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RESEARCH ARTICLE

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Heterologous Expression of Laccase1 from *Cryphonectria parasitica* in *Saccharomyces cerevisiae*

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ABSTRACT

Laccases are enzymes capable of oxidizing phenolic compounds and are important tools in different industrial processes. Heterologous expression of laccases is of great interest in biotechnological applications but achieving high expression levels is challenging. Three different laccases have been identified in the chestnut blight fungus *Cryphonectria parasitica*, among which a tannic acid-inducible laccase (laccase3) was successfully expressed using *Saccharomyces cerevisiae*. To obtain high and stable expression of fungal laccases, we cloned the gene encoding an extracellular laccase (Laccase1) of *C. parasitica* into a yeast episomal vector, used the resulting vectors to transform *S. cerevisiae*, and optimized the culture conditions of the selected transformants for Laccase1 production. We also tested the significance of the signal peptide of Laccase1 in the secretion of expressed Laccase1 and compared it with the widely used rice amylase signal peptide. Among the four constructs tested using a yeast episomal vector, full-length Laccase1 containing an endogenous signal peptide, showed the highest laccase activity. Interestingly, the stability of the recombinant vector expressing laccase was lower than that of the mock transformant, suggesting a detrimental effect of the Laccase1-expressing vector on host cells. Thus, we optimized the culture conditions to produce Laccase1 and the resulting optimum culture conditions identified through one-factor-at-a -time (OFAT) were 2% sucrose; 3% yeast nitrogen base without amino acid; pH 5.0; and 30°C. The laccase activity was found to be 2.2 U/mL in optimal culture conditions, resulting in a 6.5-fold increase compared to the conventional culture medium.

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1. Introduction

Laccases (EC 1.10.3.2) are multicopper-binding oxidoreductases that are naturally present in various plants and fungi [1,2]. Fungal laccases are important for morphogenesis, delignification, and pathogenic interactions, and their mechanism of action involves the oxidation of multiple phenolic compounds [3–5]. Because of their ability to oxidize a variety of compounds and produce water as a reaction by-product, these enzymes have become a highly valuable tool, referred to as "green catalysts," which highlights their properties to only produce water as the reaction by-product [6]. Currently, laccases are used in several industrial processes, such as bioremediation, pulp bleaching, dye decolorization, and pollutant removal [7–9]. These enzymes have also been employed as catalysts in producing anti-cancer medications and developing biosensors [10,11]. Due to the remarkable industrial applicability of laccase enzymes, a significant amount of research has been conducted on various recombinant laccases expressed in hosts such as bacteria, yeast, fungi, and plants [12–16]. Yeast is an ideal tool for developing new enzymes with desirable properties. However, large-scale biotechnological applications are hindered by the challenging heterologous expression of laccases [17].

Currently, the industry requires safer alternatives to reduce environmental pollutants and toxic compounds, which has led to an increasing demand for the large-scale production of laccase enzymes [18–20]. To overcome the high demand for different bioproducts at a low cost, the use of microorganisms, such as bacteria or yeast hosts, has been evaluated.

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Among these, *Saccharomyces cerevisiae*, baker's yeast, has shown remarkable advantages such as the ability to produce and secrete glycosylated eukaryotic proteins, low production cost, brief culture time, and feasible genetic manipulation [21,22]. However, the protein yields achieved with the *S. cerevisiae* expression host were not always higher than those achieved with other yeast systems, including *Pichia pastoris*, *Hansenula polymorpha*, and *Kluyveromyces lactis*, indicating the necessity for engineering strategies to increase protein production [22].

Cryphonectria parasitica is a well-known phytopathogenic fungus whose pathogenicity is associated with laccase enzyme activity. Infections of the fungus with hypovirus lead to hypovirulence and causes changes in various biological processes, including sporulation, pigment production, reproduction, and enzyme production. Through analysis of the *C. parasitica* genome, 16 putative laccase genes have been identified [23], and three laccase isozymes-extracellular *Lac1* [24], intracellular *Lac2* [25], and extracellular and tannic acid-inducible *Lac3* [24,26], have been thoroughly characterized. The activities of all three enzymes are typically suppressed by CHV1 infection. Previously, the heterologous expression of a laccase from *C. parasitica* was evaluated in *S. cerevisiae*, showing the feasibility of producing up to 45 mU/mL of LAC3 after two days of culture [27].

Laccase genes encode various isozymes that differ in kinetic properties, substrate affinities, or expression patterns [28]. *C. parasitica* is often considered a "super pathogen" owing to its abundance of progenies, fast and extensive damage, various means of dissemination, and high surviving capability. Laccase, an important virulence factor in *C. parasitica*, plays a pivotal role in the break down of phenolic compounds such as tannins, which typically provide chestnut trees with resistance against various pathogens. By oxidizing tannins, laccases convert them into low molecular-weight compounds, thus reducing or eliminating their protective toxicity. This process weakens the defenses of tree, offering an eco-friendly yet destructive approach to infection. It effectively oxidizes phenolic compounds, making it a promising tool for wastewater treatment and soil remediation.

Among three well-characterized laccases of *C. parasitica*, *Lac1* has attracted attention because of its constitutive expression and broad-spectrum biocatalytic activity, allowing the fungus to survive in various host environments, insinuating the high potential of this enzyme for the biodegradation of plant biomass and phenolic compounds. However, the expression pattern of *Lac1* in *S. cerevisiae* has not been evaluated. The main objective of this study was to evaluate the heterologous expression of *Lac1* in *C. parasitica* using an *S. cerevisiae* expression system.

2. Materials and methods

2.1. Strains and culture media

All plasmids used in this study were maintained in *E. coli* Top 10, which was maintained in LB broth or agar supplemented with appropriate antibiotics at 37 °C. The obtained plasmids were verified using restriction enzyme analysis and sequencing to confirm the presence of the genes of interest.

The *Saccharomyces cerevisiae* 2805 strain *(MATα pep4::HIS3 prb 1-δ Can1 GAL2 his3 ura3-52*) was used as a host for Laccase1 expression [29]. The host strain was maintained in YEPD media (yeast extract 10 g/L, peptone 20 g/L, and D-glucose 2 g/L), whereas the transformants were maintained in uracil dropout media (*ura*$^-$) (yeast nitrogen base 6.7 g/L, 2% glucose, and uracil synthetic dropout supplemented with amino acids at 1.92 g/L). For the liquid culture of the Laccase1-based transformants, a single colony of each of the transformant strains was inoculated into 5 mL of *ura*$^-$-selective liquid culture and grown for 48 h with continuous agitation at 200 rpm and 30 °C. From this culture, 250 µL was inoculated into 5 mL of *ura*$^-$-selective broth and cultured for 16 h under similar conditions. Thereafter, 1.5 mL of this culture was inoculated into 40 mL of *ura*$^-$-selective medium and cultured for 8 d to measure the enzymatic activity. The culture was grown at 30 °C and 200 rpm; 1.2 mL of culture was collected every day and centrifugated for 3 min at 13,000 rpm, and 360 µL of the clear supernatant was collected for measurement of laccase activity.

