

Effect of pretreatment of hydrothermally processed rice straw with laccase-displaying yeast on ethanol fermentation

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Abstract A gene encoding laccase I was identified and cloned from the white-rot fungus *Trametes* sp. Ha1. *Laccase I* contained 10 introns and an original secretion signal sequence. After *laccase I* without introns was prepared by overlapping polymerase chain reaction, it was inserted into expression vector pULD1 for yeast cell surface display. The oxidation activity of a laccase-I-displaying yeast as a whole-cell biocatalyst was examined with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and the constructed yeast showed a high oxidation activity. After the pretreatment of hydrothermally processed rice straw (HPRS) with laccase-I-displaying yeast with ABTS, fermentation was conducted with yeast codisplaying endoglucanase, cellobiohydrolase, and β -glucosidase with HPRS. Fermentation of HPRS treated with laccase-I-displaying yeast was performed with 1.21-fold higher activities than those of HPRS treated with control yeast. The results indicated that pretreatment with laccase-I-displaying yeast with ABTS was effective for direct fermentation of cellulosic materials by yeast codisplaying endoglucanase, cellobiohydrolase, and β -glucosidase.

Keywords Biorefinery · Laccase · Cell surface engineering of yeast · *Trametes* sp. · Lignin

Introduction

The establishment of a sustainable society will be necessary in the absence of fossil resources (Gerngross and Slater 2000), and biorefinery may play a key role in meeting future energy needs. Biorefinery substitutes carbon sources extracted from plants for fossil resources (Kamm and Kamm 2004). Bioethanol, a representative biorefinery product, is currently the most widely used biofuel. Grain biomass is used as a raw material for bioethanol because the main component is starch, which can be easily hydrolyzed to glucose. The use of grain biomass competes with the food supply, however, raising new problems (Abe and Takagi 1991). Meanwhile, cellulosic biomass does not compete with food supply and is thought to be a potential material, but the lignin in cellulosic biomass inhibits its use as a carbon source.

Lignin, one of main components in cellulosic biomass, is one of the most abundant organic polymers on earth, and lignin monomers—e.g., coniferyl alcohol and sinapyl alcohol—are useful compounds for foods and medicines (Sinha et al. 2008). Lignin is a cross-linked persistent macromolecule (Labat and Gonçalves 2008); thus, lignin degradation is required to exploit the useful monomers. Similarly, lignin inhibits the use of cellulose as a carbon source. Therefore, lignin degradation requires a substantial investment in infrastructure and technique if cellulose and hemicellulose are to be used in cellulosic biomass (Octave and Thomas 2009). In nature, lignin is mainly degraded by wood-decaying fungi. White-rot fungus, which is one of these fungi, selectively degrades lignin by secreting lignin-degrading enzymes—e.g., laccase (Lebrun et al. 2011; Xiao et al.

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2006). Laccase, a multi-copper enzyme, oxidizes large spectral substrates via one-electron extraction to generate radicals (Smith et al. 1998; Sulistyaningdyah et al. 2004). Fungal laccases are thought to participate in several biological pathways, including lignin degradation (Baldrian 2006). It has been demonstrated that several laccases have paper pulp biobleaching potential through lignin degradation (Herpöel et al. 2002; Ibarra et al. 2006; Sigoillot et al. 2004). Typically, laccase degrades lignin with a mediator—e.g., 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Ibarra et al. 2006). The lignin degradation by white-rot fungi and purified laccase is certainly reliable because both degradation systems occur in nature; however, the process costs time and money. The white-rot fungi *Trametes* sp. secretes laccases, and laccase I from *Trametes* sp. Hal has high oxidation activity and thermostability (Nakatani et al. 2010; Shimizu and Nakatani 1996). The nucleotide sequence encoding laccase I from *Trametes* sp. Hal is undetermined, however.

The cell surface engineering of yeast *Saccharomyces cerevisiae* is useful in the molecular breeding of yeast for various practical applications (Kondo and Ueda 2004). The engineering has three major advantages; the displaying yeast can be used as a whole-cell biocatalyst; the enzymes are accumulated on the yeast cell surface; and glucose can be used for the fermentation without contamination. First, the engineering enables 10^4 – 10^5 functional proteins to be displayed on a yeast cell surface, and the displaying yeast can be used as the whole-cell biocatalyst without separation and purification (Shibasaki et al. 2001). Second, the distance between each enzyme on the cell surface is close because of accumulation of enzymes on the cell surface, and it is possible that the synergistic reaction easily occurred in the displaying system. For example, the enzymes on a scaffold protein such as a cellulosome show a higher activity than the free enzymes since the distance between enzymes in cellulosome is close (Jeon et al. 2011). The synergistic reaction could be effective for each degrading activity by the cellulase and lignin-degrading enzyme on the yeast cell surface. For the cellulases, cellulose was synergistically degraded to glucose by three cellulases, i.e., endoglucanase (EG); cellobiohydrolase (CBH), β -glucosidase (BG), because the distance of three cellulases could be closed. In fact, the yeast codisplaying EG, CBH, and BG can produce ethanol from cellulose (Fujita et al. 2004). On the other hand, the engineering has the advantage for the degradation of lignin. Previously, although lignin degradation was used with free enzymes in the enzymatic method, the degradation was difficult because the degradation with radical reactions by the lignin-degrading enzymes is activated with synergistic effect (Smith et al. 1998;

Sulistyaningdyah et al. 2004). The synergistic effect probably occurred in the close distance between the enzymes. Third, the cellulases-displaying yeast can degrade cellulose and take in the degraded glucose directly. Accordingly, there was no contamination in the medium containing cellulases-displaying yeast (Fujita et al. 2004).

