Current Topics in Toxicology
Vol. 17, 2021

Original Article

Phenol degradation by macrofungi from Peninsular Malaysia

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ABSTRACT

Phenol contamination is a worldwide issue and thus an effective way to remove the presence of phenol in environment is needed. In this study, green and cost effective mycoremediation for phenol degradation was tested using common edible macrofungi, namely *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes*. These macrofungi were cultured in Bushnell Haas Broth (BHB) with different concentrations of phenol (0.3, 0.6, 0.9, and 1.2 µL/mL). BHB with no phenol added served as negative control. All the microfungi were cultured for 15 days. Dry biomass (g) of the mushrooms were weighted and enzyme (laccase and manganese peroxidase) activities were assayed using spectrophotometry with $\lambda = 450$ nm for laccase, and $\lambda = 610$ nm for manganese peroxidase (MnP). Results showed the biomass and enzymes' activities decreased as the phenol concentration increased. This suggests despite phenol's toxicity to the macrofungi, these macrofungi were still able to tolerate and biodegrade phenol by using laccase and MnP. *A. bisporus* recorded the highest MnP activity and highest production of biomass, while *P. ostreatus* produced the highest laccase activity. *A. bisporus* was found to be the best candidate for use in phenol degradation based on the highest biomass weight and highest MnP activity.

KEYWORDS: phenol degradation, phenol tolerance, mycoremediation.

INTRODUCTION

Phenol is one of the most utilized chemical substances in the world. It has been widely used in many different industries for producing products like pesticides, vaccine, and skincare products. The accumulation of phenol in our environment has reached an alarming level [1].

Phenol is a reactive substance. Other chemical groups, e.g. chlorophenols, methylphenols and alkylphenols may replace hydrogen atoms at the phenyl ring to form more high toxicity substances. These substances will accumulate in the adipose tissue of the food source and affect human health *via* food chain [2, 3]. According to the Agency for Toxic Substances and Disease Registry (ATSDR) and Environmental Protection Agency (EPA), inhalation and skin contact of phenol will lead to respiratory tract irritation, throat swelling, trachea inflammation and ulceration, whereas ingestion of phenol causes vomiting, diarrhoea and damage to liver and kidney [1, 4]. Thus, phenol has become one of the most concerned pollutants in many countries [5].

In order to solve the issue of phenol contamination, several methods have been developed that remove phenol from the environment [6]. However, high operation cost, energy consumption, and the generation of new wastes limit the usage of these methods [7]. Therefore, environmental-friendly and cost effective mycoremediation is a good alternative to remove phenol pollutants from the environment [8].

The white rot fungi are known to degrade lignin (complex phenolic compound) by secreting various extracellular enzymes like laccase, manganese peroxidase and lignin peroxidase [9]. They are able to bio-remediate phenol [10], which shares similar metabolic pathway with lignin [11]. In this study, the ability of three white rot fungi *Agaricus bisporus* (white button mushroom), *Pleurotus ostreatus* (grey oyster mushroom) and *Lentinula edodes* (shiitake

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mushroom) to degrade phenol was determined. The tolerance and ability of each macrofungi to degrade phenol were determined.

METHODS

Preparation of mushrooms culture

The macrofungi *A. bisporus* (white button mushroom), *P. ostreatus* (grey oyster mushroom) and *L. edodes* (shiitake mushroom) were purchased from Nilai fresh market in Nilai, Malaysia. Tissues from inner part of fresh macrofungi cap was picked using sterilized forceps, and transferred to potato dextrose agar (PDA). The tissues were incubated at 25 °C for 1 week [12]. The culture work was conducted in aseptic conditions.

Phenol tolerance study

Bushnell Haas Broth (BHB) is a media without carbon source. BHB media was prepared together with addition of phenol at different concentrations (0, 0.3, 0.6, 0.9, and 1.2 µL/mL). El-Zaher *et al*. [13] confirmed that most of the white rot fungi could tolerate the presence of phenol up to 1.0 µL/mL. The media was used to determine the ability of macrofungi to survive with the only carbon source from phenol.

