

Utilization of Organic Wastes for Laccase Production by *Pleurotus ostreatus*

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Abstract

Production of laccase was achieved by using white-rot fungus, *Pleurotus ostreatus*, grown on economical and cost-effective materials. The fungus was cultivated in liquid medium containing mung bean pomace, soybean pomace and wastewater treatment sludge as sole carbon source for 15 days. In addition, different concentrations of an inducer, benzyl alcohol, were tested to induce laccase production. The results revealed that the highest laccase activity was equal to 1.859 ± 0.129 U/mL in mung bean pomace containing medium, and the lowest laccase activity was 0.234 ± 0.019 U/mL in soybean pomace medium. Addition of 10 mm benzyl alcohol enhanced laccase production up to 2.952 ± 0.080 U/mL in the mung bean pomace medium at day 15 of cultivation. Level of laccase production increased correspondingly with increasing concentration of the inducer. The high level of laccase production in mung bean medium could contribute to high C/N ratio and excessively high levels of carbon and nitrogen contents in soybean suppressed laccase production.

Keywords: Organic wastes, Inducer, Benzyl alcohol, Laccase production, *Pleurotus ostreatus*

1 Introduction

Laccase is an oxidoreductase enzyme which plays an important role in biodegradation. Laccase is recognized for its catalyzing role in a broad range of toxic and recalcitrant substrates, phenolic and non-phenolic compounds, such as 3,4-dimethylphenols, 4-ethylphenol, 2,4-dichlorophenol, bisphenol A (BPA), anthracene, benzo[a]pyrene (BaP), Polycyclic Aromatic Hydrocarbon (PAH), synthetic dyes, lignin [1]–[5]. Nowadays, laccase has potential to be applied in various industrial and biotechnological applications such as textile, food, wood processing, and pulp and paper industry [6]. Therefore, optimizing a method for

efficient laccase production is important and will be beneficial in several applications. [7].

Laccase is particularly widespread in white-rot ligninolytic basidiomycete fungi and different species of fungi produce laccase in variable quantity. Wood-degrading fungi such as *Trametes versicolor*, *Trametes ochracea*, *Trametes hirsuta*, *Cerena maxima*, *Pleurotus eryngii*, *Lentinus tigrinus* are common laccase producers [8]. Several factors can affect laccase production such as species and strains of fungi, medium composition, type of substrates, carbon to nitrogen ratio, and culture conditions [9]. One of the important factors influencing laccase production is the type of substrate or carbon source used during cultivation. Some carbon sources

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can stimulate and increase level of laccase production, while certain carbon sources can only support fungal growth but cannot enhance laccase production. Moreover, addition of appropriate number of natural and synthetic inducers, some heavy metals, lignin and aromatic compounds like cotton stalk, veratryl alcohol, *o*-toluidine, benzyl alcohol and copper sulfate can help stimulate the level of laccase production [9]–[12].

For these reasons, this research aims to apply economical and cost-effective materials for laccase production. The usage of organic wastes is beneficial in terms of recycling waste materials and reducing the costs for enzyme production. In this study, we used *Pleurotus ostreatus*, one of the most extensively studied white-rot fungus in the basidiomycetes that is fast growing and can be cultured on a wide range of substrates [7]. Various types of substrates have been used to test for laccase production, such as molasses wastewater, industrial wastes, waste from paper industry, sawdust, lignoagricultural wastes, coffee husk, eucalyptus bark, fruits, grains, and various amounts of laccase have been produced [9], [11], [13]–[15]. In this study, we cultivated *P. ostreatus* on organic wastes from food industry and wastewater treatment plant which were mung bean pomace, soybean pomace, and sewage sludge and compared levels of laccase production from these materials as well as investigated different concentrations of an aromatic inducer (benzyl alcohol) on laccase production.

2 Methods

2.1 Preparation of fungus

The fungus, *P. ostreatus*, was grown on Potato Dextrose Agar (PDA) and incubated at 27°C for 5 days. Then, one mycelium bit (7 mm diameter) was transferred to the center of petri dish containing freshly prepared PDA and incubated at 27°C for 5 days to obtain the active mycelium. After that, the active mycelium bits were transferred to liquid medium for laccase production.