Sed1 is a major stress-induced and structural-glycosylphosphatidylinositol cell wall glycoprotein in stationary-phase cells (Park et al. 2005), and it is known that *S. cerevisiae* BY4741 ($\Delta sed1$) can display enzymes more efficiently than the wild type in yeast cell surface engineering (Kotaka et al. 2010; Kuroda et al. 2009).

In this study, cloning and sequence determination of laccase I were carried out to construct a lignin conversion system. The laccase-I-encoding gene was successfully expressed on the yeast cell surface, and the laccase-I-displaying yeast functioned as a whole-cell biocatalyst. Furthermore, hydrothermally processed rice straw (HPRS), which consists mainly of cellulose and lignin, was practically selected as the substrate for fermentation, and the effect of pretreating it with laccase-I-displaying yeast with ABTS on fermentation efficiency was evaluated.

Materials and methods

Strains, media, and reaction buffer

Escherichia coli strain DH5 α (F^- , $\phi 80dlacZ\Delta M15$, $\Delta[lacZYA-argF]U169$, *endA1*, *hsdR17*[r_k^- , m_k^+], *supE44*, *thi-1*, λ^- , *recA1*, *gyrA96*, *relA1*, *deoR*) was used as the host for recombinant DNA manipulation, and the bacteria were grown in Luria–Bertani medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] sodium chloride) containing 100 μ g/mL ampicillin. *S. cerevisiae* strain BY4741($\Delta sed1$) (*MATa*, *his3 Δ 1*, *leu2 Δ 0*, *met15 Δ 0*, *ura3 Δ 0*, *YDR077w::KanMX4*), obtained from EUROSCARF (Frankfurt, Germany), was used for the cell surface display of laccase I.

Yeast transformants were aerobically preincubated in synthetic dextrose (SD) medium (0.67% [w/v] yeast nitrogen base without amino acids, 2% [w/v] glucose and appropriate supplements).

Yeast transformants were aerobically cultivated in SD medium with casamino acids (SDC) medium (0.67% [w/v] yeast nitrogen base without amino acids, 2% [w/v] glucose, 0.5% [w/v] casamino acids, and appropriate supplements) for the main incubation of the activity measurement. Laccase displayed on the yeast cell surface must involve Cu^{2+} because laccase is a multicopper enzyme and the medium supplies the Cu^{2+} for laccase; thus, laccase-I-displaying yeast and yeast harboring pULD1 (Kuroda et al. 2009) were incubated in the SDC medium adjusted to 0.5 mM $CuSO_4$.

The buffer used for activity measurement of ABTS was 50 mM sodium acetate buffer (pH 5.0) containing 0.5 mM CuSO₄. The buffer was called ABTS buffer.

Yeast codisplaying EG, CBH, and BG and yeast harboring pRS423, pRS425, and pRS426 fermented with HPRS in 50 mM citric acid buffer (pH 5.0) containing a yeast nitrogen base with casamino acids (0.67% [w/v] yeast nitrogen base without amino acids, 2.0% [w/v] casamino acids, and 0.003% [w/v] L-methionine). We referred to the medium as the fermentation medium.

The pretreatment of HPRS with laccase-displaying yeast was conducted in 50 mM citric acid buffer (pH 5.0) containing 0.5 mM CuSO₄. The buffer was called pretreatment buffer.

After the pretreatment, sugar was directly fermented by yeast in the pretreatment buffer. The pretreatment buffer contained no nutrients, however. Thus, another medium was added to the pretreatment buffer. This medium was called the concentrated medium (50 mM citric acid buffer [pH 5.0] containing 0.84% [w/v] yeast nitrogen base without amino acids, 2.5% [w/v] casamino acids, and 0.0038% [w/v] L-methionine). The buffer contained no glucose; thus, the pretreated HPRS was the only carbon source for fermentation. The fourfold volume of concentration medium was added to the pretreatment buffer. Afterward, the final medium concentration was as follows: 0.67% (w/v) yeast nitrogen base without amino acids, 2.0% (w/v) casamino acids, and 0.003% (w/v) L-methionine.

The white-rot fungus *Trametes* sp. Ha1 strain (FERM P-14472, Advanced Industrial Science and Technology: <http://unit.aist.go.jp/pod/cie/procedures.html>; strain no. AKU 5050, AKU, Faculty of Agriculture, Kyoto University) was grown

in potato dextrose agar medium (3.9% [w/v] potato dextrose and 2.0% [w/v] agarose) (Nakatani et al. 2010).