2.2. Plasmid construction

The sequence of *Lac1* (GenBank accession number: M73257.1) was amplified from the total RNA of *C. parasitica* EP155/2 and used to synthesize total cDNA with random hexamer primers using SuperScript IV Reverse Transcriptase IV (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The Laccase1 cDNA sequence was amplified from the total cDNA using the PCR method. *Bam*HI and *Sgr*DI restriction enzyme recognition sites were added before the start codon and after the stop codon, respectively.

The resulting full-length *Lac1* cDNA construct (LAC1), encoding 591 amino acids, was cloned into the T-blunt vector for sequence verification. Subsequently, the gene was released from the vector using *Bam*HI/*Sgr*DI and cloned into the

pYEGPD-TER vector (Figure 1A,B), which contained a constitutive *GPD* (glyceraldehyde-3-phosphate dehydrogenase) promoter and *GAL7* terminator. Constructs with a 31-amino acid signal peptide of rice amylase (ramy1A, GenBank accession no. X16509) attached to the full *Lac1* sequence (RspLAC1), mature *Lac1* sequence without a 20-amino acid endogenous signal peptide (mLAC1), and rice signal peptide (ramy1A) attached to the mature *Lac1* sequence (RspmLAC1) were obtained (Supplementary Figure S1). The sequence of mature *Lac1* was determined as previously described [26]. *Bam*HI and *Sgr*DI restriction sites were added before the start codon and after the stop codon, respectively. The resulting fusion genes were cloned into the pGEM T-Easy vector for sequence verification. Subsequently, the genes were released from the vector *via* enzymatic digestion and cloned into an expression vector (Figure 1B). Recombinant pYEGPD-TER vectors were transformed into *S. cerevisiae* 2805 using the lithium acetate method as previously described [30–32]. Briefly, competent yeast cells were prepared using lithium acetate (LiAc), and plasmid DNA was introduced into competent cells with single-stranded salmon sperm DNA in a transformation buffer containing LiAc and polyethylene glycol (PEG). Subsequently, the transformed cells were selected on *ura*$^-$-selective solid medium. The empty vector pYEGPD-TER was also transformed to create a mock transformant, which was used as the negative control.

2.3. Colony PCR and quantitative real-time PCR

The resulting yeast transformants were grown on *ura*$^-$-selective agar plates for 3 days, and candidate transformant colonies were screened using colony PCR. Colonies were picked up using a sterilized toothpick and dissolved in 10 µL of H_2O. Subsequently, 1 µL of the resulting colony suspension was added to a PCR mixture containing forward and reverse primers to clone either the full or a 1.0 kb section of the *Lac1* cDNA sequence (Supplementary Figure S2). PCR was performed for 30 cycles at the annealing temperature of 55 °C.

For quantitative real-time RT-PCR (qRT-PCR), transformants were cultured in an appropriate medium, and total yeast RNA was extracted as previously described [33]. The RNA concentration was determined using a Qubit 4 fluorometer (Thermo Fisher Scientific Inc.). To evaluate the expression levels of target genes, qRT-PCR was performed as

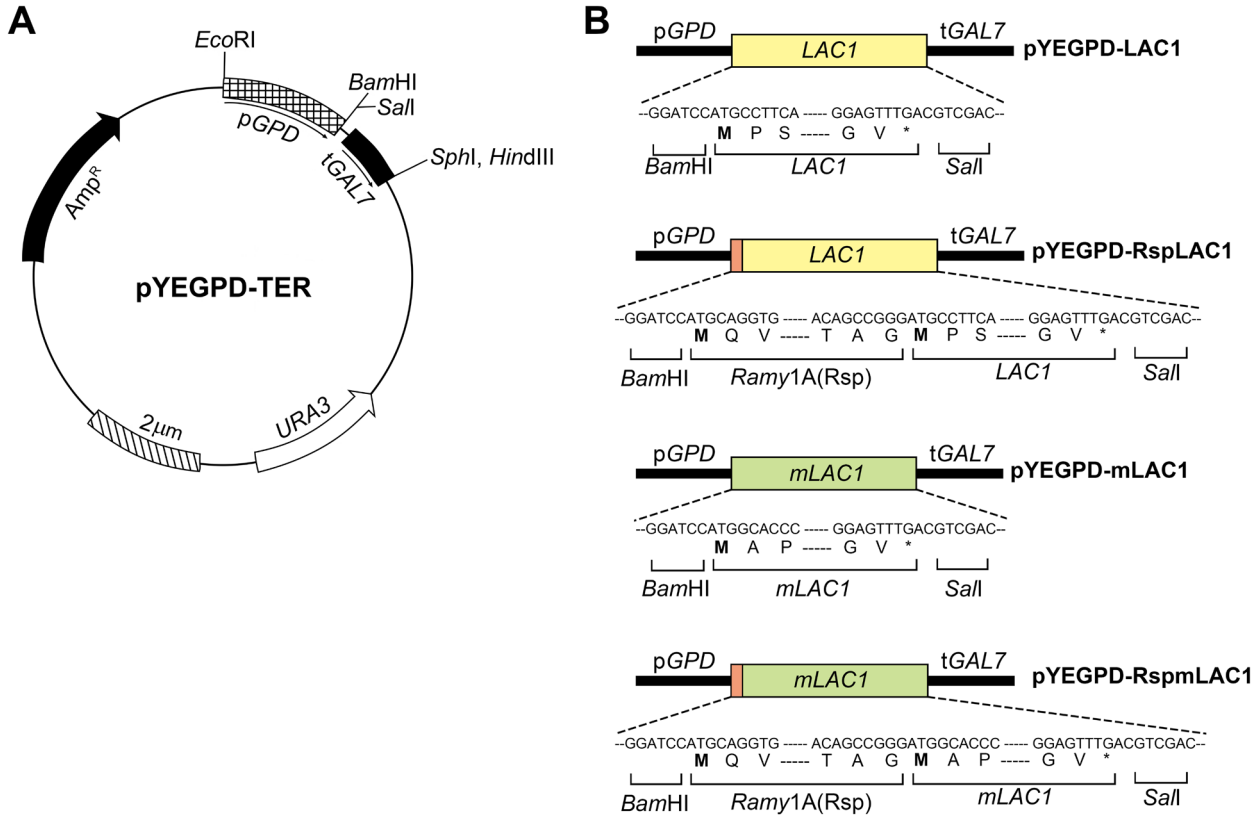

Figure 1. Schematic representations of expression vectors designed in this study. All the expression cassettes were cloned into the pYEGPD-TER vector using *Bam*HI and *Sgr*DI restriction enzymes (A). Constructs based on Laccase1 sequence full Laccase1 construct (pYEGPD-LAC1), rice amylase signal peptide attached to the full Laccase1 sequence (pYEPD-RspLAC1); mature Laccase1 constructs mature Laccase1 attached to rice amylase signal peptide (pYEGPD-RspmLAC1) and mature Laccase1 only (mLAC1) (B).

previously described [34]. The transcript levels were measured relative to the amount of *GPD* in *S. cerevisiae*, which served as an internal control. The primer pairs used for each target gene are listed in Table 1.

