Research Article

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A Preliminary Study on the Newly Isolated High Laccase-producing Fungi: Screening, Strain Characteristics and Induction of Laccase Production

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Abstract: A simple separation method was used in this study to directly separate laccase-producing fungi from withered plant materials. A laccase-producing filamentous fungus was isolated and purified. The strain was highly similar to the species in genus *Trametes* by ITS sequence analysis, and therefore named *Trametes* sp. MA-X01. The addition of cupric ions and aromatic compounds to the liquid medium could induce the laccase synthesis in *Trametes* sp. MA-X01. Copper-induced laccase activity increased in a dose-dependent manner. The highest laccase activity (2138.9 ± 340.2 U/L) was obtained by adding 2.5 mM Cu^{2+} to the culture medium, which was about 7 times higher than that of the control group. The induction degree of aromatic compounds was different. For the present study, the highest laccase activities were obtained by adding vanillic acid (1007.9±59.5 U/L) or vanillin (981.6±77.2 U/L) to the medium, which were 3.5 and 3.4 times higher than the laccase activity of the control group, respectively.

Keywords: Laccase; Fungi; Screening; Genus *Trametes*

1 Introduction

Lignin, a complex aromatic polymer, is also one of the important elements constituting the plant cell wall. Structurally, lignin can greatly increase the strength and the anti-degradation component of the cell wall, as well as, improve its antiviral capability and pest resistance [1]. The structure of lignin is complex, and the composition of lignin varies from species to species. The molecular weight and linkage motifs vary not only according to plant species but also to the environmental factors [2]. Of industrial interest is lignin cross-linked with cellulose, hemicellulose and pectin components, conferring mechanical strength to the cell wall and by extension the plant as a whole, and resistant to degradation. Lignin is insoluble in water and alcohol, and difficult to be hydrolyse with acid or enzymatic methods. Lignocellulose is the most abundant renewable biomass on earth. It has long been recognized as an alternative source for producing renewable fuels and chemicals [3, 4] but the conversion of lignocellulosic biomass is still a significant challenge.

As the principal decomposers in ecosystems, fungi play a key role in the carbon cycle in nature. Wood-decay fungi can be classified as brown-rot, soft-rot and white-rot fungi. These microorganisms can produce different enzymes or possess the abilities to break down woods and colonize different environmental niches. The brown-rot fungi can decompose cellulose and hemicellulose in wood, but the lignin cannot be degraded completely. Similar to brown-rot, soft-rot fungi secrete cellulase from their hyphae to break down cellulose in the wood, leaving the lignin intact. By contrast, the white-rot fungi can synthesize a variety of degrading enzymes, the lignocelluloses are efficiently degraded, thereby resulting in the white appearance of the wood. Due to the complexity and diversity of the lignin structure, the degradation by white-rot fungus requires the synergistic action of many enzymes. Fungal extracellular class II peroxidases (PODs) including manganese peroxidases (MnPs), lignin peroxidases (LiPs), and versatile peroxidases (VPs) are thought to play an active role in lignin depolymerization [5]. These peroxidases are heme-

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containing enzymes that catalyze a number of oxidative reactions and hydroxylations, using H_2O_2 as the electron acceptor. In addition to PODs, lignin degradation by white-rot fungi involves several other enzyme families, such as dye decolorizing peroxidases (DyP), heme-thiolate peroxidases (HTP) [6, 7] and laccase [8].

