

A Newly Isolated Wood-rot Fungus for Laccase Production in Submerged Cultures

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A wood-rot fungus was isolated and investigated for its laccase production in submerged cultures. Based on sequence comparison and phylogenetic analysis of the 18S rDNA genes with reference taxa, the wild-type strain HLS-2 with high laccase production was identified as *Psathyrella candolleana* with GenBank Accession no. HQ215597. The production of laccase by *P. candolleana* HLS-2 can be enhanced by adding several agricultural wastes to the medium. Among them, wheat bran had the most significant effect and the highest laccase activity of nearly 12000 U/L. Extracellular laccase formations by this fungus can also be induced by veratryl alcohol, guaiacol, vanillic acid, coumaric acid, 2-2'-azinobis(3-ethyl- benzthiazoline-6-sulfonate) (ABTS), and copper sulfate. The maximal laccase activity, approximately 23000 U/L, was obtained from the shake-flask culture containing 1 mM Cu²⁺ without phenolic or aromatic inducers. Laccase activity of the extracellular liquid was stable at pH 8.0 and retained over 90% activity at 4 °C after 40 days.

Keywords: Laccase; White-rot fungi; *Psathyrella candolleana*; Agricultural residues

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INTRODUCTION

Lignocelluloses, the most plentiful organic substances on the globe, are becoming more important resources because they can be converted into energy products or materials to substitute for fossil resources. However, lignin, one of the dominant components of woody plants, is insoluble in solvents and recalcitrant to microbes (Fernaund *et al.* 2006). Degradation of lignin is one of the critical factors in many technical processes involving wood, such as pulping and bleaching in the papermaking industry. Conventional methods of delignification based on harsh chemicals and conditions have caused serious pollution to the environment by releasing chlorinated wastewater and sulfur-containing gas (Eugenio *et al.* 2008; Lara *et al.* 2003). New delignification technologies, especially environmentally friendly biotechnologies employing enzymes, have received much attention from researchers. To date, the enzymes that degrade lignin have been discovered in fungi, insects, and in a few bacteria (Revankar and Lele 2006; Telke *et al.* 2011; Gorman *et al.* 2012). The most well known lignin-degrading enzymes are produced from fungal origins, especially those belonging to the class of white-rot basidiomycetes, which are the only known microorganisms that degrade lignin completely to carbon dioxide and water in nature (Revankar and Lele 2006). The lignin-degrading enzymes produced by these types of organisms mainly include lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Gassara *et al.* 2010).

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) is a multicopper enzyme that can oxidize phenols or aromatic amines by reducing molecular oxygen to

water, with the involvement of the copper centers that play a crucial role in catalytic oxidation (Madhavi and Lele 2009). This enzyme is remarkably non-specific regarding substrates, and its zone of application can be widened even further in the presence of an aromatic electron transfer or radical-forming mediators, such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), N-hydroxybenzotriazole (HBT), and N-hydroxyacetanilide (NHA). Therefore, laccase has the potential to be implemented in various fields, such as pulp biobleaching, discoloration of textile dye, wastewater treatment, polymer syntheses, *etc.* (Couto and Herrera 2006; Widsten and Kandelbauer 2008).

