), and no yeast cells (Lane 5).

Fig. 4. HPLC analysis of gentiobiose (middle line) and degradation products from laminarin by Gly5M-displaying yeast cells (top line) or control cells (bottom line). The *arrow* indicates the peak of gentiobiose.

Fig. 5. TLC analysis of degradation products from gentiobiose. Standards are glucose (G) and gentiobiose (G2) (Lane 1). The reactions were performed by BG-displaying yeast cells

(Lane 2), control yeast cells harboring pULD1 (Lane 3), and no yeast cells (Lane 4).

Fig. 6. Ethanol production from laminarin by yeast coculture system. (a) Ethanol productions were examined on different initial inoculation ratios of Gly5M- and BG-displaying yeast cells at 24 h. (b) Ethanol production was measured up to 96 h when the initial OD₆₀₀ values of Gly5M- and BG-displaying yeast cells were 50:0 (closed circle), 35:15 (closed triangle), 0:50 (open triangle), or when the control cells were inoculated instead (open circle). All of the total initial OD₆₀₀ values were adjusted to 50. Results are the means of 3 independent experiments, and error bars represent the standard errors.

Fig. 7. Total sugar concentrations in fermentation media of 50:0, 35:15, 0:50, and control cells after 24 h of fermentation. Results are the means of 3 independent experiments, and error bars represent the standard errors.

Fig. 1. Motone et al.

(a)

(b)

Fig. 2. Motone et al.

Fig. 3. Motone et al.

Fig. 4. Motone et al.

Fig. 5. Motone et al.

(a)

(b)

Fig. 6. Motone et al.

Fig. 7. Motone et al.