2.4. Laccase assay

The laccase transformants were grown on *ura*⁻-selective media supplemented with tannic acid 0.5% [27] and grown for 2–5 d at 30 °C. Three laccase transformants for each laccase construct with the highest coloration observed on the border of the colonies were selected for subsequent liquid culture analysis.

Measuring laccase activity in culture filtrate using 2,6-dimetoxyphenol (DMOP) as a substrate has long been established because of specificity over other substrates [35,36], and has also been used in a previous study on the heterologous expression of laccase in *S. cerevisiae* [27]. Laccase activity in the culture filtrate was therefore evaluated using a spectrophotometric assay with a substrate solution consisting of 10 mM DMOP in sodium tartrate buffer at pH 3.4 [37]. A laccase unit was defined as an A468 increase of 1.0/min at 25 °C, as described by Rigling et al. [37]. The total reaction volume consisted of 300 µL, comprising 60 µL of clear supernatant of culture broth after centrifugation and 240 µL of substrate solution. Laccase activity was measured daily for 8 days. For comparison, we measured the laccase activity of the culture filtrates of selected transformants using ABTS as a substrate [38].

To measure intracellular laccase activity, the total soluble protein was prepared from the disrupted cells of transformants as previously described [39], and laccase activity was measured.

2.5. Optimization of culture media and conditions

The experiment for optimizing culture conditions was carried out using 50 mL of modified *ura*⁻-selective medium in a 250 mL flask. Colonies from the *ura*⁻-selective media plate were inoculated into *ura*⁻-selective broth, and pre-cultures for overnight at 30 °C and 150 rpm. Thereafter, the pre-culture medium corresponding to 1% of the final culture volume was inoculated into the *ura*⁻-selective based dropout culture medium and incubated for 24 h. The laccase activity in the culture broth was measured.

To optimize the medium composition, six different carbon sources (glucose, lactose, fructose, sucrose, maltose, and glycerol) were tested. To select the optimal carbon source for laccase production, 2% of each carbon source was used in a modified *ura*⁻-selective medium. Six nitrogen sources, yeast extract, YNB without amino acids, peptone, tryptone, beef extract, and soy peptone, were tested by adding different nitrogen sources to the medium with the selected carbon source. Once the carbon and nitrogen sources were determined, optimum concentrations were selected.

Additionally, optimum culture conditions such as pH and temperature were determined. Various pH values in the range of 4.0–9.0, adjusted with HCl or NaOH, were tested for optimum pH conditions. For temperature, tests were conducted in the range of 20 °C–30 °C.

Table 1. Oligonucleotide primers used in this study.

Primers	Sequence (5′–3′)
Lac1-cDNA-F	CGCGGATCCATGCCTTCATTCTTTCGAGC
Lac1-cDNA-R	CTACGTCGACGTCAAACTCCCGAATCATCCT
Lac1-BamHI-ATG	CGCGGATCCATGCCTTCATTCTTTCGAGC
Lac1-SgrDI-stop	CTACGTCGACGTCAAACTCCCGAATCATCCT
ramyIA-F	CGAGGATCCATGCAGGTGCTGAACACC
ramyI-fLac1-ov-F	AACTTGACAGCCGGGATGCCTTCATTCTTT
ramyI-fLac1-ov-R	AAAGAATGAAGGCATCCCGGCTGTCAAGTT
ramyI_mLac1-ov-F	AACTTGACAGCCGGGGCACCCTCGCTTCT
ramyI_mLac1-ov-R	AGAAGCGAGGGTGCCCCGGCTGTCAAGTT
BamHI-mLac1-F	TATGGATCCATGGCACCCTCGCTTCTC
qRT_Lac1-F	GCTCGTAAATGGTAACATTCTG
qRT_Lac1-R	GATCGTTGTCCCATTGTACTTG
qRT_GPD-F	CAACCGTCGATGTCTCCGT
qRT_GPD-R	TTCAGCGGCAGCCTTAACA

Underlines indicate restriction enzyme sites.

2.6. Statistical analysis

All enzyme activities were analyzed by ANOVA using the SPSS software ver.18.0 (IMB Corp., Armonk, NY, USA). The significance of all effects was determined using Duncan's test at $p = .01$.

3. Results

3.1. Construction of expression vectors

To express the Laccase1 gene, a full-length Laccase1 gene (LAC1), a mature LAC1 (mLAC1), and two fusion gene constructs of the rice amylase1 signal peptide (Rsp) fused to LAC1 (RspLAC1) and the endogenous signal peptide replaced with Rsp (RspmLAC1) were PCR-amplified. All the PCR amplicons were cloned and sequenced for further verification.

For yeast expression, the pYEGPD-TER vector, which is a high-copy number episomal vector containing a 2 µ yeast replication origin that has been used to express various types of heterologous proteins in yeast [32,33,40], was employed. The *Bam*HI and *Sgr*DI-digested target genes were cloned into *Bam*HI and *Sal*I-digested pYEGPD-TER vectors. The resulting recombinant vectors were sequenced and used to transform the *S. cerevisiae* 2805 strain. The primers used in this study are listed in Table 1.

3.2. Analysis of transformed S. cerevisiae

At least 20-30 putative transformants for each construct were selected on *ura*⁻-selective media and the presence of the expression vector was analyzed using colony PCR as described in Materials and Methods. To amplify the *Lac1*-containing vector, a set of primers covering the full *Lac1* gene and a set of primers covering the internal region of the mLAC1 gene were used for the mLAC1-containing vector (Table 1). Colony PCR using DNA preparations from the putative transformants revealed the presence of 1.7 kb amplicon for *Lac1* and 1.0 kb amplicon for the mLAC1 genes (Supplementary Figure S2), which indicated that the corresponding expression plasmids were successfully transferred into the recipient yeast.

3.3. Analysis of laccase expression; plate assay

Because Bavendamn media using the tannic acid-supplemented malt extract agar are commonly used for laccase activity [37] and we also demonstrated that recombinant yeast expressing laccase3 showed a characteristic color change around the colony on tannic acid-supplemented MEA [27], we tested the expression of laccase1 using the tannic acid-supplemented *ura*⁻-selective media.

A plate assay to observe the color changes around the colonies as an indicator of laccase activity was applied to the tannic acid-supplemented *ura*⁻-selective media (Figure 2). All transformants, except those transformed with the expression vector mLAC1 showed distinctive color changes around the colonies grown on the tannic acid-supplemented *ura*⁻-selective media. These results indicated that the plate assay using *ura*⁻-selective media supplemented with tannic acid was able to express laccase, and three, but not all four, constructs were able to express and secrete the laccase enzyme.