Isolation of genomic DNA and cloning of the laccase-I-encoding gene

Mycelia of *Trametes* sp. Ha1 were grown on a potato dextrose agar plate at 30°C for 1 week. The mycelia were frozen in liquid nitrogen and ground to a powder using a mortar. After grinding, genomic DNA (gDNA) was isolated from the powder using Blood & Cell Culture DNA Kit (Qiagen, Hilden, Germany). A partial amino acid sequence of laccase I from *Trametes* sp. Ha1 has been revealed (Nakatani et al. 2010). Based on the laccase genes from *Phaseolus coccineus* (Hoshida et al. 2001) and *Trametes* sp. AH28-2 (Xiao et al. 2006), which secrete laccases similar to laccase I from *Trametes* sp. Ha1, primers Lac F and Lac R (Table 1) were designed for the cloning. After polymerase chain reaction (PCR) amplification with the designed primers, the DNA fragment of *laccase I* was inserted into the *Hinc*II site of pUC19, and the DNA fragment was analyzed through sequencing. The DNA sequence of *laccase I* has been submitted to the GenBank database (accession number JF825479).

Construction of the fusion gene for display of laccase I

To recognize introns and a secretion signal sequence of *laccase I*, the laccase-I-encoding gene was compared with the laccase gene of *Trametes* sp. AH28-2. Overlapping PCR was performed to synthesize *laccase I* without introns using designed 22 primers Lac F, Lac R, Exon F, and Exon R (see

Table 1 Oligonucleotide primers for *laccase I*

Primer	Sequence	Primer	Sequence ^a
Lac F	5'-ATGTCGAGGTTCAGTCTCTTCTCGCCTTC-3'	Exon 7 F	5'-GGGGAAACGGTACCGTTTCCGCCTCGTCTC-3'
Lac R	5'-CTACTGGTCGTTGACATCGAGCGCGTCG-3'	Exon 7 F	5'-CGGCATTCAACACGAAGGAGTACCGCTGCG-3'
Exon 1 F	5'-ATGTCGAGGTTCAGTCTCTTCTCGCCTTC-3'	Exon 8 F	5'-CTCCTTCGTGTTGAATGCCGACCAGGATG-3'
Exon 1 R	5'-AGCGGTCACCTTGTATCCCGCAGCAGGG-3'	Exon 8 R	5'-GGGAGCCGGGAGCCATGGTGTCGAGCGGG-3'
Exon 2 F	5'-GGGTAACAAGGGTGACCGCTTCCAATC-3'	Exon 9 F	5'-CACCATGGCTCCCGGCTCCCGGTGCGCCG-3'
Exon 2 R	5'-CGTGCCAGTGAATACTCGTGCTCTTGAGC-3'	Exon 9 R	5'-TGGTACCGTTGAAGTTGAAGGCCATGTTG-3'
Exon 3 F	5'-CACGAGTATTCACTGGCACGGCTTTTTC-3'	Exon 10 F	5'-CTTCAACTTCAACGGTACCAACTTCTTC-3'
Exon 3 R	5'-AGAATGTGCCGGCCTGATCCGGAACCTGG-3'	Exon 10 R	5'-CAAAGGCGTGACCGTGCAAGTGGAAGGGG-3'
Exon 4 F	5'-GGATCAGGCCGGCACATTCTGGTACCACAG-3'	Exon 11 F	5'-CTTGACCGGTACGCGCTTTGCCGTCGTTTCG-3'
Exon 4 R	5'-CGGTGTCGTCGTTGTGACGTCATACAAGC-3'	Exon 11 R	5'-CTACTGGTCGTTGACATCGAGCGCGTCG-3'
Exon 5 F	5'-CGTCGACAACGACGACACCGTGATCACGC-3'	Lac-KEX2 F	5'-GTCACGAAGGGGAAAAAGTACCGTTTCCGC-3'
Exon 5 R	5'-CGGCACCCAGCGGGAATGCTGGTCCGAGC-3'	Lac-KEX2 R	5'-GCGGAAACGGTACTTTTCCCTTCGTGAC-3'
Exon 6 F	5'-AGCATTCCTCGTGGTGCCGACGCAACGC-3'	Lac-BamHI F	5'-ATGCAGGATCCGCCATTGGGCCCACCGCTGACCTC-3'
Exon 6 R	5'-GGAAACGGTACCGTTTCCCTTCGTGACG-3'	Lac-XhoI R	5'-ATGCAGCTCGAGCTGGTCGTTGACATCGAGCGCGTCG-3'

^a Underlining indicates the restriction sites for the enzymes

Table 1); Exons F and R were used to amplify for exon synthesis, and the number of the primers indicated the number of exons; Lac F and Lac R were used for amplification of full-length *laccase I* with each exon. The synthesized *laccase I* was inserted into the *HincII* site of pUC19. *Laccase I* has the –Lys–Arg– that *S. cerevisiae* Kex2 protease recognizes (Bulter et al. 2003). Site-directed mutagenesis was performed with primers Lac-KEX2 F and Lac-KEX2 R to replace the –Lys–Arg– cleavage of *laccase I* with –Lys–Lys– (see Table 1). The resulting plasmid was named pUC-Lac I. To display laccase I on the yeast cell surface, a cassette vector pULD1 for α -agglutinin-based display of proteins was used (Kuroda et al. 2009). For insertion into pULD1, *laccase I* without a secretion signal sequence was amplified from pUC-Lac I using PCR with primers Lac-BamHI F and Lac-XhoI R (see Table 1), and the amplified DNA fragment was inserted into the *BglII*–*XhoI* section of pULD1. Consequently, the fusion gene encoding the glucoamylase secretion signal, laccase I, FLAG tag, and 3' half of α -agglutinin was constructed under the control of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. The constructed plasmid was named pULD-Lac I. The control plasmid was pULD1-s (Kuroda et al. 2009).