2.2 Preparation of organic wastes

Three organic wastes which were mung bean pomace, soybean pomace, and sewage sludge were dried at 60°C for 48 hours, then crushed to small sizes and passed through a 2 mm sieve. Each organic waste was used

as a sole carbon source in liquid medium for fungal cultivation. Analysis of carbon and nitrogen contents was performed using CHN analyzer (Leco, UK).

2.3 Medium used for laccase production

Liquid medium used for laccase production was adapted from M. Tišma *et al.*, [11]. The components were as following (grams per liter): peptone 0.6, yeast extract 0.5, KH₂PO₄ 0.8, Na₂HPO₄ 0.2, MgSO₄•7H₂O 0.5, CaCl₂•2H₂O 0.05, citric acid 0.15, and 20 grams of the previously prepared organic wastes. Three pieces of active mycelium bits grown on PDA were transferred into 250 mL flasks containing 125 mL of the sterile medium, and incubated at 27°C, 110 rpm for 15 days. Every three days, laccase activity was monitored by collecting 1 mL of culture medium in aseptic condition, and centrifuged at 12,000 rpm for 10 minutes. Then, the mycelium-free supernatant was analyzed for laccase activity. Enzyme assays were done in triplicate, and a blank reaction was performed using liquid medium without fungus inoculation.

2.4 Effects of inducer on laccase production

An inducer used in this study was 5 and 10 mm of benzyl alcohol. The inducer was added to the culture medium at day 3, then continuously cultured till day 15. The culture medium and culture conditions were the same as those grown at regular condition or without the inducer. The laccase activity was monitored every three days using method described above.

2.5 Laccase activity assay

Laccase activity was performed using a colorimetric assay by testing ability of laccase in oxidizing 2.0 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid, ABTS) in acetate buffer, pH 4.5 [16]. Each reaction contained the crude enzyme and ABTS solution at a ratio of 1:6. To test the laccase activity, the reaction was incubated at 25°C for 5 minutes, stopped the reaction by adding 100 µl of 1% Sodium Dodecyl Sulfate (SDS) and measured the laccase activity at absorbance of 420 nm using UV-vis spectrophotometer. Calculation of laccase activity used the following formula and one unit of the enzyme (U) represented the oxidation of 1 µmol ABTS per minute at 25°C using the extinction

coefficient ($\epsilon_{420\text{nm}}$) of $0.036 \mu\text{M cm}^{-1}$ [17].

$$\text{Enzyme activity} \left(\frac{\text{U}}{\text{L}} \right) = \frac{A_{420} \times \text{Total Volume (mL)}}{\epsilon b \times \text{Time (min)}} \times \frac{1}{\text{Enzyme Volume (mL)}} \times \text{DF}$$

3 Results and Discussions

3.1 Laccase production on organic waste medium

Laccase production was conducted by culturing *P. ostreatus* in submerged liquid media mixed with mung bean, soybean, and sewage sludge as sole carbon source for 15 days. The result revealed that *P. ostreatus* showed the highest laccase activity on mung bean containing medium with the value of 1.859 ± 0.129 U/mL, followed by the sewage sludge and soybean medium with laccase activity of 0.719 ± 0.107 and 0.234 ± 0.019 U/mL, respectively (Figure 1). The level of laccase activity gradually increased after day 6 and the highest value was found at day 15. Laccase production from these organic wastes was much higher than that produced from carbon source like glucose. We found that small amount of laccase was produced on the medium containing glucose with the maximum value of only 0.0009 ± 0.0001 U/mL (data not shown).

Several studies have shown that different compositions and number of substrates yield variable amounts of laccase production. Laccase activity of *P. ostreatus* (Fr.) Kumm. was investigated on four substrates which were alder sawdust, rye straw, hemp and flax shive [18]. The results showed that the highest laccase activity of 1,520 neat/kg was observed on hemp shive, while the best mycelial growth was obtained on the flax shive [18]. This evidence can be an indication that some carbon source can merely support mycelial growth, but cannot activate laccase production. Similar result was observed in our study in the way that glucose could support mycelial growth well; however, laccase activity was relatively low comparing to other substrates used in this study. Furthermore, amount of substrate used is also an important factor. Laccase activity of *P. ostreatus* ASI2344 was significantly increased by adding appropriate amount of apple pomace [15]. By adding 2.5% apple pomace, laccase activity was approximately 280%, and 90% higher than that of *P. ostreatus* grown with addition of 5% apple pomace and without apple pomace, respectively [15]. In addition, substrates also affected on different