Interestingly, a nutrient-rich medium, such as YEPD supplemented with tannic acid, could not discern the characteristic color changes around the colonies, which suggested that selection pressure by *ura*⁻-selective media is a prerequisite for determining laccase expression. Incubation in a tannic acid supplemented *ura*⁻-selective medium was required to observe the characteristic color change as an indicator of laccase activity.

Based on a plate assay using tannic acid-supplemented *ura*⁻-selective media, the three transformants, which showed the most distinctive color changes were selected for each construct and used for further analysis of enzyme production in liquid media. Three transformants with the mLAC1 construct that showed no discernible color change were randomly selected for further analysis.

3.4. Estimation of laccase expression; liquid culture

The three transformants showing the most intense color change for each construct were cultured on *ura*⁻-selective liquid media, and culture filtrates for each strain were used for the laccase assay. Laccase activity increased as the culture progressed, peaked at 6 days of culture, and then gradually decreased (Figure 3). No significant differences were observed between the strains. However, significant differences in laccase activity were observed among the transformants with different vector constructs. Transformants carrying the LAC1 construct showed the highest activity at all time points, followed by transformants carrying the RspmLAC1 construct, whereas transformants with the RspLAC1 construct showed the lowest activity. No activity was observed in the transformants carrying the mLAC1 construct. The greatest activity of the transformant with the LAC1 construct was 0.35 U/ml on day 6 after culture. Because many heterologously expressed laccase activities were assessed using the ABTS oxidation method, we showed the laccase activity of selected transformants using it for a wider comparison of laccase yield from previous studies (Supplementary Figure S3).

Because no activity was observed with the culture filtrate of the mLAC1 construct, we tested whether laccase was expressed intracellularly. Total soluble protein, representing intracellular soluble protein, was prepared from disrupted cells of the transformants containing the mLAC1 construct, and laccase activity was measured. No laccase activity was observed during the preparation of the total soluble proteins. These results suggested that the expression, but not secretion, of laccase1 using the mLAC1 construct hindered the activity.

3.5. Plasmid stability and cell growth of recombinant

We observed laccase activity using selective media but not nutrient-rich nonselective media such as YEPD; therefore, we measured the stability of the laccase-expressing plasmids. Plasmid stability was measured by comparing the colony-forming units (CFUs) on nonselective YEPD with those on *ura*⁻-selective media after plating the same number of cells grown in nonselective YEPD liquid media for 3 days. As shown in Table 2, more than 60% of transformants with the LAC1 construct did not grow on the *ura*⁻-selective media. When transformants were

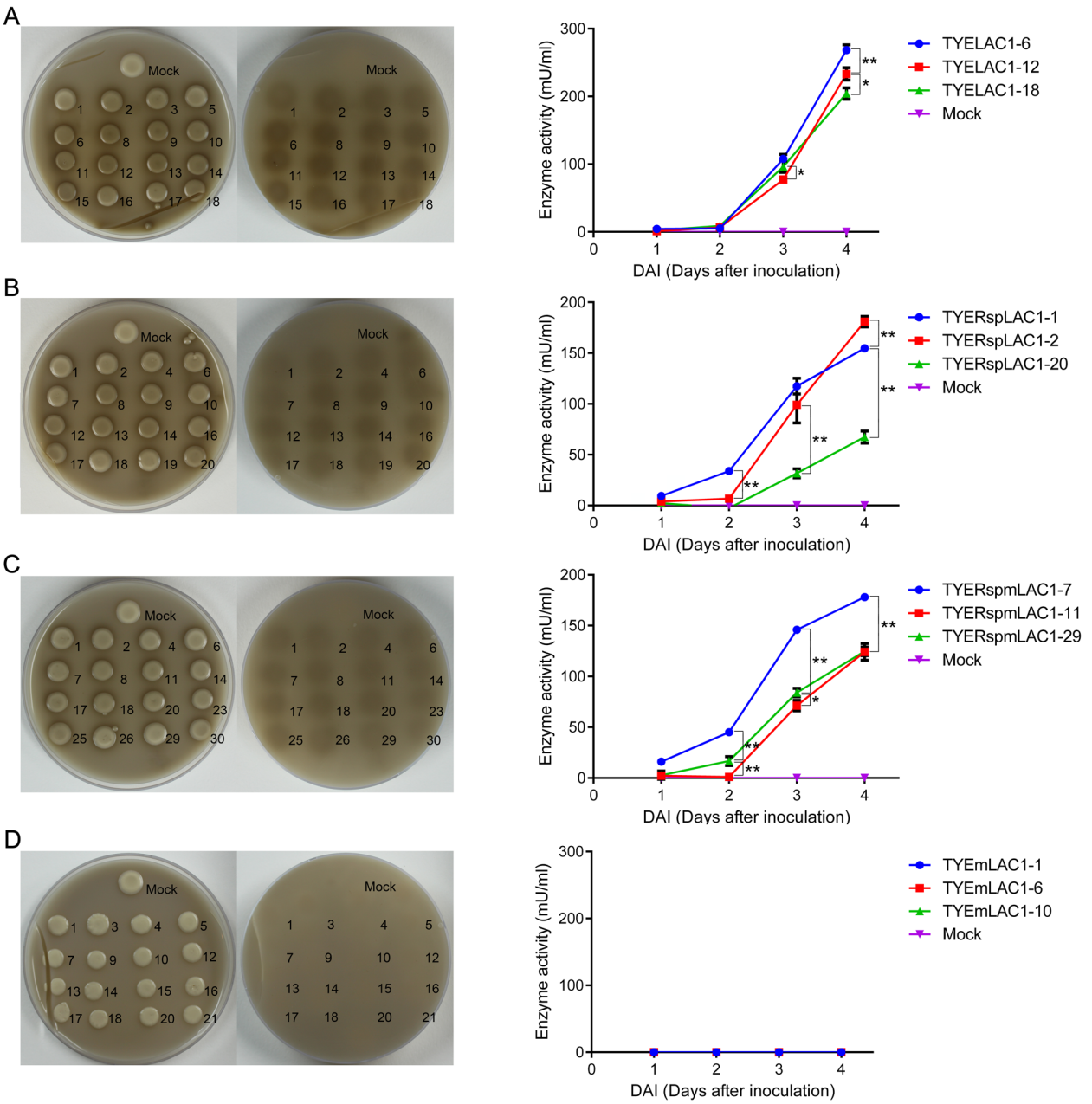

Figure 2. Analysis of laccase activity. Plate assay using tannic-acid supplemented media (left panels). Brown staining was observed around colonies of transformants, whereas no color was observed around the mock transformant. Line graph shows the laccase activity measurement of the three selected transformants during the 4 days of culture (right panels). (A) From the 1-19 pYEGPD-LAC1 transformants, number 6, 12, and 18 were selected for growth in liquid culture. (B) pYEGPD-RspLAC1 #1, 2, and 20 transformants were selected for growth on liquid culture. (C) The pYEGPD-RspmLAC1 transformants #7, 11, and 29 were selected for further analysis. (D) pYEGPD-mLAC1 transformants did not show brown coloration on plate assay. Error bars represent mean ± SD. Student's *t*-test ($n = 3$) was used to compare data between two groups, $^{**}p < .01$ and $^{*}p < .05$.