The successful application of laccase in the bioremediation processes mentioned above is based both on high reactivity and productivity enzymes. It is essential to explore the laccase-producing organisms and establish the ideal conditions for manufacturing high levels of enzyme products. However, extracellular laccase is constitutively produced in small amounts by basidiomycete fungi, especially wild-type strains of white-rot fungi. To achieve high production of laccase, fungi are often stimulated with a wide variety of inducing substances, which are mainly aromatic or phenolic compounds related to lignin or lignin derivatives, such as ferulic acid, 2,5-xylidine, p-anisidine, and veratryl alcohol (Revankar and Lele 2006). Copper as a trace element and metal activator also has a prominent effect on laccase synthesis in white-rot fungi, especially in the strains *Pleurotus ostreatus* (Hou *et al.* 2004), *Funalia trogii* (Birhanli and Yesilada 2006), and *Trametes pubescens* (Galhaup *et al.* 2002). A common method used in molecular biology for enhancing laccase production is overexpression of this enzyme in a suitable host, such as yeast, which combines a high capacity for growth, the easy manipulation of unicellular organisms, and a eukaryotic organization enabling post-translational modifications (Huang *et al.* 2011; Klonowska *et al.* 2005). However, contrary to other oxidoreductases, like glucose oxidase that is produced industrially by recombinant strains of filamentous fungi, laccase is difficult to overexpress heterologously in an active form due to the fact that laccase is a glycosylated enzyme whose sugar moieties are involved in the stabilization process against proteolysis (Couto and Herrera 2007). The utilization of natural solid substrates, especially lignocellulosic residues and by-products from agricultural and food industries, is also a common approach for laccase production employing fungi. Substrates such as barley bran, wheat bran, cane bagasse, rice straw, wheat straw, soybean meal, *etc.* are being used in both solid-state and submerged fermentation. Such residues and by-products are widely available and renewable, as well as rich in carbon, nitrogen, and minerals, which makes them suitable for the growth and metabolism of microorganisms. Most of these materials contain cellulose, hemicelluloses, and lignin, which act as inducers to the ligninolytic enzymes (Couto and Herrera 2007; Couto and Sanroman 2005).

Hence, the main objective of the present work was to isolate and identify a new strain of white-rot fungi, and to investigate the effects of inducers and lignocellulosic substrates on laccase formation by this fungus. The stability of laccase from this fungus was also determined.

EXPERIMENTAL

Chemicals

2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was purchased from Sigma-Aldrich (Germany). All other chemicals (including culture media ingredients)

used in this study were of analytical quality and were obtained from commercial sources in China. Wheat bran, cane bagasse, corn cobs, rice straw, wheat straw, and orange peel were collected from Shandong province, China.

Screening and Isolation of Newly Collected Fungal Strain

The rotted wood samples with fungus were collected from a subtropical rain forest in the south of China in the summer. The tissue culture technique was employed for the isolation (Revankar and Lele 2006). The fruiting bodies of various mushrooms grown on the rotted wood were removed and thoroughly washed under running tap water. Individually, each fruiting body was cut into small pieces (about 2 mm × 2 mm) and washed three to four times with sterilized water. They were then subjected to surface sterilization by treatment with 75% alcohol under aseptic conditions for 30 s and thoroughly washed with sterilized water. These sterilized pieces were then inoculated in PDA (2% potato dextrose agar) plates. After 5 days of incubation at 28 °C, distinct and predominant fungal colonies were isolated from plates and transferred to the same medium until pure colonies were obtained. Upon performing a qualitative assay using guaiacol as a substrate, the PDA plates were supplemented with 0.02% guaiacol. The white-rot fungi were grown and evaluated for laccase production on these plates (Thurston 1994).

DNA Extraction , PCR Amplification, and Sequence Data Analysis

DNA extraction was performed by the methodology described by Florez *et al.* (2007). Mycelium, removed from the agar plate, was ground with liquid nitrogen to a powder. The powder was resuspended in 500 µL of a lysis buffer (200 mM, Tris-hydrochloride, 25 mM EDTA, 250 mM NaCl, and 0.5% SDS pH 7.5), and the mixture was incubated for 30 min at 50 °C. Added to this solution was 100 µL of 1.5 mol/L NaCl, and incubation proceeded for 5 min at room temperature. Cell debris were eliminated by centrifugation and the clear lysate transferred to a new tube. The DNA was purified by phenol:chloroform:isoamyl alcohol (24:24:1) extractions and precipitated with one volume of isopropyl alcohol. The DNA was then washed twice with ethanol at 70% and suspended in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA).