The macrofungi were then transferred into BHB media with different concentrations of phenol for 20 days' incubation. BHB media were filtered and screened for laccase and manganese peroxidase enzymatic activities after incubation. All filtrated BHB media containing laccase and manganese peroxidase enzymes were centrifuged at 6500 *g* for 10 min at 4 °C. The supernatant was kept for enzyme assay [14]. The biomass was determined *via* the filtration of the media as well. Mushrooms were grown in BHB without phenol as negative control.

Laccase activity assay

Laccase activity was determined by oxidation of guaiacol [15]. The reaction mixture was prepared by adding 1 mL of supernatant to 1 mL guaiacol (2 mM) and 1 mL of sodium acetate buffer (10 mM, pH 5.0). The mixture was incubated for 30 s at 25 °C before taking the optical density (OD) reading at λ = 450 nm. Negative controls were used as blank. All enzyme activities were measured for three different samples (n = 3).

The absorbance reading was recorded and the laccase activity (IU) was calculated using Equation 1. The final absorbance value was calculated with Equation 2.

$$\text{Laccase enzymes activity (IU)} = \frac{A_{450} \times \text{Total mixture volume (mL)}}{1\ \text{mL supernatant} \times 0.5\ \text{min} \times e} \quad \text{(Eq. 1)}$$

where,

e is extinction coefficient for guaiacol (0.6740 µM/cm)

$$\text{Final absorbance value } (A_{450}) = A_{450} \text{ test sample} - A_{450} \text{ blank} \quad \text{(Eq. 2)}$$

Manganese peroxidase assay

Manganese peroxidase (MnP) activity was determined by oxidation of phenol red in the presence of hydrogen peroxide [16]. 500 µL of supernatant was added to a mixture containing 50 µL of manganese sulfate (2.0 mM), 200 µL of bovine albumin (0.5% w/v), 50 µL of hydrogen peroxide (2 mM) in sodium succinate buffer (0.2M, pH 4.5), 100 µL sodium lactate (0.25 M), and 100 µL of phenol red (0.01% w/v). The mixture was incubated for 30 s at room temperature.

The mixture was then measured at λ = 610 nm using a UV-vis spectrophotometer. Negative control was used as blank. The absorbance reading was recorded and the MnP activity was calculated using the Equation 3. Final absorbance value was calculated using Equation 4.

$$\text{Manganese Peroxidase Enzymes Activity (U/L)} = \frac{A_{610} \times 10^6}{0.5\ \text{mL supernatant} \times 0.5\ \text{min} \times e} \quad \text{(Eq. 3)}$$

where,

e is the extinction coefficient of oxidized phenol (4460 $L, m^{-1} \cdot cm^{-1}$)

$$\text{Final absorbance value } (A_{610}) = A_{610} \text{ test sample} - A_{610} \text{ blank} \quad \text{(Eq. 4)}$$

RESULTS AND DISCUSSION

Laccase activity

The highest laccase activity was observed in *P. ostreatus* at 0.3 µL/mL phenol (Figure 1). The

activity dropped dramatically after that. Similar trend was observed for the other two species of macrofungi. Ferdeş *et al*. [17] reported a similar trend of laccase activity for the same macrofungi exposed to phenolic dye (alizarin).

Park *et al*. [18] identified that the presence of 12 laccase genes in the mycelium of *P. ostreatus* contributed to the high laccase activity in that macrofungi. In addition to that, laccase genes in *P. ostreatus* are linked to stress response element (STRE) in their promoter sequence, namely *Lcc1*, *Lcc3*, *Lcc5* and *Lcc6*. This contributes to high laccase activities.

L. edodes also contained 12 laccase genes, but only three of these genes *Lcc1*, *Lcc5* and *Lcc6* were expressed in mycelium [19]. Other genes were expressed in *L. edodes* fruiting bodies and primordia stages [20]. *A. bisporus* had two (*Lcc2* and *Lcc4*), while *L. edodes* had one laccase (*Lcc1*) gene with STRE in their promoter regions [21].

Manganese peroxidase activity

The highest MnP activity was produced by *A. bisporus* exposed to 0.3 µL/mL phenol (Figure 2). The activity of the enzyme reduced thereafter, with the increase of phenol concentration. Bonnen *et al*. [22] reported similar trend in their bisphenol degradation experiment where the *Agaricus* sp produced high MnP activity.