grown on the *ura*–selective media, more than 50 and 40% of transformants with the RspLAC1 and RspmLAC1 constructs, respectively, did not produce colonies on the selective media. In contrast, mock transformants with the vector only showed that less than 15% of transformants did not show CFUs on the selective media. Interestingly, transformants with the mLAC1 construct showing no laccase activity showed a similar number of CFUs as mock transformants, suggesting that the presence of a larger episomal plasmid than in mock transformants was not the only reason for plasmid stability. As a control, transformants grown in *ura*$^{-}$-selective liquid showed no significant difference in CUFs between the selective and nonselective plates. These results explain why laccase activity was detected in culture filtrates grown in *ura*$^{-}$-selective liquid media but not in nonselective YPED broth.

3.6. Selection of carbon and nitrogen sources

To determine the optimal carbon source, the selected *Lac1* transformants were cultured in a medium containing each of the six different carbon sources and

assayed for laccase activity in the culture broth (Figure 4A). Among the six carbon sources, glucose, fructose, and sucrose showed significant laccase activity, whereas almost no laccase activity was observed with the other three carbon sources. Among the three laccase-positive carbon sources, the sucrose-containing medium showed the highest activity (0.556 U/mL), followed by those containing glucose (0.337 U/mL) and fructose (0.239 U/mL).

Among the six tested nitrogen sources, the medium containing YNB without amino acids showed significant laccase activity (0.539 U/mL), whereas only limited activity was observed in the medium containing either peptone or beef extract (Figure 4B). No discernible activity was observed with the other three nitrogen sources.

We then tested the optimal concentration of sucrose, which ranged from 5 to 60 g/L. As shown in Figure 4C, the laccase activity increased as the concentration increased, peaked at 20 g/L, and then gradually decreased as the concentration increased. The highest enzymatic activity using 2% sucrose was 0.543 U/mL. In addition, the optimal concentration of the selected YNB without amino acids ranging from 6.7 to 60 g/L was determined using a medium containing 2% sucrose as the carbon source (Figure 4D). Laccase activity increased as the concentration of YBN without amino acids increased up to 30 g/L, and then gradually decreased thereafter. The highest activity was found to be 1.399 U/mL in the medium containing 20 g/L sucrose and 30 g/L YBN without amino acids.

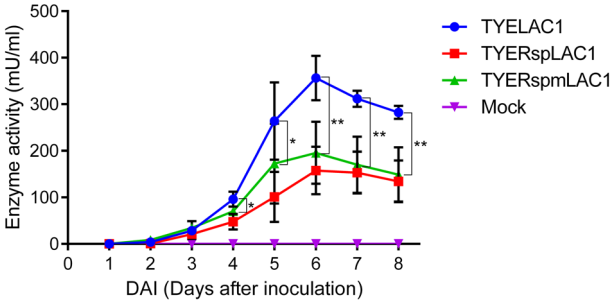

Figure 3. Laccase activity on different Laccase1-based transformants. The previously selected transformants with higher laccase activity values were grown on *ura*⁻-selective media for 8 d and laccase activity was measured daily. Error bars represent mean ± SD. Student's *t*-test ($n = 3$) was used to compare data between two groups, $^{**}p < .01$ and $^{*}p < .05$.

3.7. Effect of temperature and pH

After the optimal medium composition was established, the optimal culture conditions, such as pH and temperature, were determined. Various pH values ranging from 4.0 to 9.0 adjusted with HCl and NaOH were tested for the optimal pH condition (Figure 5A). Among the pH values tested, pH 5.0 showed the optimal condition with an activity of 1.830 U/mL. Laccase activity gradually decreased as the pH increased to 7.0. Decreased laccase activity and severe growth defects were observed above pH 7.0 (Figure 5A). Temperatures ranging from 20 °C to 40 °C were also tested with the optimized culture medium at pH 5.0 (Figure 5B). Laccase activity increased as the temperature increased and peaked at 30 °C; thereafter, the activity dramatically decreased at 35 °C and 40 °C due to the growth defect. The highest activity at 30 °C was 2.196 U/mL followed by 2.030 U/mL at 25 °C and 0.328 U/mL at 20 °C (Figure 5B). Therefore, the highest laccase activity of the *Lac1* transformant was 2.196 U/mL using the *ura*⁻-selective optimal media (yeast nitrogen base 30 g/L, 2% sucrose, uracil synthetic dropout supplemented with amino acids 1.92 g/L, pH 5.0) at 30 °C. This optimization process improved productivity by more than six times compared to the original *ura*⁻-selective media.

4. Discussion

Laccase expression using gene constructs containing the signal peptide was successful, whereas the gene construct encoding the mature peptide alone showed no enzymatic activity. Quantitative real-time RT-PCR (qRT-PCR) revealed that a significant amount of *mLac1* transcripts accumulated (Supplementary Figure S4), suggesting that transcriptional control was not the reason for the lack of expression. *In silico* analysis (http://www.unafold.org/) of the secondary structure of the putative mLAC1 transcript

Table 2. Plasmid stability measured by comparing the CFUs on nonselective YEPD with those on *ura*⁻-selective media.

	CFUs[a]/plate	
Strain[b]	YEPD	*ura-*
Mock	89.6 ± 12.7[c]	75.6 ± 10.5
LAC1	77.2 ± 16.6	20.0 ± 5.0
RspLAC1	94.6 ± 10.1	23.6 ± 6.0
RspmLAC1	102.0 ± 20.0	36.0 ± 3.1
mLAC1	94.8 ± 15.0	81.0 ± 5.3

[a]Colony-forming units per plate.
[b]For each strain, 100 cells, cultured in nonselective YEPD liquid media for 4 days, were spread on the media.
[c]Values are shown as the means ± standard deviation.