The PCR amplification of 18S rDNA gene of the isolates was carried out using upstream primer with sequence 5'-TCCTGCCAGTAGTCATATGC-3' and downstream primer with sequence 5'-TGATCCTTCGCAGGTTTCAC-3' in a 50 µL reaction volume (Guo *et al.* 2003). The thermal cycling program was as follows: 5 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 56 °C, 2 min extension at 72 °C, and a final 7 min extension at 72°C. PCR products were sequenced by Life Technologies Corporation (Shanghai, China).

The sequence was used as a query sequence to search for similar sequences from GenBank using BLAST program. The most similar reference sequences with query sequences were obtained and used for subsequent phylogenetic. The complete 18S rDNA sequences were aligned using Clustal X program. The bootstrapped, unrooted tree was structured by the neighbour-joining method from the distance data generated by multiple alignment of the nucleotide sequences in MEGA4.0 software.

Culture Conditions for Laccase Production

A mycelium suspension was obtained by the inoculation of several plugs from the fungus-growing zone on 2% potato dextrose agar, in 50 mL of growth medium in cotton-

plugged Erlenmeyer flasks (300 mL). The growth medium for the fungal culture contained (per liter): 4 g potato extract, 20 g glucose, 1 g peptone, 3 g KH_2PO_4 , and 3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. This was incubated at 28 °C at constant agitation (150 rpm). After 3 days a dense mycelial mass was formed. It was separated from the culture medium, suspended in an equal volume of sterilized water and then homogenized in a blender. The blended mycelium suspension was stored at 4 °C until it was used as inocula for the culture in the shake-flasks (Borras *et al.* 2008).

The effect of lignocellulosic residues, wheat bran, cane bagasse, corn cobs, rice straw, wheat straw, and orange peel on laccase production by the fungus was examined in the above-mentioned growth medium with a substrate concentration of 1% (w/v) (Arora and Gill 2000). $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and several putative phenolic and aromatic laccase inducers such as veratryl alcohol, guaiacol, ferulic acid, syringaldehyde, vanillic acid, coumaric acid, and ABTS with the concentration of 1 mM were added to the growth medium together with 1% (w/v) wheat bran at the time of inocula transfer (Birhanli and Yesilada 2006). All cultures were incubated in 80 mL of the medium in 300 mL Erlenmeyer flasks at 28 °C and 150 rpm. Every flask was inoculated with 20 mL of a homogenized mycelium suspension that was pre-cultured as described above. A control experiment was run under similar conditions without substrates or inducers. Samples from the flasks were taken periodically, centrifuged, and the clear supernatant was used for determination of laccase activity.

Analytical Determinations

Laccase activity was determined spectrophotometrically with ABTS as a substrate. The assay mixture contained 2 mL ABTS (0.5 mM) and 2 mL aliquots of appropriately diluted culture fluid. Oxidation of ABTS was monitored by following the increase in A_{420} ($\epsilon = 3.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute (Bourbonnais and Paice 1990). Glucose concentration was measured by the dinitrosalicylic acid (DNS) method using D-glucose as a standard according to Ghose (Ghose 1987). Data in subsequent sections represent arithmetic mean values of three experimental repetitions.

Laccase Stability

The stability of laccase at different pH values was analyzed by incubating the enzyme solution in 100 mM phosphate buffer (pH 5.0-8.0) or Tris-HCl buffer (pH 9.0) at 4 °C. The thermostability of laccase was evaluated in 100 mM phosphate buffer (pH 8.0) from 4 to 40 °C. The residual activity of laccase was measured periodically using the standard procedure previously described with ABTS as the substrate. The results refer to the maximum activity value obtained and are represented as percentages.