Highest MnP activity by *A. bisporus* suggested that the H_2O_2 and organic acid production level in *A. bisporus* was the highest when compared to other species when exposed to phenol [23]. Both H_2O_2 and organic acid production are cofactors for MnP activity in almost all white rot fungi [24].

Both *A. bisporus* and *L. edodes* contain two MnP genes [25], but the MnP activity of *L. edodes* was far lesser than *A. bisporus* (Figure 2). The results might be due to increased production of H_2O_2 and organic acid in *A. bisporus*, which led to high MnP production, because *A. bisporus* contains 22 aryl alcohol oxidase and nine glyoxal oxidase-encoding genes but *L. edodes* only contains eight aryl alcohol oxidase and three glyoxal oxidase genes. Therefore, low level of H_2O_2 and organic acid production in *L. edodes* could not induce the high expression level of MnP when compared to *A. bisporus*. *P. ostreatus* produced the least MnP, as the macrofungi did not encode aryl alcohol oxidase genes in its genome [26]. Thus, it loses a crucial advantage on increasing its MnP level.

Mushroom growth

Macrofungi *A. bisporus* produced the highest biomass with phenol concentration of 0.3 µL/mL phenol (Figure 3). Similar trend was observed in the biodegradation of phenolic compund 2-ethylhexyl-phthalate [27].

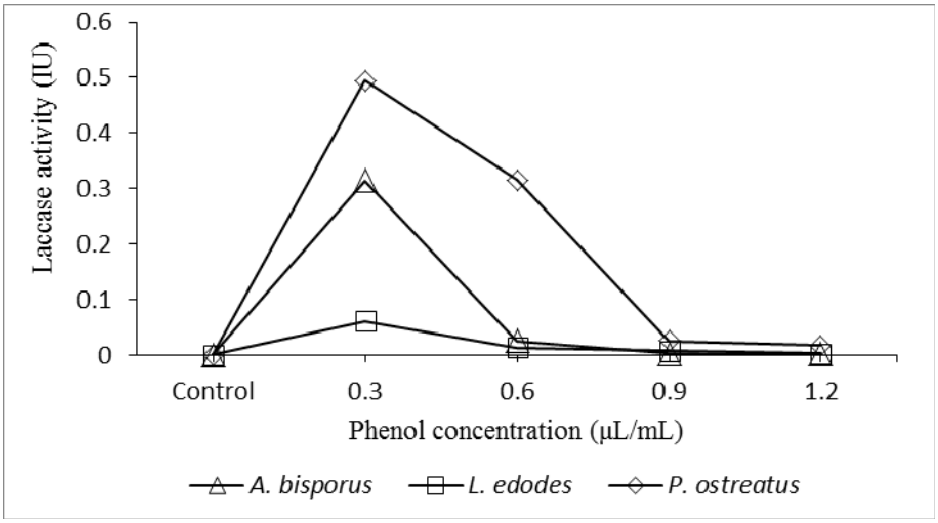

Figure 1. Laccase activity (IU) of *A. bisporus*, *L. edodes* and *P. ostreatus* observed after 15 days of incubating in the phenol-containing BHB media. Standard deviation for all reading was < 5 % for all tests.