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), unlike other lignin degrading enzymes, belong to the superfamily of multi-copper oxidases (MCO) [8]. Even though the model lignin degrading organism *Phanerochaete chrysosporium* does not produce a typical laccase [9], laccases were found in almost all white-rot fungi and thought to play an important role in lignin degradation. Laccases catalyze a single-electron oxidation with the concomitant four electron reduction of molecular oxygen to water [10]. In addition, laccase will degrade various structures of natural and synthetic chemicals, such as phenols, aromatics, and aliphatic amines; the only by-product is water. Therefore laccases attract much research attention not only for their biological functions, but also their potential use in industry and biotechnology [11, 12]. Furthermore, significant differences in the substrate specificity, catalytic capacity, reaction conditions, and expression regulation are observed on the different kinds of laccase [13]. Finding new laccase resources in order to meet the demands of practical use has become more attractive. This work mainly aimed to discover native laccase-producing fungi. The participation of laccase in the degradation of lignocelluloses was adopted to set up a screening method to directly separate the laccase-producing fungi from the withered plant materials.

2 Materials and methods

2.1 Medium for screening and culture conditions

Potato dextrose agar (PDA) medium containing 200 g/L potato extract, 20 g/L D-glucose, 3 g/L KH_2PO_4, 1.5 g/L $MgSO_4$ and 20 g/L agar were used to isolated fungi. ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) or guaiacol was added to the medium to the final concentration of 0.02% to be an indicator. PDA medium was also used for regular mycelia growth. After 7 days of growth at 25°C, the mycelia were stored at 4°C, and the stocks were transferred to fresh PDA every three months.

Liquid PDB medium (PDA medium without agar) was used in shaken cultures. The pre-cultures were prepared by homogenisation of an agar plug sized about 4 cm^2 containing mycelia in 100 ml of PDB medium in a 250 ml flask for 7 days at 25°C with agitation at 160 rpm. The pre-cultures were homogenised again and subsequently, 5 ml of homogenised pre-cultures were transferred to 100 ml fresh liquid PDB medium in 250 ml flasks, followed by incubation at 25°C, with shaking (160 rpm).

2.2 Sample collection and fungi isolation

Samples were collected from the campus of Anhui Science and Technology University, which is located in Anhui Province in China. Withered plant materials, such as dry leaves, hay, corn stalks, or other dry and decaying plant materials, were collected. The soil on the sample surface was removed first, and then samples were cut into small pieces and directly placed on the screening culture medium, incubated at 25°C for observation. After 1–3 days, the fungi on the surface of the plant material was adhered to the medium and grew. Laccase catalyzes the oxidation of ABTS in the medium to form a green complex, and sometimes it can produce a purple region due to the rapid generation and accumulation of the color material. The color in the medium indicates the detection of laccase. The mycelia grown on/near the colored area were separated for the secondary screening.

2.3 ITS amplification and identification of the strain

Total genomic DNA was extracted from the 7-day fresh mycelia using the plant genome DNA extraction kit (Tiangen Biochemical Technology Co., Ltd.). The regular PCR extension was implemented by primer ITS1-F: 5'-CTTGGTCATTTAGAGGAAGTAA-3'; and ITS4-B: 5' -CAGGAGACTTGTACACGGTCCAG-3'. The PCR program was as follows: 5 min at 94°C; 30 rounds of 30 s at 94°C, 30 s at 55°C, and 40 s at 72°C; and final extension for 10 min at 72°C. The fragments were submitted to a sequencing service (General Biosystems, Inc., China). The sequence was aligned using the BLAST software in the NCBI database. Based on the results of the Blast search, 15 sequences with the highest similarity were selected to perform the phylogenetic analysis and construction of an unrooted tree using the software MEGA version 7.0.

2.4 Enzyme assay

The fermented broth was centrifuged at 3000 r/min (Eppendorf 5810R) for 5 min; and the supernatant

contained the crude enzyme. Laccase activity was determined at room temperature using ABTS as a substrate. The reaction mixture contained 1.8 ml of 0.2mol/L sodium acetate buffer (pH = 4.5), 0.2 ml of 1 mmol/L ABTS and 1 ml of diluted crude enzyme. After incubation for 1 min at room temperature, laccase activity was determined as the increase in the absorbance at 420 nm [ε = 36000 L/(mol·cm)]. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute.