RESULTS AND DISCUSSION

Screening and Identification

Laccase is remarkably non-specific regarding substrates, which can be divided into three groups: ortho-, meta-, and para-substituted compounds with a lone electron pair. Thurston (1994) stated in a review that guaiacol and syringaldazine, ortho-substituted compounds, are typically referred to as the best substrates for most laccases. Thus, the isolates belonging to the group of basidiomycetes fungi, collected from a

subtropical rain forest in the south of China, formed a brown-colored zone around and above the isolated colony on the PDA plate containing guaiacol as a substrate, which was a characteristic of extracellular laccase production on a solid medium by filamentous fungi (Madhavi and Lele 2009; Thurston 1994). Figure 1 and Table 1 present the growth and discoloration of 19 collected strains after 3 days of cultivation, in which laccase activities were found on the plates containing guaiacol.

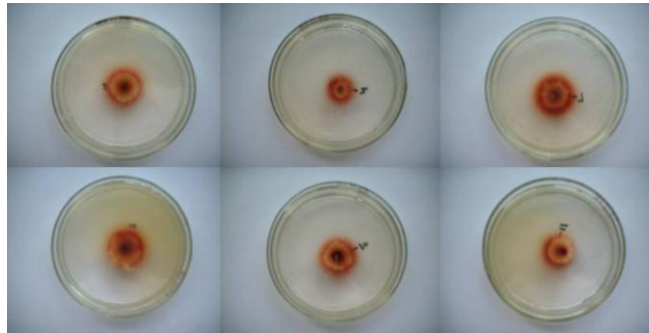


Fig. 1. Plate assay method for laccase test showed the brown zone developed by isolated strains using guaiacol as substrate in the medium after 3 days of incubation

Table 1. Growth and Discoloration of Several Screening Strains on the Plate Containing Guaiacol for 4 Days Culture

Strain	Diameter of colony zone (cm)	Diameter of brown zone (cm)
HN-2	1.7	2.1
HN-4	2.1	2.6
HN-9	2.4	2.8
HN-11	1.7	2.0
HLS-1	1.5	2.8
HLS-2	2.1	3.3
HLS-5	1.3	3.2
HLS-6	2.2	3.1
HLS-8	1.1	2.0
Xylh-3	1.6	2.3
Xylh-7	2.0	2.6
XZ-6	1.7	2.5
XZ-9	1.5	2.3
LZ-2	1.8	2.1
LZ-7	1.6	2.4
JZ-1	1.5	2.6
JZ-6	1.3	2.3
JZ-8	2.0	2.5
KM-5	1.2	2.7

Preliminary experimentation of several screening strains in a shake-flask culture showed that the maximal laccase activity was obtained using the growth medium. Among the seven isolates, the strain designated HLS-2 was found to be the best laccase producer with laccase activity of about 4600 U/L after 6 days of cultivation (Fig. 2).

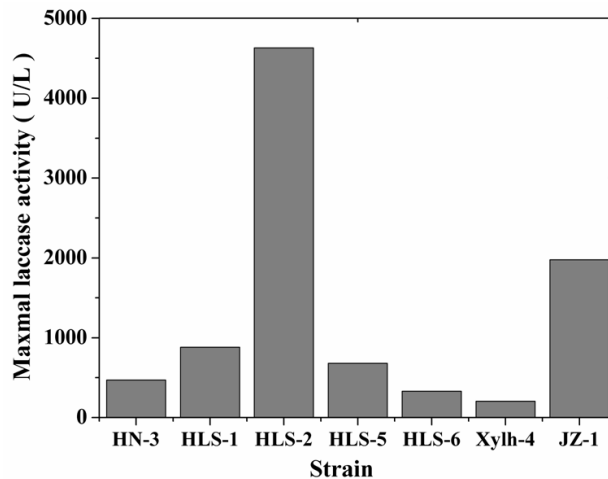


Fig. 2. Comparison of laccase production by several screened strains in the shake-flask culture after 6 days of cultivation

