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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.


## 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 only as the 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).

Recently, degradation of alginate, which is one of the major components of brown macroalgae, and bioethanol production from its monosaccharide 4-deoxy-L-erythro-5-hexoseulose-erythro-5-hexoseulose uronic acid were demonstrated (Enquist-Newman et al., 2014; Takagi et al., 2016b; Wargacki et al., 2012). Moreover, many studies have developed the ways to utilize other carbohydrates composing brown macroalgae such as mannitol, cellulose, and hemicellulose (Chujo et al., 2015; Lin and Tanaka, 2006). However, the complete utilization of macroalgae cannot be realized

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without the exploitation of the algal storage glucan, laminarin. Although several studies have reported degradation of laminarin by laminarinase (β-1,3-endoglucanase or β-1,3-exoglucanase), laminarin was broken down incompletely and resulted in the accumulation of oligosaccharides due to the lack of harnessing a β-glucosidase (Kawai et al., 2006; Kumagai and Ojima, 2010). Furthermore, while ethanol fermentation of laminarin has been attempted with several microbes (Adams et al., 2009; Adams et al., 2011; Horn et al., 2000a, 2000b; Lee and Lee, 2011; Mountfort and Rhodes, 1991), the yeast *Saccharomyces cerevisiae*, which is one of the most beneficial hosts for bioethanol production, has not been engineered for direct ethanol fermentation of laminarin.

In this study, we focused on a marine bacterium *Saccharophagus degradans* 2–40 with a powerful ability to degrade marine biomass (Andrykovitch and Marx, 1988; Weiner et al., 2008). This bacterium has multi-component enzyme systems to degrade at least 10 different kinds of carbon polysaccharides, including cellulose, hemicellulose, agar, pectin, alginate, and laminarin (Hutcheson et al., 2011). Therefore, we screened laminarinases derived from *S. degradans* to be displayed on the *S. cerevisiae* cell surface based on the result of qualitative proteome analysis. The technology of yeast cell surface display has several advantages (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 6,000*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 ($OD_{600}$) in triplicate.

*Escherichia coli* strain DH5α (F⁻, ϕ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F) U169, *deo*R, *rec*A1, *end*A1, *hsd*R17 ($r_\kappa^-$, $m_\kappa^+$), *pho*A, *sup*E44, λ⁻, *thi*-1, *gyr*A96, *rel*A1; 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 μg/mL ampicillin.

*S. cerevisiae* strain BY4741/Δ*sed1* (Kuroda et al., 2009) (*MAT*a, *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 6,000*g* for 10 min at 4 °C. Harvested cells were resuspended in 700 μL 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-250 T 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*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 long 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 $\leq 0.01$ 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