2.5 Polyacrylamide gel electrophoresis

To identify the number of isoenzymes, the crude enzyme was subjected to native polyacrylamide gel electrophoresis (Native PAGE). It was performed at an alkaline pH under nondenaturing conditions. The separating and stacking gels contained 10% and 4% acrylamide respectively, and the pH of the separating and stacking gel were 8.8 and 6.8, respectively. The electrode reservoir solution contained 25 mM Tris and 200 mM glycine (pH 8.4). 30 μL crude enzyme was loaded onto the gel, and the gel was electrophoresed under 60 V at 4°C for 6 h. After that the gel was rinsed with ddH_2O, and incubated in 0.2M sodium acetate buffer (pH = 4.5) with 2mM ABTS to visualize the laccase bands.

2.6 Determination of the mycelial dry weight

The mycelia were separated from the liquid medium by centrifugation at 3000 r/min (Eppendorf 5810R) for 5 min and washed three times with ddH_2O. The mycelia were dried at 60°C to constant weight (n = 5).

2.7 Data analysis

All samples were prepared in triplicate and the data presented in figures are mean values; the error bars represent the standard deviation. Data calculation and error analysis were performed using the software Excel 2007.

3 Results

3.1 Screening fungal species for laccase producing

In view of the role of laccase in the degradation of lignocellulose, we tried to directly separate the laccase-producing fungi from the plant materials. As shown in Figure 1A, fungi that attached to the plant surface could grow on to the medium. The green or purple color reaction in the medium indicates the presence of laccase, meanwhile, the mycelia growing on/near the color were considered putative positives. The mycelia were isolated using a sterile toothpick for further purification and secondary screening.

In this paper, one isolated strain was chosen to be studied. As shown in Fig. 1B, this fungus grew well in the PDA medium, with white and dense flat colonies. The laccase produced during the growth of the mycelia, which made the purple color reaction for the medium containing ABTS, whereas red color reaction was observed for the medium containing guaiacol (Fig. 1B). The colored region was greater than the diameter of the colony, which may explain the high yield and good activity of laccase produced in this strain.

3.2 Identification and phylogenetic studies of the strain

The PCR amplification sequence of the ITS in this fungi consisted of 771 nucleotides. BLAST results indicated that the fungi was highly similar to the species in genus *Trametes*, with 99% similarity with the ITS sequence of *Trametes versicolor*. Based on the results of the Blast search, 15 sequences with the highest similarity were selected to perform the phylogenetic analysis. The resulting trees showed that the strain was different from other *Trametes versicolor*, the position in the phylogenetic tree was clustered to *Trametes* sp.Y-H1 (Fig. 2). We named the isolated fungi as *Trametes* sp. MA-X01. The ITS sequence of *Trametes* sp. MA-X01was submitted to GenBank, the accession number is MF687399.

3.3 Mycelial growth and laccase production in liquid PDB medium

The strain *Trametes* sp. MA-X01 mycelia can grow well in liquid PDB medium, as shown in Figure 3. On the 3rd day