Construction of plasmids for simultaneous display of EG, CBH, and BG

All primers used for the plasmid construction are listed in Table 2. PCR was carried out with KOD-plus-DNA polymerase (Toyobo, Osaka, Japan).

To produce three kinds of cellulases in yeast BY4741 ($\Delta sed1$), the three plasmids for cellulase display were slightly modified using the plasmids constructed by Murai et al. (1998), Fujita et al. (2002), and Fujita et al. (2004). To produce EG, CBH, and BG in high quantity on the cell surface of yeast BY4741($\Delta sed1$), which can efficiently display proteins (Kuroda et al. 2009; Kotaka et al. 2010), the three plasmids were reconstructed for codisplay of EG, CBH, and BG. The *Trichoderma reesei* EG gene on pEG23u31H6 (Fujita et al. 2002) was amplified with PCR using EG-*BglII* F and EG-*XhoI* R primers. The amplified DNA of EG fragment was inserted into the *HincII* site of pUC19. The resulting plasmid was named pUC-EG. The EG gene has mutations; thus, reverse mutation was conducted for the EG gene on pUC-EG, and the resulting plasmid was named pUC-EGcomp. Cassette vector pRS425display was constructed using pRS425 and pULD1 (Kuroda et al. 2009). The sequence encoding the GAPDH promoter, secretion signal of glucoamylase, multicloning

Table 2 Constructed plasmids and primers for cellulase

Plasmid or primer	Feature or sequence
Plasmids	
pEG23u31H6	Cell surface display of endoglucanase II with RGSHHHHHH-tag, and <i>URA3</i>
pUC-EG	Sequence manipulation of endoglucanase II
pUC-EGcomp	Complete of sequence manipulation of endoglucanase II
pRS425display	Cassette vector for cell surface display of proteins with FLAG-tag, and <i>LEU2</i>
pEG	Cell surface display of endoglucanase II with FLAG-tag, and <i>LEU2</i>
pBG211	Cell surface display of β -glucosidase with <i>HIS3</i>
pBG211HHHH	Cell surface display of β -glucosidase with HHHHHH-tag, and <i>HIS3</i>
pBG	Cell surface display of β -glucosidase with RGSHHHHHH-tag, and <i>HIS3</i>
pFCBH2W3	Cell surface display of cellobio- β -hydrolase II with FLAG-tag, and <i>TRP1</i>
pRS425display-s	Cassette vector for cell surface display of proteins with Strep-tag, and <i>LEU2</i>
pRS426display	Cassette vector for cell surface display of proteins with Strep-tag, and <i>URA3</i>
pCBH	Cell surface display of cellobiohydrolase II with Strep-tag, and <i>URA3</i>
Primers	
EG- <i>BglII</i> F	5'-CAGTAAGCTTAGATCTCAGCAGACTGTCTGGGGCCAGTGTG-3'
EG- <i>XhoI</i> R	5'-CAGTTCCTAGACTCGAGACCGAAAGAACGCTCTGTGCTCGACTGGTTCCTAC-3'
CBH- <i>BglII</i> F	5'-CAAGCTTGCTCAAGCGTCTGG-3'
CBH- <i>NheI</i> R	5'-AGTGCTAGCCAGGAACGATGGGTTTGC GTTTG-3'
pRS425disp-strep F	5'-TGGTCTCATCCTCAATTGAAAAAGCTGGAGGTTTCGAGCGC-3'
pRS425disp-strep R	5'-AGATCCACCCTCGAGGCTAG-3'
pBG211-HHHH F	5'-CACCATCACCATCACCATGATGAACTGGCGTTCTCTCCTCCTTTC-3'
pBG211-RGSHHHH F	5'-AGAGGATCCACCATCACCATCACCATGATGAACTGG-3'
pBG211-HHHH R	5'-AGAGATCTCCGCGGCAGAAACGAG-3'

EG endoglucanase, BG β -glucosidase, CBH cellobiohydrolase

site, FLAG-tag, and 3' half of α -agglutinin from pULD1 was inserted into the *SalI* and *SacII* sections of pRS425 (American Type Culture Collection, Vancouver, USA) after digestion with *SalI* and *SacII*. The resulting plasmid was named pRS425display. After digestion with *BglII* and *XhoI*, the EG gene on pUC-EGcomp was inserted into the *BglII*–*XhoI* section of the multicloning site of pRS425display. The constructed plasmid for cell surface display of EG was named pEG.

A plasmid was constructed to display the CBH fused with strep-tag as follows. First, the DNA fragment, which was exchanged from FLAG-tag to strep-tag, was amplified with PCR using pRS425disp-strep F and pRS425disp-strep R primers based on pRS425display (see Table 2), and the PCR product was reacted to digest and remove pRS425display (the template vector) via *DpnI* digestion. The DNA fragment was phosphorylated using T4 polynucleotide kinase and ligated itself. The resulting plasmid was named pRS425display-s. The sequence encoding the GAPDH promoter, secretion signal of glucoamylase, multicloning site, strep-tag, and 3' half of α -agglutinin on pRS425display-s was inserted into the *SalI* and *SacII* sections of pRS426 after digestion with *SalI* and *SacII*. The resulting plasmid was named pRS426display. The *T. reesei* CBH gene on pFCBH2w3 (Fujita et al. 2004) was amplified with PCR using CBH-*BglII* F and CBH-*NheI* R primers (see Table 2). The amplified CBH gene digested with *BglII*, and *NheI* was inserted into the *BglII*–*NheI* section of the multicloning site of pRS426display. The resulting plasmid for cell surface display of CBH was named pCBH.