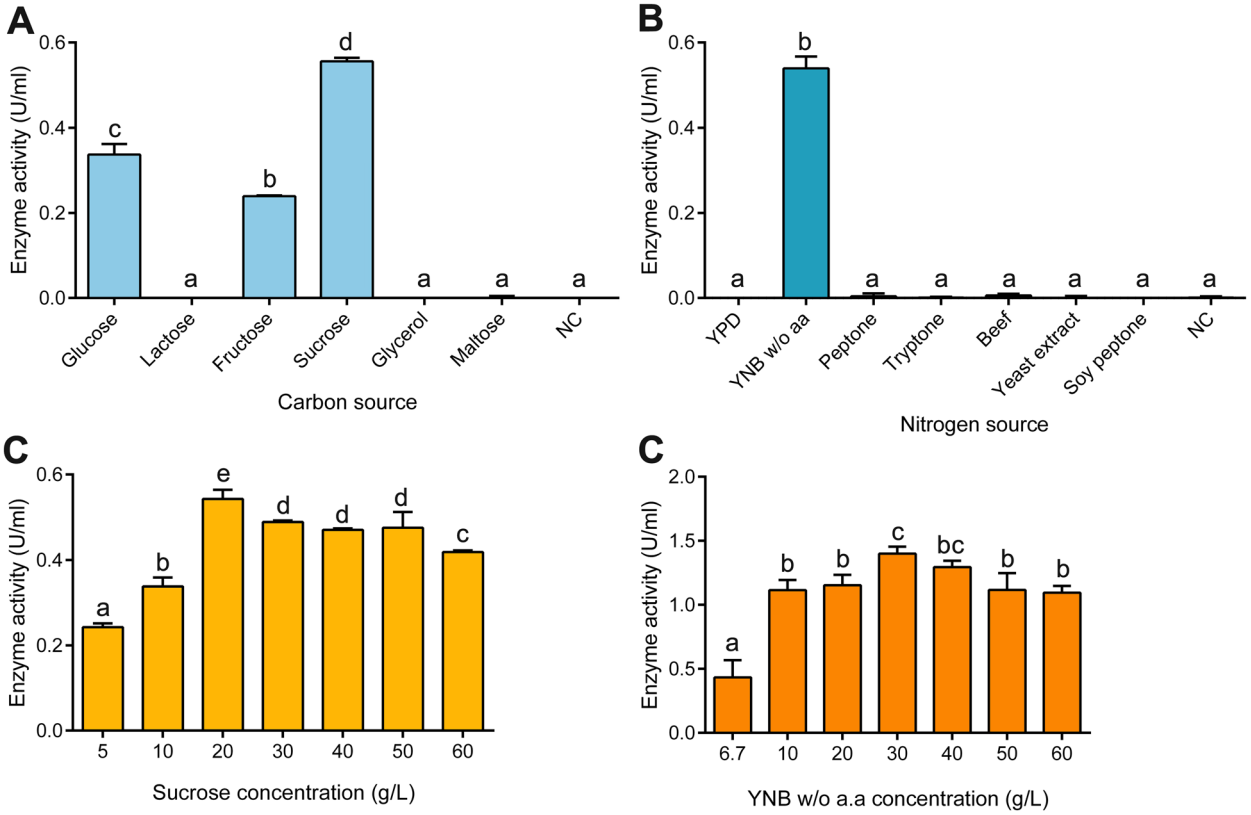

Figure 4. Optimization of media composition to enhance laccase activity of strain. Laccase activity using different carbon (A) and nitrogen sources (B). Laccase activity at different concentrations of the selected carbon (C) and nitrogen (D) sources. Enzyme activity was measured 24 h after the main culture. All enzyme activities were analyzed by ANOVA using SPSS software ver.18.0 (IMB Corp., Armonk, NY, USA). The significance of all effects was determined using Duncan's test at $p = .01$.

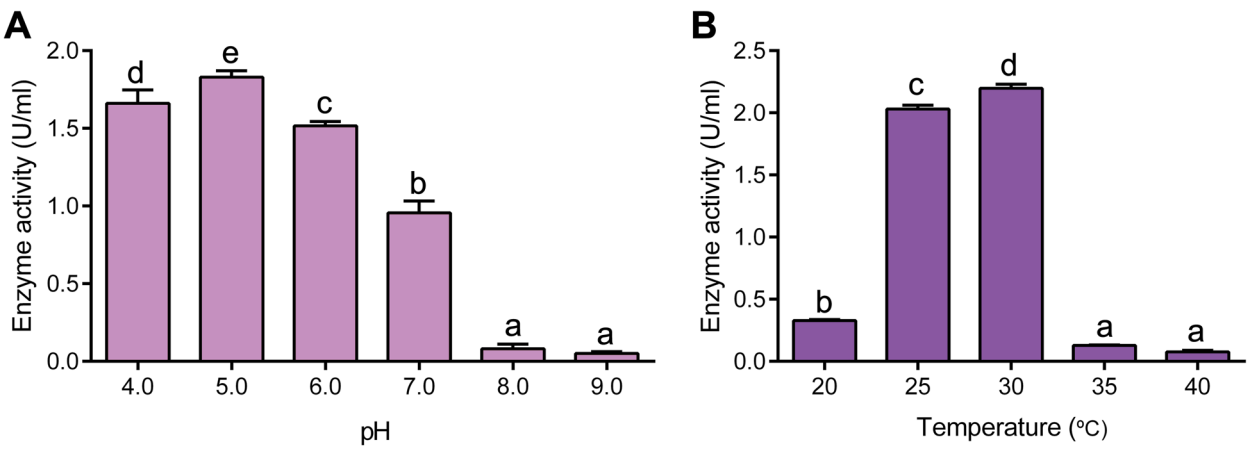

Figure 5. Optimization of culture conditions for laccase activity. Laccase activity depending on various pH ranges (A) and temperatures (B). Enzyme activity was measured 24 h after the main culture. All enzyme activities were analyzed by ANOVA using SPSS software ver.18.0 (IMB Corp., Armonk, NY, USA). The significance of all effects was determined using Duncan's test at $p = .01$.

revealed no distinctive inhibitory structure around the initiation codon or gene body. No significant difference was observed in the secondary structures of the LAC1 and mLAC1 transcripts. In addition, the analysis of laccase activity using the intracellular protein extract of the corresponding mLAC1 transformant showed no enzyme activity. Moreover, based on the successful expression of laccase using other constructs, the inhibitory C-terminal extension of laccase is unlikely to result in no enzymatic activity [13,41]. Thus, although poor translation and/or post-translational processes, such as proteolytic degradation, might be the reason for the lack of enzyme activity, the absence of a distinctive inhibitory structure for translational initiation and the persistence of laccase activity during liquid culture, that is, the maximal activity around 5 or 6 d after incubation, suggest that there might be an

intrinsic reason for the mLAC1 gene construct requiring further studies.

The stability of the episomal recombinant plasmid of the transformant was assessed by comparing the CFUs on nonselective YEPD with *ura*$^-$-selective media corresponding to cells grown in nonselective YEPD broth. Plasmid instability was remarkable in cells expressing laccase compared to strains showing no laccase activity, such as the mock and mLAC1 transformants. These results indicate that the metabolic burden due to the expression of laccase, and not the presence of a large plasmid, is the reason for plasmid instability. These results are consistent with those of a previous study showing the deleterious effect of laccase on *S. cerevisiae* cell growth [27].