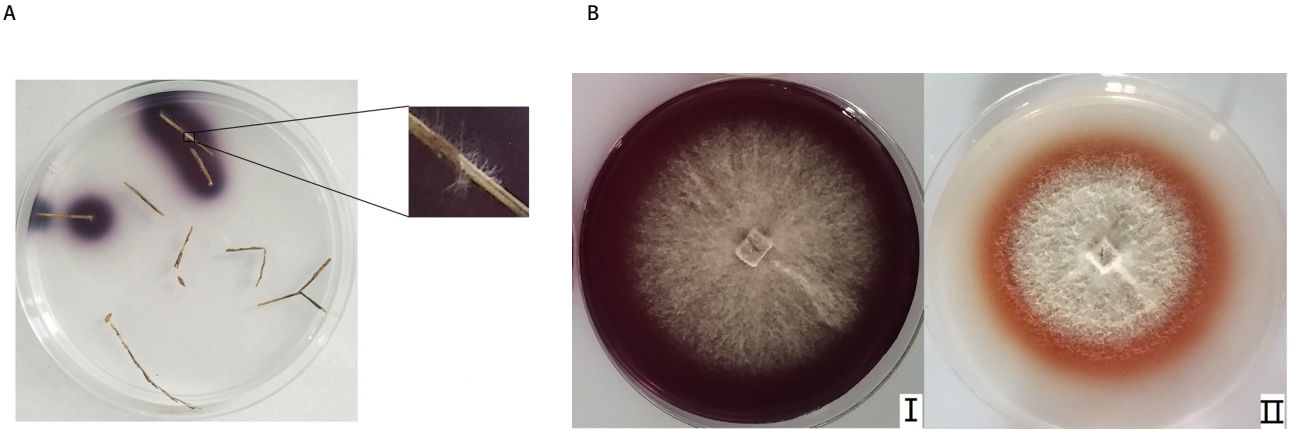


Figure 1. A: Isolation of laccase-producing fungi. The wither plant materials were placed on the PDA medium containing ABTS. The mycelial fungi on the surface of the material grew on the medium. Laccase catalyzes ABTS to produce green matter, and if it accumulates quickly it will appear violet. The mycelia grew on/near the colored region was separated. B: Colony morphology of *Trametes* sp. MA-X01 grown on the PDA medium and color reaction in the medium agar containing ABTS (I) and Guaiacol (II).

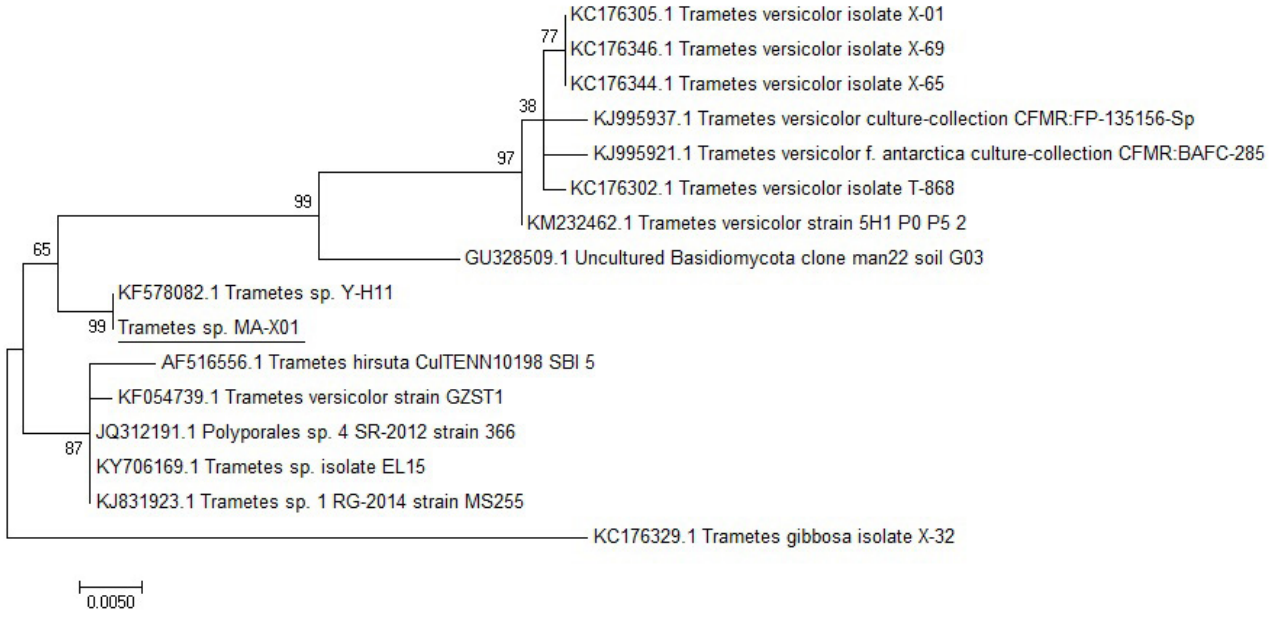


Figure 2. Phylogenetic tree constructed based on ITS sequences.