To construct pBG, the –Arg–Gly–Ser–His–His–His–His– encoding gene was fused with the 5' end of the BG gene. The DNA fragment of pBG211 containing the gene encoding –His–His–His–His– was amplified with PCR using pBG211-HHHH F and pBG211-HHHH R primers (see Table 2) based on pBG211 (Murai et al. 1998), and the PCR product was reacted to digest pBG211 (the template vector) with *DpnI*. The DNA fragment was phosphorylated using T4 polynucleotide kinase and ligated itself. The resulting plasmid was named pBG211HHHH. In the same way, the DNA encoding –Arg–Gly–Ser– was fused with the 5' end of the DNA encoding –His–His–His–His– of pBG211HHHH as follow. The DNA fragment was amplified with PCR using pBG211-RGSHHHH F and pBG211-HHHH R primers based on pBG211HHHH (see Table 2). The product was digested with *DpnI* and ligated itself. The resulting plasmid for the cell surface display of BG was named pBG.

Yeast transformation

Plasmid introduction into BY4741(Δ *sed1*) was carried out using the lithium acetate method (Ito et al. 1983) with a

YEASTMAKER yeast transformation system (Clontech Laboratories, CA, USA). The transformants were isolated on a selective SD medium plate at 30°C for 2–3 days.

Immunofluorescence labeling of yeast cells and cell observation using fluorescence microscopy

Immunofluorescence labeling of cells was carried out according to the previously described method (Kobori et al. 1992). Mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, MO, USA) was used as a primary antibody at a dilution rate of 1:300. A mixture of cells and the antibody were incubated at room temperature with gentle shaking for 1.5 h, and the cells were washed with phosphate-buffered saline (PBS; pH 7.4). As the secondary antibody, Alexa Fluor 488 anti-mouse IgG (Invitrogen, CA, USA) was used at a dilution rate of 1:300 at room temperature with gentle shaking for 1.5 h. The cells were washed with PBS (pH 7.4) and, after immunofluorescence labeling, observed with an inverted microscope IX71 (Olympus, Tokyo, Japan) through a U-MNIBA2 mirror unit with a BP470–490 excitation filter, a DM505 dichroic mirror, and a BA510–550 emission filter (Olympus). Live images were obtained using Aqua Cosmos 2.0 software (Hamamatsu Photonics, Shizuoka, Japan) to control a digital charge-coupled device camera (Hamamatsu Photonics).

Activity measurement of laccase I displayed on the yeast cell surface

After precultivation in SD medium at 30°C for 24 h, yeast cells were aerobically incubated in SDC medium at 30°C for 60 h. The cells incubated in the medium were collected via centrifugation at 5,000×g for 5 min at 4°C and washed with PBS (pH 7.4). The cell suspension was adjusted to an OD₆₀₀ of 5.0 in ABTS buffer. The control yeast harboring pULD1 was prepared the same way. ABTS (360 μ M; Sigma-Aldrich) in 50 mM acetate buffer (pH 5.0) was used as the substrate for the activity measurement of laccase-I-displaying yeast. The oxidation activity of laccase was detected by measuring the absorbance at 420 nm ($\epsilon=3.6 \times 10^4$ cm^{−1} mol^{−1} L) derived from ABTS oxidation (Childs and Bardsley 1975).

Pretreatment of HPRS using laccase-I-displaying yeast with ABTS

After precultivation in SD medium at 30°C for 48 h, yeast cells were aerobically cultivated in SDC medium at 30°C for 60 h. Laccase produced on yeast cell surface was supplied with Cu²⁺ from the medium. The cells incubated in the medium were collected via centrifugation for 5 min at 5,000×g and 4°C and washed with PBS (pH 7.4). The cell

suspension was adjusted to an OD₆₀₀ of 3.0 in the pretreatment buffer. The control yeast harboring pULD1 was prepared the same way. After the OD₆₀₀ adjustment, 2% (w/v) HPRS and 360 µM ABTS were added to the reaction buffer, and pretreatment was conducted for 24 h at 30°C.

Detection of the reactant after pretreatment

A high-performance liquid chromatography (HPLC) system for detection consisted of a LC-20AD pump (Shimadzu, Kyoto, Japan), a CTO-20A column oven (Shimadzu), a SPD-M20A UV detector (Shimadzu), a CHEMCOBOND ODS-W column (4.6×250 mm, 5 µm; Chemco Scientific, Osaka, Japan), and a 7725 injector (Rheodyne, CA, USA). The reactants were determined from the chromatographic data monitored at UV 254 nm and processed using LC Solution software (Shimadzu). The mobile phase consisted of water and acetonitrile, and the column oven was set to 40°C. The following gradient procedure was used: starting at sample injection, 1.0% acetonitrile for 2 min; a linear gradient from 1.0 to 50% for 10 min; and 100% acetonitrile for 2 min. The flow rate was 1.0 mL/min.