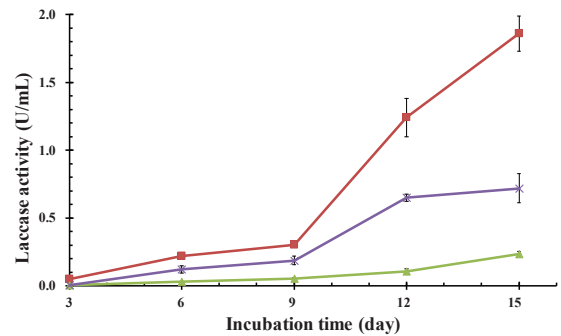


Figure 1: Laccase production on different organic wastes of *P. ostreatus* (■ mung bean; ✕ soybean; ▲ sewage sludge).

mycelial morphology such as compact or freely dispersed forms, and the freely dispersed forms with the addition of paper industrial waste could enhance laccase production [11]. However, our result found that the mycelial morphology was similar in every substrate used that was the mycelium was clumped together even on glucose.

The result from this study revealed that *P. ostreatus* produced the highest laccase activity on mung bean medium. This could be attributed to availability of carbon and nitrogen contents and carbon to nitrogen ratio also played a vital role in laccase production [9]. Appropriate nitrogen concentration is an important nutritional factor for high laccase production in *P. ostreatus* HP-1, while excessively high or low nitrogen concentrations suppress laccase production [13]. The strain HP-1 yielded laccase activity of 2,142, 3,120 and 1,629 U/g of substrate with 2, 20, and 200 mm combination of organic and inorganic nitrogen sources, respectively [13]. Similar results were obtained with our study in the way that excess availability of carbon (50.086%) and nitrogen (5.196%) contents in the soybean pomace might suppress laccase production (Table 1), whereas proper levels of carbon and nitrogen contents as well as high C/N ratio (12.445) in the mung bean pomace could enhance laccase production.

Table 1: Carbon and nitrogen contents of the substrates used in the experiment

| Substrates | Carbon (%) | Nitrogen (%) | C/N Ratio |
|------------------|------------|--------------|-----------|
| Mung Bean Pomace | 42.314 | 3.400 | 12.445 |
| Soy Bean Pomace | 50.086 | 5.196 | 9.639 |
| Sewage Sludge | 20.349 | 3.334 | 6.103 |

On the contrary, there was a study which found that addition of nitrogen source could increase the activity of lignocellulolytic enzyme, soluble proteins and the productivity of the fungus [14]. By adding nitrogen source like rice bran, *P. ostreatus* could produce the highest laccase activity up to $16 \mu\text{M min}^{-1} \text{kg}^{-1}$ of substrate on coffee husk supplemented with 20% rice bran. Without addition of the rice bran, the highest laccase activity of $11 \mu\text{M min}^{-1} \text{kg}^{-1}$ of substrate was observed [14].

3.2 Influence of inducer on laccase production

Influence of inducer on laccase production was performed by adding 5 and 10 mm benzyl alcohol at day 3 of incubation and further cultured until day 15 of the experiment. The result revealed that *P. ostreatus* still produced the highest level of laccase activity on the mung bean medium, followed by the sewage sludge and soybean. The highest laccase activity of *P. ostreatus* fed on the mung bean was equal to 2.357 ± 0.076 and 2.952 ± 0.080 U/mL after adding 5 and 10 mm benzyl alcohol, respectively as shown in Figure 2(a) and 2(b). By adding 10 mm benzyl alcohol on mung bean medium could increase level of laccase production by 58.8%. This result could imply that 10 mm benzyl alcohol was not toxic to the cells because the level of laccase was increased with increasing concentrations of the benzyl alcohol. Increasing in laccase production in response to increasing concentrations of the inducer was also observed in the soybean and sewage sludge. However, addition of benzyl alcohol to the medium containing glucose could not stimulate laccase production. The laccase activity was still lower than 0.001 ± 0.0001 U/mL after adding 10 mm of benzyl alcohol (data not shown).

