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Heterologous expression of *Trametes versicolor* laccase in *Saccharomyces cerevisiae*

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ABSTRACT

Laccase is used in various industrial fields, and it has been the subject of numerous studies. *Trametes versicolor* laccase has one of the highest redox potentials among the various forms of this enzyme. In this study, we optimized the expression of laccase in *Saccharomyces cerevisiae*. Optimizing the culture conditions resulted in an improvement in the expression level, and approximately 45 U/L of laccase was functionally secreted in the culture. The recombinant laccase was found to be a heavily hypermannosylated glycoprotein, and the molecular weight of the carbohydrate chain was approximately 60 kDa. These hypermannosylated glycans lowered the substrate affinity, but the optimum pH and thermo-stability were not changed by these hypermannosylated glycans. This functional expression system described here will aid in molecular evolutionary studies conducted to generate new variants of laccase.

1. Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), a multi-copper oxidase, is one of the major extracellular components of the lignin-degradation system of *Trametes versicolor* [1,2]. Laccase has been studied extensively because of its ability to degrade lignin, and it can also degrade various persistent organic pollutants, such as chlorophenols [3,4] and textile dyes [5]. The enormous potential of laccase as a biological catalyst for environmental purification has been recognized. *T. versicolor* produces several laccases, and laccase III is the isoenzyme that is secreted at the highest level. In our studies, the laccase III gene was overexpressed by self-cloning [6] and was expressed in plants for the remediation [7] of soil contamination and to reduce the lignin content in rice plants [8]. Furthermore, laccase is currently used in practical applications in the textile and food industries [9], and it is useful as a biocathode in enzymatic biofuel cells [10]. Thus, laccase has garnered considerable attention across diverse research fields.

Developments in evolutionary molecular engineering provide powerful tools for the functional modification of laccase. For the evolutionary approach, an efficient heterologous laccase expression system is essential. The heterologous expression of active laccase has been studied in many microbes, including *Saccharomyces cerevisiae* [11–13], *Phichia pastoris* [14], and *Aspergillus niger* [15]. In previous studies, when *P. pastoris* was used as a host, high expression levels of laccase were achieved [14]. However, for direct evolutionary engineering, highly efficient homologous transformation is needed. *S. cerevisiae* has a highly homologous recombination activity. In a homologous region of approximately 25 bp, homologous recombination occurs, and DNA fragments are ligated. Gibson [16] showed that at least 38 overlapping single-stranded oligonucleotides and a linear double-stranded vector were assembled in one transformation event in *S. cerevisiae*. Based on these results, *S. cerevisiae* was selected as a host for this study. Furthermore, *T. versicolor* laccase was chosen for the expression target because it is a form of the enzyme with the highest redox potential. The redox potentials of *T. versicolor* laccase (785 mV) [17] and *Pycnoporus cinnabarinus* (750 ± 20 V) [18] are higher than those of other laccases, such as those from *Melanocarpus albomyces* (460 ± 10 mV) [12] and *Myceliophthora thermophile* (450 ± 10 mV) [18,19]. To the best of our knowledge, there has been no report on

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the efficient expression of *T. versicolor* laccase in *S. cerevisiae* or the characterization of the recombinant laccase. Here, we optimized the secretory production of laccase by recombinant *S. cerevisiae* and characterized the enzyme.

2. Materials and methods

2.1. Strains and reagents

Escherichia coli JM109 (Takara Bio) was used as the host cell for the manipulation of DNA. *Saccharomyces cerevisiae* BY2777 (*MATa*, *prb1*-1122, *prc1*-407, *pep4*-3, *ura3*-52, *leu2*, *trp1*) was used for the heterologous expression host. The enzymes used to manipulate DNA or protein were obtained from New England Biolabs, Takara Bio, Promega, and Nippon gene, unless otherwise stated. Compounds used for cell media were purchased from Sigma-Aldrich, Wako, and Difco.

2.2. Plasmid construction

The laccase III cDNA (*cvl3*; GenBank accession no. D13372) cloned from *Trametes versicolor* (IFO1030) was cloned to yield pTFL3. The laccase III cDNA and the signal peptide (21 amino acids from the N terminus) cassette were PCR amplified using a forward primer (5′- tattaagcttgcatgcctgcaggtcgactctagaggatgtcgaggtttcac tctcttctc-3′**)** and reverse primer (5′-agaattcgagctcttactggtcgct cgggtcgagcgcatc-3′) and pTFL3 as a template. The fragment was cleaved with *Hin*dIII and *Eco*RI and cloned into the *Hin*dIII and *Eco*RI sites of pYES2 (Thermo Fisher Scientific K.K.) to yield the expression plasmid pTA946 as shown in Fig. 1. The laccase III cDNA with its native signal sequence was under the control of the *GAL1* promoter and a *CYC1* terminator.