Fermentation with HPRS cellulose by yeast codisplaying EG, CBH, and BG

Yeast codisplaying EG, CBH, and BG was cultivated and collected as follows: precultivation of yeast occurred aerobically in SD medium at 30°C for 48 h, the yeast was aerobically cultivated in SDC medium at 30°C for 60 h, and the cells were collected via centrifugation at 5,000×g at 4°C for 5 min and washed with PBS (pH 7.4). The cell suspension was adjusted to an OD₆₀₀ of 10, and 2% (w/v) HPRS was added to the fermentation medium. Cellulase SS (Nagase ChemteX Corporation, Kyoto, Japan) was added to the fermentation medium at the concentration adjusted at 1 FPU/g HPRS. CO₂ gas was bubbled into the reaction vessel for 2 min to displace O₂. The fermentation was conducted semiaerobically with a magnetic stirring bar rotating at 130 rpm using a Rexim magnetic stirrer (AS ONE, Osaka, Japan) at 30°C. Five hundred microliters of reaction medium were collected as a sample and filtered for the detection of fermentation using Ultrafree-MC Centrifugal Filter Units (Millipore, Massachusetts, USA).

Yeast fermentation after pretreatment

After the pretreatment of HPRS by laccase-I-displaying yeast, the fourfold volume of the concentrated medium was added to the pretreatment buffer. To degrade and ferment the cellulose of HPRS, yeast codisplaying EG, CBH, and BG was added to the pretreated buffer. The codisplaying yeast was cultivated and collected as follows: yeast

codisplaying EG, CBH, and BG was aerobically cultivated in SDC medium at 30°C for 60 h after precultivation in SD medium at 30°C for 48 h, and the cells were collected via centrifugation at 5,000×g at 4°C for 5 min and washed with PBS (pH 7.4). The cell suspension was adjusted to an OD₆₀₀ of 10 in the fermentation medium. To facilitate cellulose degradation, cellulase SS was added at the concentration adjusted at 1 FPU/g HPRS. CO₂ gas was bubbled into the reaction vessel for 2 min to displace O₂. The fermentation was semiaerobically conducted with a magnetic stirring bar rotating at 130 rpm using a Rexim magnetic stirrer (AS ONE) at 30°C. Five hundred microliters of reaction medium were collected as a sample and filtered for detection of fermentation using Ultrafree-MC Centrifugal Filter Units (Millipore).

Evaluation of fermentation for ethanol production

The HPLC system for the evaluation consisted of a LC-20AD pump (Shimadzu), a CTO-20A column oven (Shimadzu), a RID-10A detector (Shimadzu), a YMC-Pack Polyamine II column (4.6×250 mm) (YMC Co. Ltd., Kyoto, Japan), and a 7725 injector (Rheodyne). The concentration of produced ethanol was determined from the chromatographic data monitored at the RID-10A and processed using LC Solution software (Shimadzu). The mobile phase consisted of water and acetonitrile, and the column oven was set at 30°C. The eluent composition was 90% acetonitrile as isocratic. The flow rate was 1.0 mL/min.

Results

Cloning of the laccase I gene from *Trametes* sp. Ha1

For the cloning of *laccase I*, gDNA was isolated from mycelia of *Trametes* sp. Ha1 as described above. The gene encoding laccase I (2,108 bp) was amplified with PCR using designed primers (see Table 1) and cloned. After the sequence determination of the cloned gene, the gene encoding laccase I was compared with *lac A* of *Trametes* sp. AH28-2. Based on the amino acid sequence of *lac A*, the gene encoding laccase I was found to contain 10 introns and an original secretion-signal sequence. Laccase-I-encoding DNA without introns from *Trametes* sp. Ha1 showed 97.3% DNA sequence homology with *lac A* of *Trametes* sp. AH28-2 (Xiao et al. 2006) and 86.7% DNA sequence homology with *lignolytic phenoloxidase* of *Trametes hirsuta* (Kojima et al. 1990). Furthermore, the amino acid sequence of laccase I was compared with *lac A* and *lignolytic phenoloxidase*. The sequence homologies were 99.2% and 98.7%, respectively (Fig. S1).

Display of laccase I on yeast cell surface

Laccase-I-encoding DNA without introns was prepared by overlapping PCR for the cell surface display of laccase I on yeast. The Kex2 endoprotease recognition site (–Lys–Arg–) in laccase I was replaced with the nonrecognition site (–Lys–Lys–) through site-directed mutagenesis because the produced protein containing the recognition site in yeast was degraded by yeast kex2 endoprotease. To display laccase I on the yeast cell surface, *laccase I* without the original secretion signal sequence was inserted into pULD1, a cassette vector for efficient display of proteins (pULD-Lac I; Fig. 1). The constructed pULD1-Lac I was introduced into yeast. Immunofluorescence labeling with anti-FLAG antibody was conducted to detect the display of laccase I on the yeast cell surface. The green fluorescence of Alexa Fluor 488 was observed on the cell surface of the yeast harboring pULD1-Lac I, whereas the fluorescence was absent on the cell surface of yeast harboring control vector pULD1-s (Fig. 2). The codisplay of EG, CBH, and BG was detected in the same way (data not shown).