Further testing of the strain HLS-2 involved its identification using 18S rDNA gene sequencing. The genomic DNA from the isolate strain HLS-2 was extracted and purified as a template. By means of PCR, an amplicon of 866 bp was obtained. Comparison of the 18S rDNA sequence of our strain with the sequences stored in GenBank or in the proprietary fungal DNA databases, showed that the identical sequence from strain HLS-2 had many similarities to genus *Psathyrella* and *Basidiomycete*, particularly *Basidiomycete* sp. DQ304704.1, *Psathyrella candolleana* DQ465339.1, and *Psathyrella gracilis* DQ851582.1 with the homology of 99%. The bootstrapped, unrooted tree was structured by the neighbour-joining method from the distance data generated by multiple alignment of the nucleotide sequences (Fig. 3).

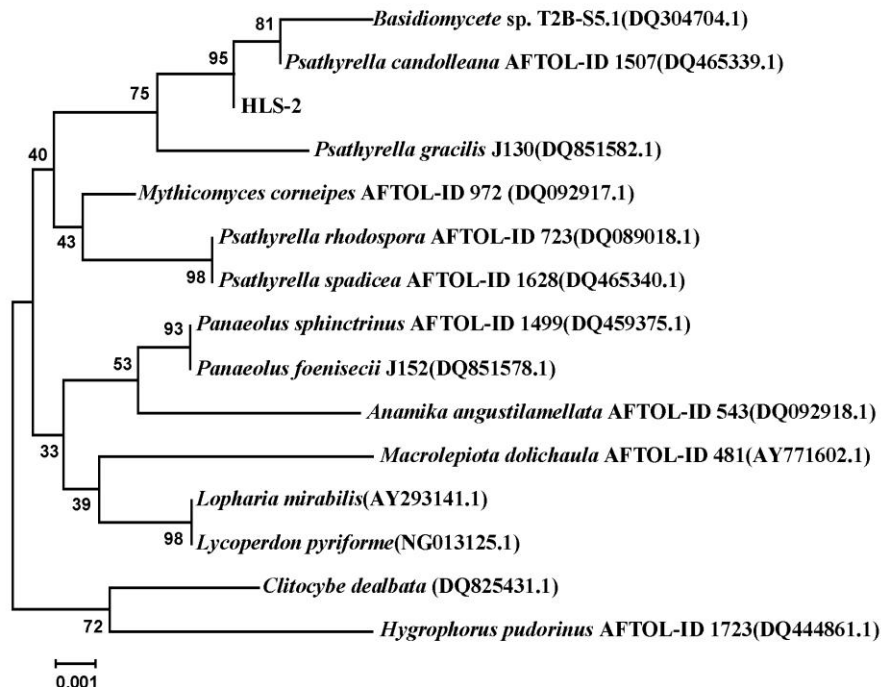


Fig. 3. Phylogenetic position of strain *Psathyrella candolleana* HLS-2

This tree generated from the 18S rDNA sequences of 15 taxa showed the relationships of strain HLS-2 with reference taxa. In this tree strain, HLS-2 formed a monophyletic clade with *Basidiomycete* sp. DQ304704.1, *P. candolleana* DQ465339.1, and *P. gracilis* DQ851582.1 with 75% bootstrap support. In this monophyletic clade, strain HLS-2 also formed a clade with *Basidiomycete* sp. DQ304704.1 and *P. candolleana* DQ465339.1 (95% bootstrap support). Based on the results of sequence comparison and phylogenetic analysis of the 18S rDNA genes with reference taxa, the isolate strain was identified as *Psathyrella candolleana* HLS-2 (GenBank Accession no. HQ215597). *P. candolleana*, a type of edible white-rot fungi, is widely distributed in China, and it usually grows from roots or wood chips of trees in summer and autumn. Laccase from other genera of family, such as *Pleurotus* (Bettin *et al.* 2011), *Trametes* (Iandolo *et al.* 2011), and *Pycnoporus* (Vikineswary *et al.* 2006), have been extensively studied, whereas none have been reported from *P. candolleana*. Hence, this is the first report of laccase production from the genus *Psathyrella*.