of cultivation, the mycelia entered the logarithmic phase, and then showed fast proliferation. The average growth rate was about 1.67 g/L per day from the 3rd to the 9th day of cultivation, followed by a subsequent reduction in the growth rate, averaging 0.75 g/L per day from the 10th to the 14th day of cultivation. After 14 days of cultivation, the biomass reached 16.96 ± 1.45 g/L.

With the mycelial growth, laccase protein was secreted into the medium, and the activity continuously increased (Figure 3). On the 14th day of cultivation, the laccase activity reached 313.5 ± 51.0 U/L.

3.4 Effect of copper concentration on laccase production and mycelial growth

Copper is an essential element that involved in many biological functions in the cell, while it becomes toxic when in excess. Copper, the cofactor of laccase, also induces the laccase activity in almost all the fungi. Thus both the toxic effect of copper on the mycelial growth and inducing effect on laccase activity were evaluated. The variation of the colony diameters in the presence of different concentrations of copper were shown in Fig.

4A. When the strain grew in the PDA without Cu^{2+}, the colony diameter reached to almost 83.3 ± 3.7 mm in the 7th day. The mycelia growth was slightly inhibited when 1mM Cu^{2+} was added to the medium, the colony diameter was approximately 79.2 ± 4.2 mm. The mycelia growth decreased with the Cu^{2+} concentration in the medium

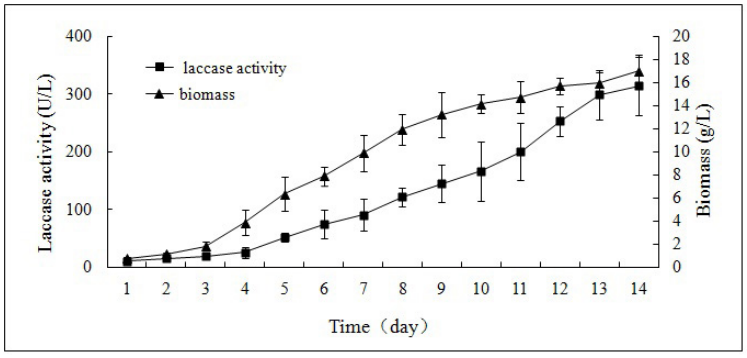
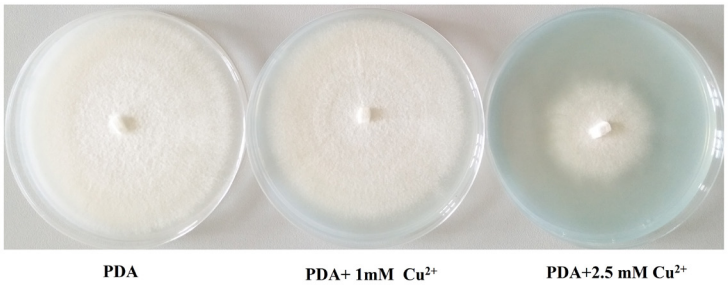


Figure 3. Time course of laccase activity and biomass accumulation of *Trametes* sp. MA-X01 cultivated in PDB medium.