Culture media, that is, YEPD vs. *ura*$^-$-selective media, showed a great difference in the laccase activity of the transformants even with the same construct. Our previous study on *Lac3* expression demonstrated that the expressed laccase was specifically labile in the culture media of YEPD because of the protease activity in the culture broth of the same parental host strain *S. cerevisiae* 2805 [42]. Our results, which showed no activity in YEPD broth, are in good agreement with those of a previous study [27]. Similarly, our attempts to optimize the culture components showed that media supplemented with YEPD, yeast extract, or peptone alone did not support laccase production. These results suggest that laccase expression increased in chemically defined media under selective culture conditions during the optimization process, while laccase expression remained constant in complex media. Although other studies on laccase production in yeast demonstrated that organic nitrogen sources, such as peptone and yeast extract, showed better laccase activities than simple synthetic nitrogen sources, such as NH_4Cl, NH_4NO_3, and KNO_3, due to buffering effects on cultured cells [43], our results showing the near absence of laccase activity using organic nitrogen sources are likely to be strain-specific. Compared to simple synthetic nitrogen sources, complex media, such as YEPD, require more protease activity for yeast nutrient uptake. Thus, it is highly likely that proteolytic degradation in nutrient-rich complex media is the limiting factor for laccase expression. Thus, plasmid stability and medium composition in nutrient-rich complex media such as YEPD are important factors for the expression of cloned laccase genes, even in successful transformants.

Optimization of Laccase1 production resulted in 2.2 U/mL under the conditions of 2% sucrose; 3% yeast nitrogen base without amino acid; pH 5.0; and 30 °C. Since strain preferences for different nutrient sources have been reported [44,45], our optimal conditions to produce laccase1 were likely strain-specific. However, the enzymatic activity of Laccase1 in this study was significantly higher than the optimized laccase3 production (0.3 U/mL). Laccases contain a broad spectrum of substrates, including various phenolic and aromatic amines. However, the oxidizing efficiency of each laccase varies depending on its substrate [46]. Considering the enzymatic efficiency of the tannin-specific laccase3, the significant increase in enzyme activity by recombinant Laccase1 might be attributed to the enzymatic efficiency of recombinant Laccase1 rather than a larger amount of protein product of the *Lac1* gene.

5. Conclusion

This study demonstrated that heterologous laccase1 was successfully expressed in *S. cerevisiae* and optimal expression conditions were determined. We optimized the composition of growth medium and culture conditions for the heterologous expression of the laccase1. Though this optimization study, we were able to produce 2.2 U/mL of laccase1 enzyme, making it possible to produce large amounts of a novel recombinant laccase1 that has previously been difficult to produce on a commercial scale.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

References

[1] Baldrian P. Fungal laccases-occurrence and properties. FEMS Microbiol Rev. 2006;30:215–242. doi: 10.1111/j.1574-4976.2005.00010.x.

[2] Umar A. Screening and evaluation of laccase produced by different *Trichoderma* species along with their phylogenetic relationship. Arch Microbiol. 2021;203(7):4319–4327. doi: 10.1007/s00203-021-02420-5.
[3] Giardina P, Faraco V, Pezzella C, et al. Laccases a never-ending story. Cell Mol Life Sci. 2010;67(3):369–385. doi: 10.1007/s00018-009-0169-1.
[4] Khatami SH, Vakili O, Movahedpour A, et al. Laccase: various types and applications. Biotechnol Appl Biochem. 2022;69:2658–2672. doi: 10.1002/bab.2313.
[5] Umar A, Ahmed S. Optimization, purification and characterization of laccase from *Ganoderma leucocontextum* along with its phylogenetic relationship. Sci Rep. 2022;12(1):2416. doi: 10.1038/s41598-022-06111-z.
[6] Riva S. Laccases: blue enzymes for green chemistry. Trends Biotechnol. 2006;24(5):219–226. doi: 10.1016/j.tibtech.2006.03.006.
[7] Dana M, Khaniki GB, Mokhtarieh AA, et al. Biotechnology and industrial applications of laccase: A Review. J Appl Biotechnol Rep. 2017;4:675–679.
[8] Mate DM, Alcalde M. Laccase engineering: from rational design to directed evolution. Biotechnol Adv. 2015;33(1):25–40. doi: 10.1016/j.biotechadv.2014.12.007.
[9] Alshiekheid MA, Umar A, Ameen F, et al. Biodegradation of chromium by laccase action of *Ganoderma multipileum*. J King Saud Univ Sci. 2023;35(10):102948. doi: 10.1016/j.jksus.2023.102948.
[10] Mohit E, Tabarzad M, Faramarzi MA. Biomedical and pharmaceutical-related applications of laccases. Curr Protein Pept Sci. 2020;21(1):78–98. doi: 10.2174/1389203720666191011105624.
[11] Castrovilli MC, Tempesta E, Cartoni A, et al. Fabrication of a new, low-cost, and environment-friendly laccase-based biosensor by electrospray immobilization with unprecedented reuse and storage performances. ACS Sustain Chem Eng. 2022;10(5):1888–1898. doi: 10.1021/acssuschemeng.1c07604.
[12] Kumar R, Kaur J, Jain S, et al. Optimization of laccase production from *Aspergillus flavus* by design of experiment technique: partial purification and characterization. J Genet Eng Biotechnol. 2016;14(1):125–131. doi: 10.1016/j.jgeb.2016.05.006.
[13] Bulter T, Alcalde M, Sieber V, et al. Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. Appl Environ Microbiol. 2003;69(2):987–995. doi: 10.1128/AEM.69.2.987-995.2003.
[14] Gao N, Liu CX, Xu QM, et al. Simultaneous removal of ciprofloxacin, norfloxacin, sulfamethoxazole by co-producing oxidative enzymes system of *Phanerochaete chrysosporium* and *Pycnoporus sanguineus*. Chemosphere. 2018;195:146–155. doi: 10.1016/j.chemosphere.2017.12.062.
[15] Zhang Y, Wu L, Wang X, et al. The cotton laccase gene GhLAC15 enhances *Verticillium* wilt resistance via an increase in defence-induced lignification and lignin components in the cell walls of plants. Mol Plant Pathol. 2019;20(3):309–322. doi: 10.1111/mpp.12755.
[16] Umar A, Yuan W, Lu J, et al. Fungal-plant interaction: a pathogenic relationship between *Ganoderma segmentatum sp.* nov. and *Vachellia nilotica*. Front Microbiol. 2024;15:1411264. doi: 10.3389/fmicb.2024.1411264.
[17] Piscitelli A, Pezzella C, Giardina P, et al. Heterologous laccase production and its role in industrial applications. Bioeng Bugs. 2010;1(4):252–262. doi: 10.4161/bbug.1.4.11438.
[18] Kastberg LB, Ard R, Jensen MK, et al. Burden imposed by heterologous protein production in two major industrial yeast cell factories: identifying sources and mitigation strategies. Front Fungal Biol. 2022;3:827704. doi: 10.3389/ffunb.2022.827704.
[19] Umar A, Abid I, Elshikh MS, et al. Agitation role (Dissolved Oxygen) in production of laccase from newly identified *Ganoderma multistipitatum* sp. nov. and its effect on mycelium morphology. BMC Microbiol. 2023;23(1):280. doi: 10.1186/s12866-023-03009-2.
[20] Umar A. Production of laccase from filamentous fungi by submerge fermentation, examples from Pakistan. Sydowia. 2020;72:65–76.
[21] Antošová Z, Herkommerová K, Pichová I, et al. Efficient secretion of three fungal laccases from *Saccharomyces cerevisiae* and their potential for decolorization of textile industry effluent-A comparative study. Biotechnol Prog. 2018;34(1):69–80. doi: 10.1002/btpr.2559.
[22] Aza P, de Salas F, Molpeceres G, et al. Protein engineerig approaches to enhance fungal laccase production in *S. cerevisiae*. Int J Mol Sci. 2021;22(3):1157. doi: 10.3390/ijms22031157.
[23] Feng BZ, Li PQ, Fu L, et al. Exploring laccase genes from plant pathogen genomes: a bioinformatic approach. Genet Mol Res. 2015;14(4):14019–14036. doi: 10.4238/2015.October.29.21.
[24] Kim DH, Rigling D, Zhang L, et al. A new extracellular laccase of *Cryphonectria parasitica* is revealed by deletion of Lac1. MPMI. 1995;8(2):259–266. doi: 10.1094/MPMI-8-0259.
[25] Rigling D, Van Alfen NK. Extra- and intracellular laccases of the chestnut blight fungus, *Cryphonectria parasitica*. Appl Environ Microbiol. 1993;59(11):3634–3639. doi: 10.1128/aem.59.11.3634-3639.1993.
[26] Chung HJ, Kwon BR, Kim JM, et al. A tannic acid inducible and hypoviral-regulated Laccase3 contributes to the virulence of the chestnut blight fungus *Cryphonectria parasitica*. Mol Plant Microbe Interact. 2008;21:1582–1590. doi: 10.1094/MPMI-21-12-1582.
[27] Kim JM, Park SM, Kim DH. Heterologous expression of a tannic acid-inducible laccase3 of *Cryphonectria parasitica* in *Saccharomyces cerevisiae*. BMC Biotechnol. 2010;10(1):18. doi: 10.1186/1472-6750-10-18.
[28] Kadam AA, Saratale GD, Ghodake GS, et al. Recent advances in the development of laccase-based biosensors via nano-immobilization techniques. Chemosensors. 2022;10(2):58. doi: 10.3390/chemosensors10020058.
[29] Park EH, Shin YM, Lim YY, et al. Expression of glucose oxidase by using recombinant yeast. J Biotech. 2000;81:35–44. doi: 10.1016/s0168-1656(00)00266-2.