2.3. Yeast transformation and culture condition

The lithium acetate method was used for transformation [20], and colonies complementing uracil auxotrophy were used as transformants on SC-medium (0.67% yeast nitrogen base without amino acids, 0.197% ura dropout mixture) with 2% glucose. Transformants confirmed by PCR using primers specific for *cvl3* cDNA were incubated in SC-medium with 2% raffinose at 30 °C until OD_{660} of 1.8. Then, the cells were harvested by centrifugation, and SC-medium with galactose and $CuSO_4 \cdot 5H_2O$ was added to the cells for the induction of laccase, followed by incubation at 20 °C [21]. A baffled flask (300 mL) was used to agitate 50 ml of the culture at 160 rpm. The effect of cell density on laccase activity was examined using various inoculum densities. In addition, the effects of galactose and copper concentrations in the medium on laccase expression were investigated.

2.4. Assay of laccase activity

The culture collected over time was centrifuged, and the laccase activity in the supernatant was measured by assessing the oxidation of 0.5 mM 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) in 10 mM sodium acetate buffer (pH 3.1). The absorbance was monitored using a spectrophotometer (V-550DS; Jasco) at 420 nm (ε420 nm: 36,000 M^{-1} cm^{-1}) [22] at 25 °C. Enzyme activity was measured according to the linear initial velocity. The enzyme activities are expressed as units per milliliter, where 1 unit is defined as 1 μmol substrate oxidized per min. The region where the linearity was maintained was used to measure absorbance over time.

2.5. Purification of the native and recombinant laccase

The culture filtrate of recombinant cells was filtered (0.45 μm) and concentrated by ultrafiltration (cut-off of 50 kDa). The concentrated supernatant was applied to a Sephadex-G75 column (1.5 × 8 cm) using 10 mM phosphate buffer (pH 7.0) as the eluent. Fractions with ABTS-oxidizing activity were collected and concentrated by ultrafiltration (cut-off of 10 kDa). Furthermore, the concentrated sample was applied to a TOYOPEARL DEAE-650M column (1.5 × 8 cm, TOSOH) and eluted with a linear gradient of 0–200 mM NaCl in 10 mM phosphate buffer (pH 7.0). Fractions containing laccase activity were pooled and concentrated and replaced simultaneously with 10 mM phosphate buffer (pH 7.0). Protein concentration was determined with a micro BCA protein assay using a calibration curve prepared using standard BSA solutions.

Trametes versicolor IFO1030 was pre-cultured in a liquid medium (3% glucose, 1% peptone, 0.15% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 2 mg/L thiamine hydrochloride, and 16 mg/L $CuSO_4$ $5H_2O$) at 28 °C for 1 week with orbital shaking. The pre-culture was transferred to 4 times the volume of the same medium, and it was then cultivated for 20 days at 28 °C with orbital shaking. Laccase III (native laccase: nLac) was purified from the culture filtrate of *T. versicolor* using the same method mentioned described above.

2.6. Characterization of the recombinant laccase

The deglycosylation of purified native and recombinant laccase using Endo H, PNGase F and O-glycosidase was carried out according to the manufacturer's instructions. The deglycosylation was performed under denaturing conditions and non-denaturing conditions at 37 °C for 24 h. Denatured laccases (1 μg) were deglycosylated and subjected to SDS-PAGE for the analysis of the glycosylation chain. The gels were stained with Coomassie brilliant blue (CBB). Undenatured laccases (24 ng) were deglycosylated by Endo H and separated by native-PAGE for zymography

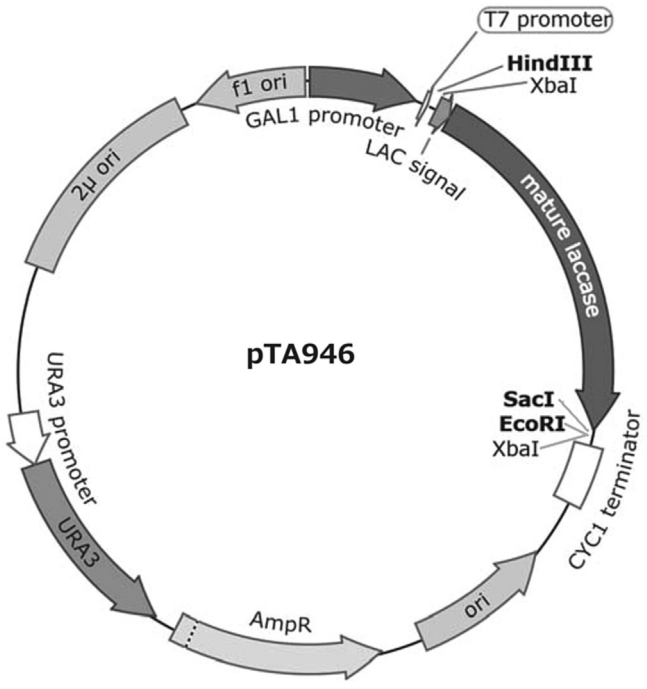

Fig. 1. Plasmid map of pTA496.