Several types of inducers like heavy metals, aromatic compounds, lignin or lignin derivatives, such as copper sulfate, veratryl alcohol, benzyl alcohol, syringaldazine could increase laccase production [11]–[12]. It was found that copper sulfate was the best inducer for *P. ostreatus* HP-1 [13]. The maximum laccase activity of 14,189 U/g of dry wheat straw was achieved using 0.28 mm copper sulfate, whereas other inducers like vanillin, gallic acid, catechol, guaiacol, ortho-dianisidine could promoted laccase production less than 7,000 U/g of dry wheat straw [13]. Another study found that *P. ostreatus* was capable of producing laccase at 0.57 U/mL without

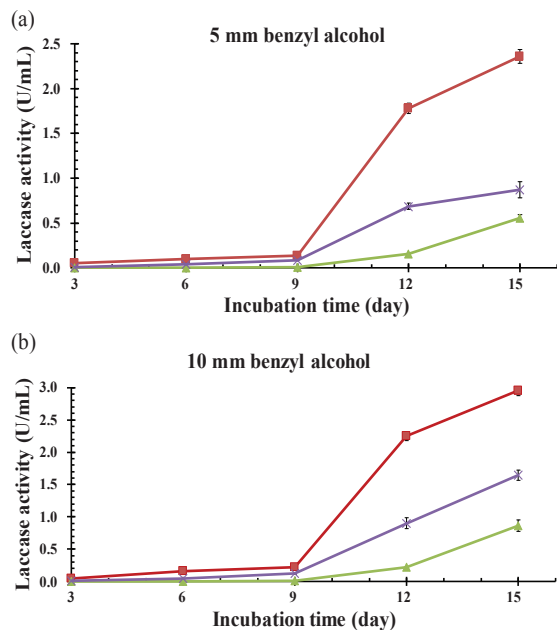


Figure 2: (a) laccase production on different organic wastes after addition of 5 mm benzyl alcohol, and (b) 10 mm benzyl alcohol (■ mung bean; * soybean; ▲ sewage sludge).

inducer. After adding several inducers, they found that the best inducer for *P. ostreatus* was 1 mm copper sulfate by increasing laccase activity up to 0.910 U/mL [19]. Nevertheless, excess concentrations of inducers could cause adverse effects on fungal growth and enzyme production because of toxicity of the inducer and inhibitory effect of the inducers at high concentrations [13]. Our result found that 10 mm benzyl alcohol was acceptable level used to induce laccase production because higher amount of laccase was produced with increasing concentrations of the inducer.

When compare laccase activity with other studies which used other fungal species, such as *T. troglia*, *T. versicolor*, and *Lentinus squarrosulus*, the levels of laccase activity of these studies were lower than the value obtained in the present study (Table 2). However, there was a study which did produce relatively high level of laccase up to 114.64 U/mL [15]; however, they had added as much as 50 mycelial plugs in 500 mL of medium. Several studies suggest that various fungal species and different strains of *P. ostreatus* produce laccase in variable quantities which could be because of genetic variability as well as cultural conditions,

temperature and pH of the medium, composition of substrate, types of inducers, and amount of inoculum used during the experiments.

Table 2: Laccase production in different studies

| Fungi | Substrates | Laccase Activity (U/mL) | References |
|--------------------------------|--|-------------------------|---------------|
| <i>P. ostreatus</i> ASI2344 | 2.5% of apple pomace | 114.64 | [15] |
| <i>P. ostreatus</i> | culture media containing glucose + 1 mm copper sulfate (inducer) | 0.910 | [19] |
| <i>T. versicolor</i> MZKI G-99 | waste from paper industry | 0.372 | [11] |
| - <i>T. troglia</i> | - pulverized apricot seed shell | 0.386 | [20] |
| - <i>T. versicolor</i> | - bulrush | 1.216 | |
| <i>L. squarrosulus</i> | NPM liquid medium | 0.064 | [21] |
| <i>P. ostreatus</i> | mung bean pomace | 2.952 | Present study |

4 Conclusions

The white-rot fungus, *P. ostreatus*, can produce the highest amount of laccase on mung bean containing medium. This might be because high C/N ratio and fiber contents in the mung bean pomace activate laccase production. Excessively high or low carbon and nitrogen contents in the soy bean and sewage sludge suppress laccase production. Moreover, addition of an inducer, 10 mm benzyl alcohol, can increase level of laccase production by 58.8%.

Acknowledgments

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