Effects of Different Lignocellulosic Residues on Laccase Production

Lignocellulosic wastes and by-products, including wheat bran, cane bagasse, corn cobs, rice straw, wheat straw, and orange peel, were used for enhancing laccase production by *P. candolleana* HLS-2 as shown in Fig. 4. Addition of wheat bran to the culture medium resulted in the highest laccase activity of nearly 12000 U/L, which was about 2.5-fold higher than that obtained in the control experiment. Orange peel, wheat straw, and rice straw cultures presented laccase activity of about 10000 U/L, whereas the values of corn cobs and cane bagasse cultures (7000 U/L and 8000 U/L) were less effective than other substrate cultures. These positive effects on laccase production were similar to those obtained from other laccase-producing fungi, such as *Trametes versicolor* (Lorenzo *et al.* 2002), *Funalia trogii* (Kahraman and Gurdal 2002), and *Pleurotus dryinus* (Elisashvili *et al.* 2006) cultivated in submerged culture with barley bran, cotton stalk, and mandarin peels, respectively.

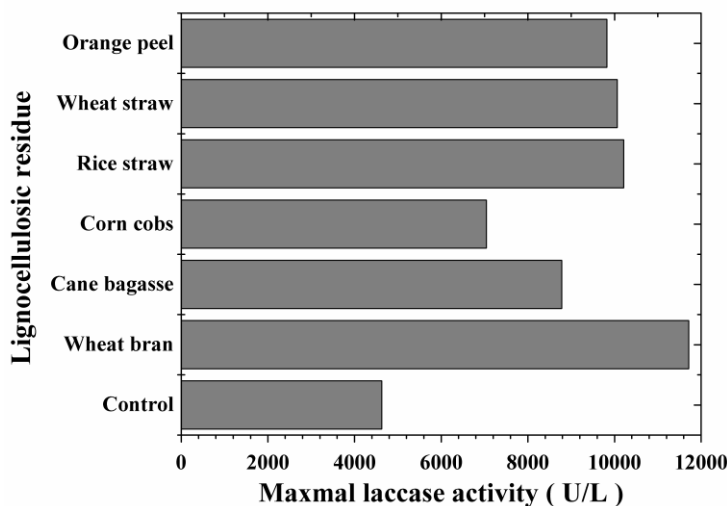


Fig. 4. Effects of different lignocellulosic residues on laccase production

The reason for the promotion of laccase activity is that these lignocellulosic wastes contained rich carbohydrates for the growth of microorganisms (Couto and Herrera 2007; Couto and Sanroman 2005), as well as low molecular weight compounds

to an extent that can stimulate the formation of lignin-degrading enzymes in fungi. For instance, ferulic acid, a common inducer utilized in laccase production by white-rot fungi, was detected in wheat bran in a concentration of 0.4% to 1.0% (Maes and Delcour 2002; Tybka *et al.* 1993). Additionally, the employed lignocellulosic residues are abundant and renewable, which are suitable for both growth substrates and inducers for laccase production by white-rot fungi.

Effects of Inducers and Cu^{2+} on Laccase Production

Enzyme formation in microorganisms is often stimulated by some low molecular weight compounds, so-called inducers. Laccase, a constitutive enzyme produced by white-rot fungi, can also be induced by phenolic or aromatic compounds related to lignin or lignin derivatives such as ferulic acid, guaiacol, and veratryl alcohol (Revankar and Lele 2006). There are some reports that copper as a micronutrient also plays a key role in laccase production by white-rot fungi. However, most of the inducers and copper are toxic to fungal growth and enzyme production when present in excess (Birhanli and Yesilada 2006). Copper sulfate and inducers were added in an equal concentration (1 mM) to the medium to investigate their effect on laccase production by *P. Candolleana* HLS-2, as shown in Fig. 5. Veratryl alcohol, guaiacol, vanillic acid, coumaric acid, ABTS, and Cu^{2+} could stimulate laccase production to a certain extent. The addition of copper sulfate (1 mM) to the culture medium resulted in the most significant effect, with a laccase activity of nearly 23000 U/L, which was about 2-fold higher than that obtained in the control experiment without inducers. These results are very interesting, since copper sulfate as an inducer not only has a positive effect on laccase production, but it is also much cheaper than phenolic or aromatic compounds.