Oxidation activity of laccase I displayed on the yeast cell surface

The oxidation activity of laccase-I-displaying yeast was measured using ABTS (Fig. 3). Oxidation of ABTS was followed by absorbance at 420 nm. Whereas control yeast showed no oxidation activity, laccase-I-displaying yeast that was cultivated for 25 h did. The result indicated that laccase I on the yeast cell surface retained oxidation activity. In

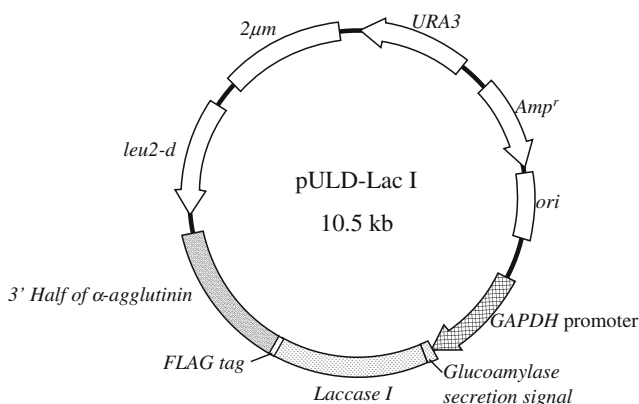


Fig. 1 Constructed plasmid for the cell surface display of laccase I. pULD-Lac I was constructed for cell surface display of *laccase I*. GAPDH promoter and terminator; glyceraldehyde-3-phosphate dehydrogenase promoter and terminator; glucoamylase secretion signal, the secretion signal of glucoamylase from *Rhizopus oryzae*; FLAG tag, epitope tag encoding DYKDDDDK; 3' half of α -agglutinin, the C-terminal 320 amino acids of *S. cerevisiae* agglutination protein, α -agglutinin

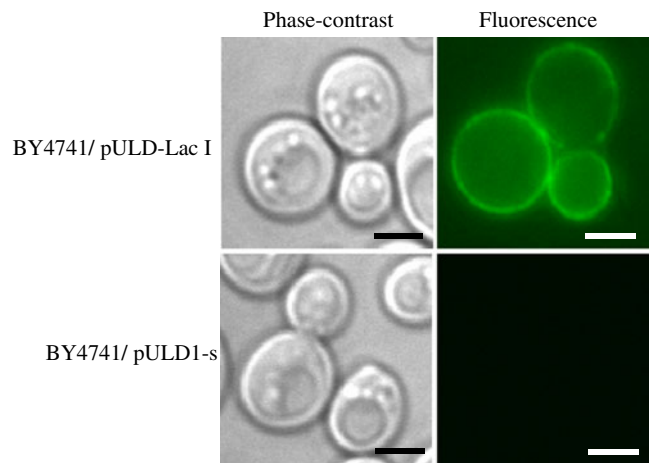


Fig. 2 Fluorescence observation of cells after immunofluorescence labeling. Yeast cells were labeled with anti-FLAG antibody and Alexa Fluor 488 anti-mouse immunoglobulin G. Phase-contrast micrograph (left column), fluorescence micrograph (right column), BY4741/pULD-Lac I (upper column), and BY4741/pULD1-s (lower column). The scale bar is 5 μ m

addition, longer cultivation of laccase-I-displaying yeast (60 h) led to higher activity. Therefore, cells with 60 h of cultivation were used in further experiments.

Activity of laccase-I-displaying yeast with ABTS as a mediator

Activity of laccase-I-displaying yeast with and without ABTS for HPRS was investigated using HPLC analysis. After the reaction was conducted following the method described as pretreatment for HPRS, the reactants were determined from the chromatographic data. The compounds showing absorption at 254 nm were produced during the

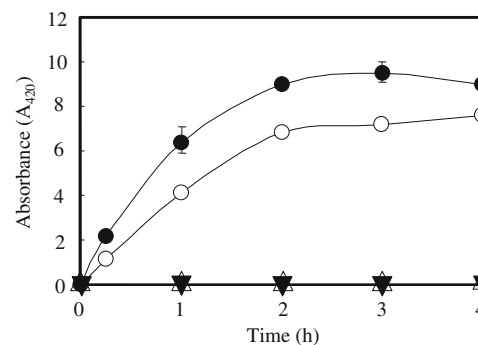


Fig. 3 Measurement of oxidation activity with ABTS of laccase I displayed on yeast cell surfaces. Oxidation of ABTS was measured at 420 nm. BY4741 (Δ sed1)/pULD1 cultivated for 25 h (open triangle), BY4741/pULD1 cultivated for 60 h (closed triangle), BY4741/pULD-Lac I cultivated for 25 h (open circle), and BY4741/pULD-Lac I cultivated for 60 h (closed circle). Values represent the means $\pm$ standard deviation of the results from three independent experiments

pretreatment only under the ABTS use condition (data not shown). To promote fermentation with HPRS, ABTS was essentially added into the pretreatment system by laccase-I-displaying yeast.

Fermentation with HPRS cellulose by yeast codisplaying EG, CBH, and BG

Yeast codisplaying EG, CBH, and BG could not directly use the HPRS cellulose without cellulase SS. On the other hand, yeast codisplaying EG, CBH, and BG had fermentation activity higher than that of control yeast (BY4741 [$\Delta sed1$]/pRS423, pRS425, and pRS426) with cellulase SS (Fig. S2). Yeast codisplaying EG, CBH, and BG was used with cellulase SS to promote yeast fermentation.

