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Short Communication

Comparative study on the determination of assay for laccase of *Trametes* sp.

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The aim of this work was to determine the sensitivity among compounds in common use for detecting laccase activity. In this study, three assay procedures to measure laccase activity of *Trametes* sp. were performed in Kirk's basal salts mediums of three dyes. In the assay methods, three substrates were employed, which were 2,2©-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), -dianisidine and guaiacol. The results indicate that laccase activity used ABTS as a substrate was significantly higher than the other two. Therefore, the ABTS method is recommended while detecting laccase activity.

Key words: ABTS, -dianisidine, guaiacol, laccase activity, *Trametes* sp.

INTRODUCTION

Laccases (benzenediol:oxygen oxidoreductase, EC1.10.3.2) are multi- copper oxidases widely distributed among plants, insects and fungi (Revankar et al., 2006). First isolated in 1883 from *Rhus venicifera*, the Japanese lacquer tree, laccases are also commonly found in fungi (Thurston, 1994). Laccases are attractive, industrially relevant enzymes that can be used for a number of diverse applications, e.g., biosensors (Freire et al., 2001), pulp bleaching (Call and Mücke, 1997; Balakshin et al., 2001), labeling in immunoassays (Kuznetsov et al.,
2001), bioremediation (Mayer and Staples, 2002) and green organic synthesis (Karamyshev et al., 2003). Fungal laccases form an important group of enzymes, as they are involved in the degradation of lignin and in removal of potentially toxic compounds (Thurston, 1994).

For detecting laccase, some assay methods including HPLC method, manometry, order spectrum method and spectrophotometry are involved (Zhu et al., 2006) . Compared with other methods, spectrophotometry is widely used owing to it's simply and sensitive characteristics. Usually, there are several compounds that have been used as substrates by spectrophotometry methods such as 2, 2'-azinobis- (3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Fernandes et al., 2005), syringaldazine (Park et al., 2006), o-dianisidine (Silva et al., 2005) and guaiacol (Arora et al., 2002). We encountered the problem regarding various substrates while studying the kinetics of laccase ing the sensitivities of in decoloration of industrial dyes, and no reports were found related to comparison of these compounds as substrates. Assay sensitivity for enzyme is largely depended upon the efficiency of substrates. Thus, sensitivity of substrates is vital to evaluate enzyme acti-vity. In this study we compared the sensitivities of the dif-ferent methods to evaluate the activity of laccase. The results can provide a reference for the substrate selection for the similar studies in the future.

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MATERIALS AND METHODS

Strain *Trametes* sp. was isolated from basidiomata collected from Zijin Mountain of Nanjing, China, and maintained on potato dextrose agar (PDA). The fungal strain was investigated to decolorize three different dyes including Fuchsin acid (triphenyl methane), Orange G (monoazo) and Congo red (disazo). The experiments were performed by cultivation in liquid medium. Each flask contained 50 ml of Kirk's basal salts (KBS) medium and 100 mg/l of each dye. Inoculums of *Trametes* sp. were prepared by removing a 6 mm diameter agar plug from the outer edge of a 7-9 days old culture grown at 28 on PDA to a Petri dish containing 20 ml potato dextrose broth (PDB) and incubated at 28 for 5 days. The final mycelia were added aseptically to each flask and shaked at 120 rpm.

Laccase activities were measured spectrophotometrically (Unico, 2100). The first method was based on the oxidation of ABTS at 420 nm with a absorbance coefficient value (= 36 000 M^{-1} cm^{-1}). The reactive mixture consisted of 1.5 ml sodium acetate buffer (1 mM, pH 5.0), 1.5 ml ABTS (0.5 mM) and 1.5 ml culture medium. The second was measured as o-dianisidine oxidation at 460 nm (= 11 300 M^{-1} cm^{-1}). The reactive mixture contained per 1 ml: 200 l citrate (100 mM)-phosphate (200 mM) buffer (pH 5.0), 100 l o-dianisidine