A



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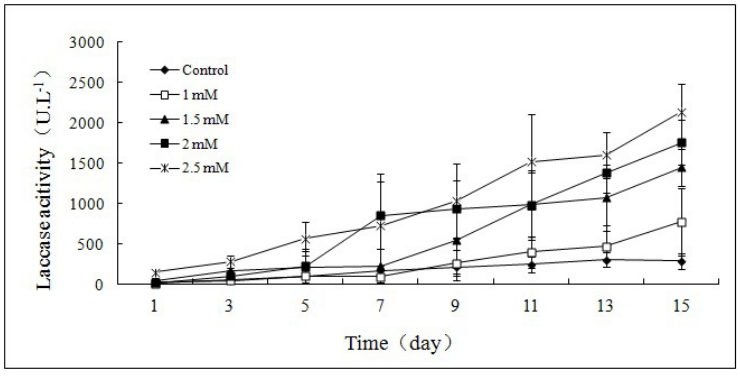

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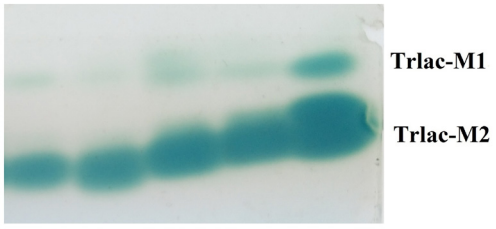

Figure 4. A: Effect of copper on the mycelial growth of *Trametes* sp. MA-X01. Fresh mycelia that grew on PDA or PDA supplemented with different concentrations of Cu^{2+} were photographed after 7 days incubation. The plates containing 0, 1, 2.5 mM Cu^{2+} are shown. B: Effect of copper concentration on the time course of laccase production in *Trametes* sp. MA-X01 C: Zymograms of laccase isoenzymes of *Trametes* sp. MA-X01. Mycelia were cultured in liquid PDB medium with different concentrations of Cu^{2+}. Electrophoresis was performed on the 9th day of cultivation. 30 µL crude enzyme was loaded onto the gel. After electrophoresis, the gels were incubated in sodium acetate buffer (pH = 4.5) with ABTS for 10 minutes to visualize the bands.

increased, and 2.5 mM Cu^{2+} concentration could inhibit the mycelia growth almost by half compared with the control, the colony diameter was only 42.4 ± 1.9 mm in the 7th day.

Based on the effect of the copper concentration on the mycelial growth in *Trametes* sp. MA-X01, Cu^{2+} at final concentrations of 1, 1.5, 2, and 2.5 mM were added to the liquid medium to determine the laccase activity. The result is shown in Fig. 4B. Although a high concentration of copper had an inhibitory effect on the growth of mycelia, the activity of laccase increased with increasing copper concentration in the medium. The highest laccase activity (2,138.9 ± 340.2 U/L) was obtained on the 15th day of cultivation after the addition of 2.5 mM Cu^{2+} to the medium, which was about 7 times higher than that of the control group.

Results of isoenzymes electrophoresis showed that under the liquid culture conditions, *Trametes* sp. MA-X01 expressed two laccase isoenzymes (Fig. 4C), namely Trlac-M1 and Trlac-M2. Trlac-M2 had a wider band than Trlac-M1, suggesting that Trlac-M2 was the major isoenzyme in the fermentation broth. Moreover, as the copper concentration increased, the width of the Trlac-M2 band gradually increased, indicating that copper induced the expression of Trlac-M2. The enhancement of the enzyme activity in the fermented liquid was mainly dependent on the increasing Trlac-M2 activity.

3.5 Effects of aromatic compounds on the laccase activity.

The effects of several aromatic compounds on the laccase activity in *Trametes* sp. Ma-X01 are shown in Fig. 5. The aromatic compounds with the final concentration of 1 mM were added into the culture medium, which has certain inducing effect on the laccase activity, but the influence was much smaller than that of cupric ions. The activity peaked to 1007.9 ± 59.5 U/L and 981.6 ± 77.2 U/L in the presence of 1mM vanillic acid and vanillin in the medium, which were 3.5 and 3.4 times higher than the control group.