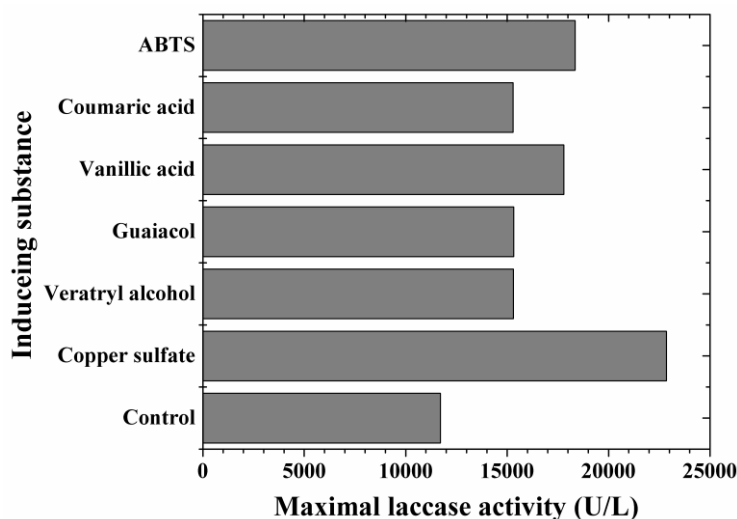


Fig. 5. Effects of several inducers and Cu^{2+} on laccase production

The positive effect of copper sulfate on laccase formation shown in the present work is in agreement with previous studies carried out with several fungal strains by Revankar and Lele (2006), who reported that the addition of 1 mM copper sulfate considerably stimulated laccase production by wild-type strains *WR-1*, *Pleurotus ostreatus* (Hou *et al.* 2004), *Trametes versicolor* (Birhanli and Yesilada 2006), and *Trametes hirsuta* (Rosales *et al.* 2007). Birhanli also reported that the addition of 0.5 mM Cu^{2+} to agitated cultures of *Funalia trogii* produced laccase activity that was more than

60-fold higher than the control. However, a concentration above 0.5 mM caused the inducing effect of copper to decrease (Birhanli and Yesilada 2006). So far, the exact mechanism for the stimulation effect caused by copper is not clear. One possible explanation could be the defensive role of laccase against oxidative stress. It is likely that laccase may have an effect in the synthesis of melanin and thus melanin could act as a physical barrier to toxic levels of copper. Most likely, Cu-induced melanin synthesis could serve as an additional mechanism to scavenge this metal from the extracellular environment (Birhanli and Yesilada 2006; Galhaup and Haltrich 2001). Hence, copper might ubiquitously be employed as an efficient inducing substance for enhancing fungal laccase production.

Thermostability and pH Stability of Laccase

For industrial applications, the desired enzyme should be stable in a wide pH and temperature range. The pH and thermal stability of laccase from *P. candolleana* HLS-2 were tested, and the results are shown in Fig. 6 and 7.