[30] Gietz RD, Schiestl RH, Willems AR, et al. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast. 1995;11(4):355–360. doi: 10.1002/yea.320110408.

[31] Ito H, Fukuda Y, Murata K, et al. Transformation of intact yeast cells treated with alkali cations. J Bacteriol. 1983;153(1):163–168. doi: 10.1128/jb.153.1.163-168.1983.

[32] Nguyen NL, Kim JM, Park JA, et al. Expression and purification of an immunogenic dengue virus epitope using a synthetic consensus sequence of envelope domain III and *Saccharomyces cerevisiae*. Protein Expr Purif. 2013;88(2):235–242. doi: 10.1016/j.pep.2013.01.009.

[33] Park SM, Mo AY, Lim JG, et al. Surface displayed expression of a neutralizing epitope of spike protein from a Korean strain of porcine epidemic diarrhea virus. Biotechnol Bioprocess Eng. 2007;12(6):690–695. doi: 10.1007/BF02931087.

[34] Park JA, Kim JM, Park SM, et al. Characterization of *CpSte11*, a MAPKKK gene of *Cryphonectria parasitica*, and initial evidence of its involvement in the pheromone response pathway. Mol Plant Pathol. 2012;13(3):240–250. doi: 10.1111/j.1364-3703.2011.00742.x.

[35] Bollag JM, Sjoblad RD, Liu SY. Characterization of an enzyme from *Rhizoctonia praticola* which polymerizes phenolic compounds. Can J Microbiol. 1979;25(2):229–233. doi: 10.1139/m79-035.

[36] Nuskern L, Tkalec M, Srezović B, et al. Laccase activity in fungus *Cryphonectria parasitica* is affected by growth conditions and fungal-viral genotypic interactions. JoF. 2021;7(11):958. doi: 10.3390/jof7110958.

[37] Rigling D, Heiniger U, Hohl HR. Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria* (*Endothia*) *parasitica*. Phytopathology. 1989;79(2):219–223. doi: 10.1094/Phyto-79-219.

[38] Gökbulut A, Ozhan O, Satilmiş B, et al. Antioxidant and antimicrobial activities, and phenolic compounds of selected Inula species from Turkey. Nat Prod Commun. 2013;8(4):475–478. doi: 10.1177/1934578X1300800417.

[39] So KK, Chun J, Luong NN, et al. Expression of an immunocomplex consisting of Fc fragment fused with a consensus dengue envelope domain III in *Saccharomyces cerevisiae*. Biotechnol Lett. 2021;43(9):1895–1904. doi: 10.1007/s10529-021-03161-7.

[40] Lim YY, Park SM, Jang YS, et al. Production of a functional mouse interferon γ from recombinant *Saccharomyces cerevisiae*. J Microbiol Biotechnol. 2003;13:537–543.

[41] Kiiskinen LL, Kruus K, Bailey M, et al. Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. Microbiology. 2004;150(Pt 9):3065–3074. doi: 10.1099/mic.0.27147-0.

[42] Jeong YS, So KK, Lee JH, et al. Optimization of growth medium and fermentation conditions for the production of Laccase3 from *Cryphonectria parasitica* using recombinant *Saccharomyces cerevisiae*. Mycobiology. 2019;47(4):512–520. doi: 10.1080/12298093.2019.1661566.

[43] Asemoloye MD, Marchisio MA. Synthetic *Saccharomyces cerevisiae* tolerate and degrade highly pollutant complex hydrocarbon mixture. Ecotoxicol Environ Saf. 2022;241:113768. doi: 10.1016/j.ecoenv.2022.113768.

[44] Iimura Y, Sonoki T, Habe H. Heterologous expression of *Trametes versicolor* laccase in *Saccharomyces cerevisiae*. Protein Expr Purif. 2018;141:39–43. doi: 10.1016/j.pep.2017.09.004.

[45] Alhomaidi EA, Umar A, Alsharari SS, et al. Evaluation of Lacc134 oxidoreductase of *Ganoderma multistipitatum* in detoxification of dye wastewater under different nutritional conditions. Microbiol Res. 2023;14(3):1398–1412. doi: 10.3390/microbiolres14030095.

[46] Kalyani D, Tiwari MK, Li J, et al. A highly efficient recombinant laccase from the yeast *Yarrowia lipolytica* and its application in the hydrolysis of biomass. PLoS One. 2015;10(3):e0120156. doi: 10.1371/journal.pone.0120156.