4 Discussion

The common procedure for isolating microorganism is that the sample is suspended in sterile ddH_2O, thereafter the suspension is serially diluted and sample dilution is plated on the medium agar. In screening laccase-producing fungi, we found that using the conventional method, the number of bacteria growing on the medium was far more than that of fungi. So we tried to separate the laccase-producing fungi from the withered plant materials directly. The result demonstrates that it is not only a simple but also an efficient method. It can avoid the interferences of most bacteria during the screening

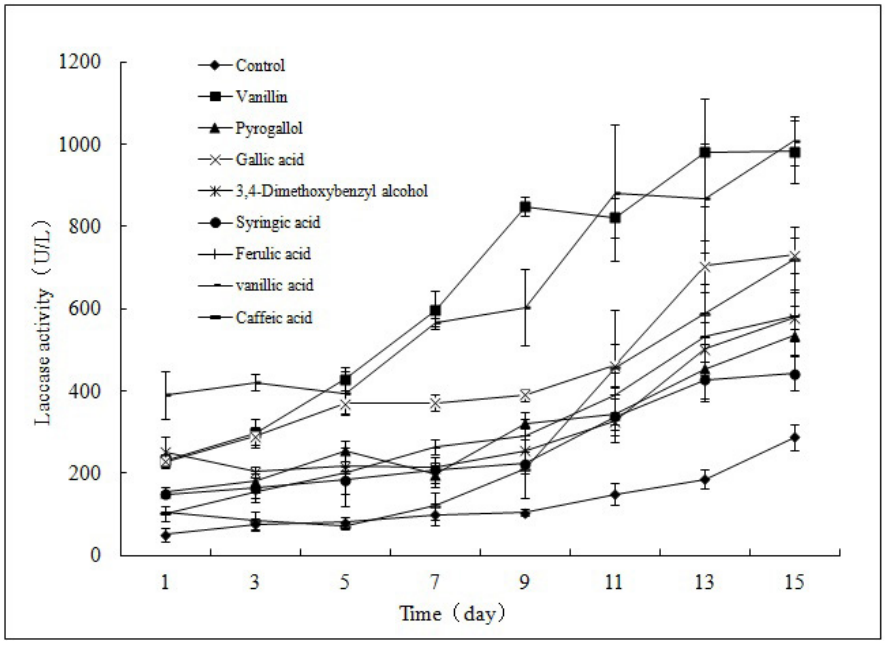

Figure 5. Effect of aromatic compounds on the laccase activities in *Trametes* sp. MA-X01. The aromatic compounds were added to the PDB medium to the final concentration of 1 mM.

process. Through the color reaction that occurred in ABTS catalyzed by laccase, enzyme-producing fungi can be detected by observation.

Although the ITS sequence of *Trametes* sp. MA-X01 has 99% similarity to that of *Trametes versicolor*, they have different position in the phylogenetic tree. In other fungal species in genus *Trametes*, laccase activity, the degree of induction and strain characteristics differed among the different strains isolated from different districts [14-18]. In this paper, the effect of copper on laccase activity in *Trametes* sp. MA-X01 was much greater than that of the aromatic compounds we tested. More interesting is the inducing effect of the cupric ion was stronger on isoenzyme Trlac-M2, while the influence on Trlac-M1 was relatively small. These data indicate that differences in the expression and regulation mechanisms of these two isoenzymes had occurred. It also can be inferred from the result of native polyacrylamide gel electrophoresis that the laccase activity in *Trametes* sp. MA-X01 mainly came from isoenzyme Trlac-M2 under the liquid culture condition.

5 Conclusion

This work introduced a simple and efficient screening method to directly separate the laccase-producing fungi from the withered plant materials. The newly isolated strain showed good ability in laccase synthesis. Cupric ion and aromatic compounds could induce the laccase activity in liquid culture condition. The effect of copper on laccase activity was dose dependent, and the inducing effect of was stronger on isoenzyme Trlac-M2. Research on newly discovered native fungi and the characteristics of the strain can provide a broad prospect for the application of laccase in industry and biotechnology.

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Conflict of interest: Authors state no conflict of interest

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