Fermentation after pretreatment of HPRS by laccase-I-displaying yeast with ABTS

After the pretreatment of HPRS by laccase-I-displaying yeast, ethanol fermentation was semiaerobically conducted with yeast codisplaying EG, CBH, and BG (Fig. 4). The results showed that the fermentation after pretreatment by laccase-I-displaying yeast with ABTS had a higher activity than that of the control, which was fermentation after pretreatment by no-display yeast with ABTS. Notably, the fermentation activity after pretreatment was 1.21-fold higher than that of the control at 24 h.

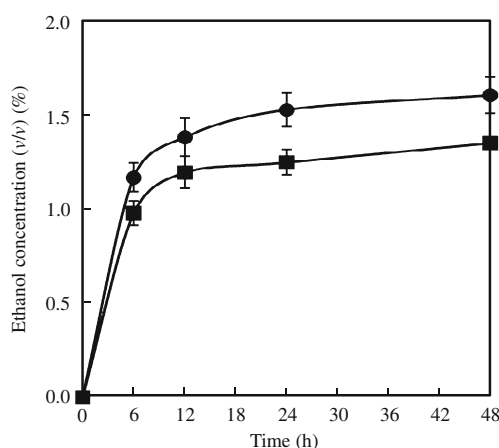


Fig. 4 Activity measurement of fermentation by yeast codisplaying endoglucanase, cellobiohydrolase, and β -glucosidase with HPRS after pretreatment by laccase-I-displaying yeast. The ethanol fermentation was evaluated using high-performance liquid chromatography as described in “Materials and methods.” The fermentation after pretreatment with laccase-I-displaying yeast and yeast producing no laccase (control) are shown as *closed circle* and *closed square*, respectively. Values represent the means $\pm$ standard deviation of the results from three independent experiments

Discussion

Laccase I from *Trametes* sp. Ha1 was successfully cloned, and its nucleotide sequence was determined completely. Comparisons of laccase I with lac A from *Trametes* sp. AH28-2 and lignolytic phenoloxidase from *T. hirsuta* showed that the amino acid sequences have high homologies (see Fig. S1), and the homology of amino acid sequence was higher than that of nucleotide sequence. Recently, evolutionary traces based on dendrograms of laccases have indicated that *Trametes* sp. is similar to *T. hirsuta* (Mohamad et al. 2008), and the high amino acid sequence homology of laccase I and lac A support an evolutionary connection. Lac A and lignolytic phenoloxidase have high oxidation activity (Kojima et al. 1990; Xiao et al. 2006); thus, laccase I was expected to as well.

Lac A from *Trametes* sp. AH28-2 has demonstrated high oxidation activity for ABTS in the secretory way (Xiao et al. 2003). LAC48424-1, the gene for which is normally expressed in *Trametes* sp. 48424, was produced in the yeast *Pichia pastoris* and purified LAC48424-1 oxidized ABTS and dyes (Fan et al. 2011). However, the concentration and the purification of the enzyme costs time and money as well. On the other hand, our laccase-I-displaying yeast was directly available as a whole cell biocatalyst, and the displaying yeast had high oxidation activity using the ABTS oxidation method (see Fig. 3). Furthermore, laccase-I-displaying yeast had higher oxidation activity when the cultivation time was longer (see Fig. 3). These results match those of another study showing that longer cultivation enhanced efficiency in a yeast display system (Kuroda et al. 2009).

Yeast codisplaying EG, CBH, and BG could not directly use HPRS cellulose for fermentation. Yeast codisplaying EG, CBH, and BG had higher fermentation activity than that of control yeast (BY4741 [$\Delta sed1$]/pRS423, pRS425, pRS426) when cellulase SS was added to fermentation medium, however (see Fig. S2). This result indicated that yeast codisplaying EG, CBH, and BG could efficiently degrade HPRS cellulose into the monosaccharide glucose after its partial degradation by cellulase SS; yeast codisplaying EG, CBH, and BG could use more glucose for the fermentation. EG, CBH, and BG on the yeast cell surface could easily access the fragmented HPRS cellulose in the presence of cellulase SS.

To enhance ethanol fermentation by yeast, HPRS was pretreated by laccase-I-displaying yeast with ABTS as a mediator. We discovered that laccase-I-displaying yeast had pretreatment activity after noting that the amount of fermented ethanol increased when laccase-I-displaying yeast was used with ABTS for pretreatment. Furthermore, the amount of lignin after pretreatment was investigated using the Klason lignin method, and the result showed that the amount of insoluble lignin did not decrease after

pretreatment with laccase-I-displaying yeast compared to that with control yeast (BY4741 [$\Delta sed1$]/pULD1) (data not shown), which means that the lignin was hardly depolymerized. The partial lignin of HPRS could be fragmented during the pretreatment, however, and cellulases could easily access the cellulose in HPRS. The hypothesis was supported by the data: The compounds were detected with HPLC at 254 nm only when laccase-I-displaying yeast and ABTS were used for the pretreatment of HPRS.

In this study, *Trametes* sp. Ha1 laccase I was cloned, and laccase I was produced and displayed on yeast cell surface. The laccase-I-displaying yeast demonstrated oxidation activity, and it was proved that the displaying yeast were effective as a pretreatment for ethanol fermentation with HPRS. Ours is the first report of the use of laccase for direct fermentation.

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