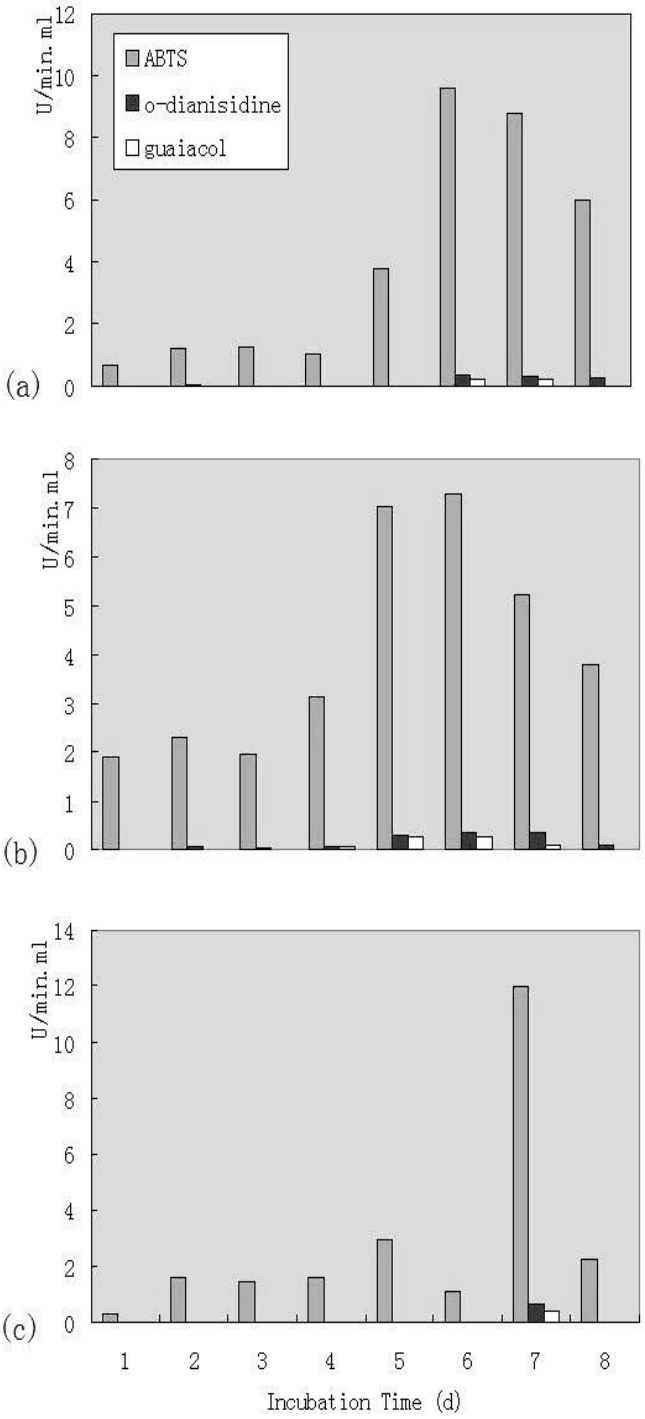

Figure 1. Laccase activity of *Trametes* sp. by three assay procedures for decoloration of three dyes: (a) Orange G (b) Congo Red (c) Fuchsin Acid.

(1 mM), 600 l culture medium and 100 l hydrogen peroxide (2 mM). The third method was measured based on guaiacol oxidation at 450 nm (=12 100 M^{-1} cm^{-1}). The reactive mixture was 3 ml acetate buffer (10 mM, pH 5.0), 1 ml guaiacol (2 mM) and 1 ml culture medium. The results used syringaldazine as a substrate were not reported because of its instability. One unit (U) of laccase activity was defined as the amount of enzyme catalyzing the production of one micromole of colored product per min per ml. The experiment was performed in triplicates. Control was done without substrates. The significance between the assay procedures was determined using analysis of variance (ANOVA) at the level of $P<0.05$.

RESULTS AND DISCUSSION

The laccase activity measured by the three methods was greatly different. By ABTS method, laccase activity could be detected in all KBS mediums with monoazo, disazo and triphenyl methane dyes (Figure 1). The lowest values were assayed on the first day in decoloration of all dyes, and then, activities of laccase increased. The highest activities were appeared after 6 or 7 days of cultivation. By the o-dianisidine and guaiacol oxidation assay, only high activity of laccase could be detected. Comparing with the ABTS method, values of laccase activity declined sharply used o-dianisidine and guaiacol as substrates. No activities were detected at the initials of incubation. In Fuchsin acid medium, the highest laccase activity was 33.31 U using ABTS (Figure 1c). Under the same conditions, the highest was 5.75 and 3.31 U using o-dianisidine and guaiacol as substrates, respectively. The similar results were obtained in the other two dyes medium (Figure 1a and 1b). In the experiment, the values used o-dianisidine was slightly higher than that used guaiacol. The compound o-dianisidine was relatively more sensitive than guaiacol.

The results clearly showed that the laccase activity assayed by the ABTS substrate was significantly higher than the other two substrates ($P<0.05$). The ABTS method was more sensitive than o-dianisidine and guaiacol methods. These results were in accord with some literatures. They showed that the catalytic efficiency of laccase from fungi tested was much lower for the substrates of o-dianisidine (Robles et al., 2000) and guaiacol (Liers et al., 2007) than that for ABTS. It also reported that enzyme inactivation by reaction products had been responsible for the lower activity of laccase toward guaiacol (Robles et al., 2000).

The great differences in the oxidation rates of the different substrates should be attributed to differences in redox potential between the enzyme and substrate (Reinhammar and Malmström, 1981). Guaiacol is a metonymy- substituted monophenol, which could be oxidized to aldehydes. o-Dianisidine is a substituted diamine and was oxidized to compounds containing disazo groups. ABTS is a non-phenolic heterocyclic compound. ABTS could be oxidized to cation radical ($ABTS^{\cdot +}$) and ABTS dication ($ABTS^{2+}$) (Johannes and Majcherezyk, 2000; Fabbrini et al., 2002). ABTS is colorless, meanwhile the $ABTS^{\cdot +}$ colors blue-green and presents absorption in the visible region (Solís-Oba et al., 2005).

Laccase is a phenol oxidase (EC1.10.3.2) that catalyzes oxidation of several substances, particularly phenols and aromatic amines (D'Acunzo et al., 2002). The catalytic properties of laccase have had a great impact on

the development of biosensors, and fungal laccase have been widely used in more and more scopes (Amitai et al., 1998). Therefore, the methodologies of evaluating laccase activity are significant in studies. The determination of the most sensitive substrate is indispensable. ABTS was found to be the most sensitive substrate among all tested compounds to evaluate the activity of laccase. Therefore, it is recommended based on this study to use ABTS assay method for detecting laccase activity during study.

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