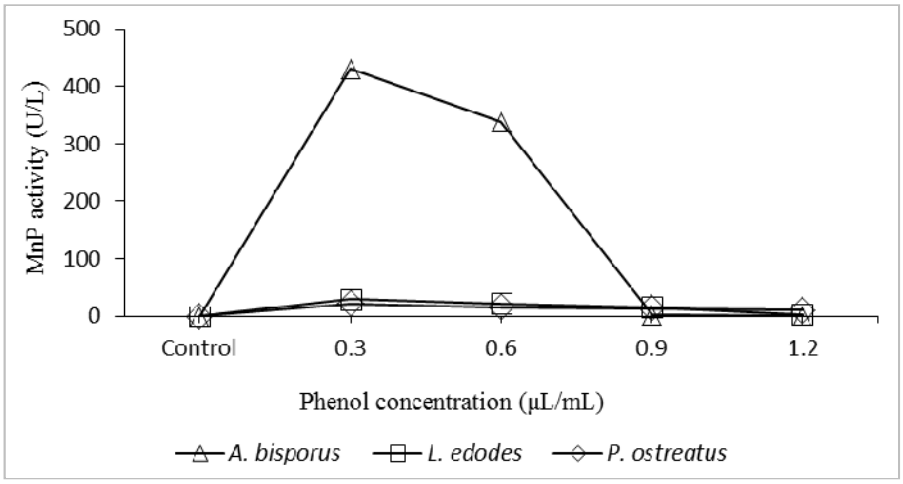

Figure 2. Manganese peroxidase activity of *A. bisporus*, *L. edodes* and *P. ostreatus* observed after 15 days of incubation in the phenol-containing BHB media. Standard deviation for all reading was < 5 % for all tests.

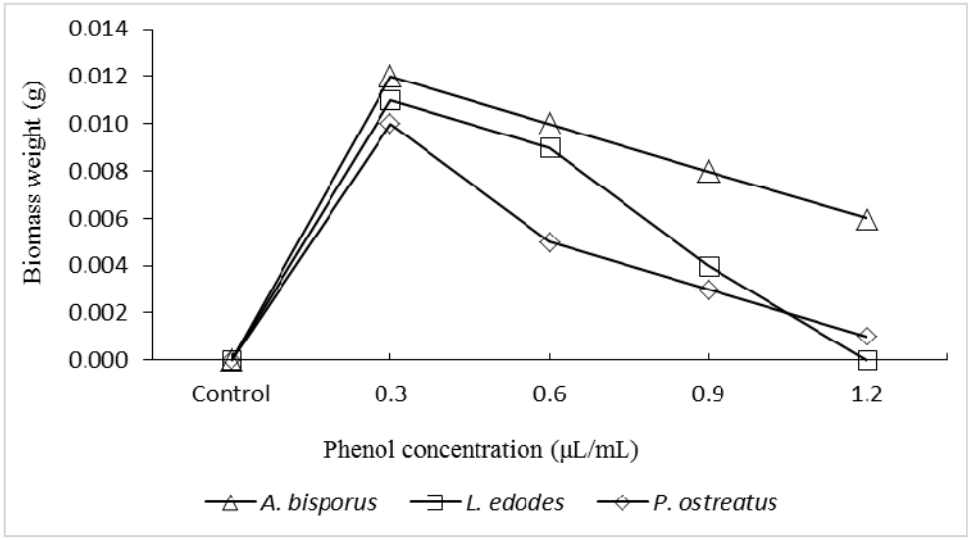

Figure 3. Biomass weight (g) of *A. bisporus*, *L. edodes* and *P. ostreatus* observed after 15 days of incubation in the phenol-containing BHB media. Standard deviation for all reading was < 5 % for all tests.

The growth of the mushrooms species is related to their laccase and MnP activities [27]. As *A. bisporus* has a higher ability to produce both laccase and MnP, it is able to degrade the phenol (antifungal agent) faster than the other two species.

L. edodes produced the least laccase and showed low MnP activities but produced higher biomass than *P. ostreatus*. The results might be due to the ability of *L. edodes* to produce pyranose oxidase to degrade phenol into 2-keto sugars, and this keto sugar would enter into Krebs cycle and produce extra ATP to support the growth of *L. edodes*. However, this laccase enzyme is only encoded in *L. edodes* and *A. bisporus* but not in *P. ostreatus* [28]. Hence, *P. ostreatus* did not have the extra advantage like *L. edodes*.

CONCLUSION

All three macrofungi showed ability to biodegrade phenol and can be used for bioremediation.

A. bisporus produced the highest biomass weight, and showed the highest MnP activities and intermediate level of laccase activity. *L. edodes* produced the intermediate biomass weight, and showed a low MnP activity and the least laccase activity, whereas, *P. ostreatus* produced the least biomass weight, and showed the least MnP activity but highest laccase activity.

ACKNOWLEDGEMENTS

This project was supported by the INTI International University Research Grant Scheme (INTI-FHLS-01-03-2018) and funded by the Biotechnology Programme.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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