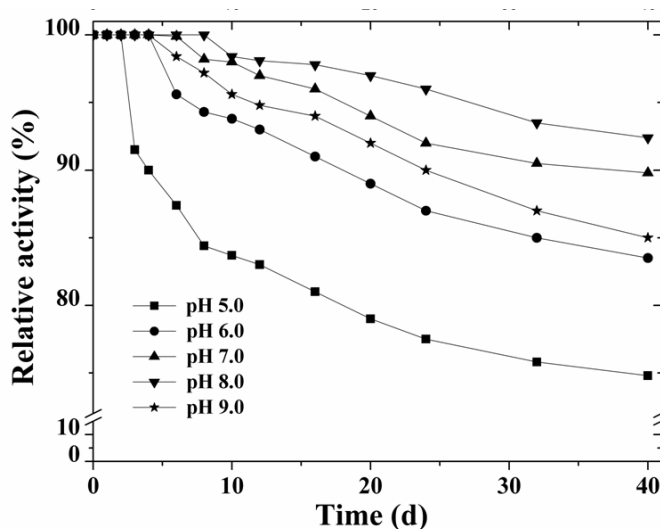


Fig. 6. Influence of pH on laccase stability at 4 °C

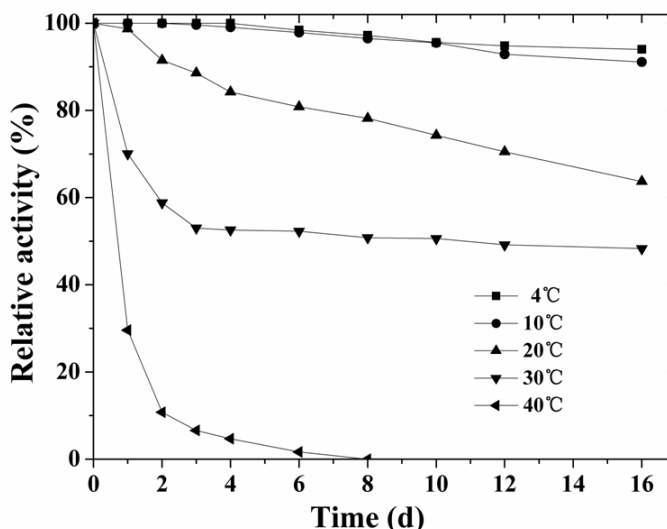


Fig. 7. Influence of temperature on laccase stability at pH 8.0

The activity of laccase remained over 90% after 40 days of incubating the extracellular fluid at pH 7.0 and 8.0. However, incubation at pH values below 6 and at pH 9.0 significantly affected laccase activity. Laccase retained more than 90% of its activity after 16 days of incubating the extracellular liquid below 10 °C at pH 8. Nevertheless, it lost approximately 40% and 50% of its activity when it was incubated at 20 °C and 30 °C after 16 days, respectively. The activity was totally lost after 8 days at 40 °C. Compared with several reported fungal laccases produced by *Trametes versicolor* (Rancano *et al.* 2003), *Pycnoporus sanguineus* (Litthauer *et al.* 2007), *Trichoderma harzianum* (Sadhasivam *et al.* 2008), and *Pleurotus sajor-caju* (Zucca *et al.* 2011), laccase from *P. candolleana* HLS-2 exhibited remarkable stability temperatures from 4 °C to 10 °C and slightly alkaline conditions, which is a great benefit for enzyme storage in industry.

CONCLUSIONS

1. A newly isolated white-rot fungus collected from a subtropical rainforest in China was identified as the genus *Psathyrella* through sequence comparison and phylogenetic analysis of the 18S rDNA genes. It was named *Psathyrella candolleana* HLS-2 with GenBank Accession no. HQ215597. The fungus is an efficient producer of laccase, such that a relatively high yield in a copper-containing medium without aromatic or phenolic compounds can be obtained.
2. The agricultural lignocellulosic residues wheat bran, cane bagasse, corn cobs, rice straw, wheat straw, and orange peel could be used both as substrates and laccase inducers in submerged cultures. Among them, wheat bran was the best for laccase production by this strain. Its laccase production was also highly stimulated by Cu²⁺ in submerged cultivations. The laccase activity in the medium with copper was about 2-fold higher than that obtained in control experiment without inducers.
3. The laccase from *P. candolleana* HLS-2 exhibited remarkable stability under lower temperatures and slightly alkaline conditions, which meant the enzyme possesses outstanding advantages for broad applications.

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