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ORIGINAL ARTICLE

Biodegradation of toxic chemicals by *Pleurotus eryngii* in submerged fermentation and solid-state fermentation

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Background/Purpose: The toxic chemicals bisphenol A (BPA), bisphenol F (BPF), nonylphenol (NP), and tetrabromobisphenol A (TBBPA) are endocrine-disrupting chemicals that have consequently drawn much concern regarding their effect on the environment. The objectives of this study were to investigate the degradation of BPA, BPF, NP, and TBBPA by enzymes from *Pleurotus eryngii* in submerged fermentation (SmF) and solid-state fermentation (SSF), and also to assess the removal of toxic chemicals in spent mushroom compost (SMC).
Methods: BPA and BPF were analyzed by high-performance liquid chromatography; NP and TBBPA were analyzed by gas chromatography.
Results: NP degradation was enhanced by adding $CuSO_4$ (1 mM), $MnSO_4$ (0.5 mM), gallic acid (1 mM), tartaric acid (20 mM), citric acid (20 mM), guaiacol (1 mM), or 2,2′-azino-bis- (3-ethylbenzothiazoline-6-sulfonic acid; 1 mM), with the last yielding a higher NP degradation rate than the other additives from SmF. The optimal conditions for enzyme activity from SSF were a sawdust/wheat bran ratio of 1:4 and a moisture content of 5 mL/g. The enzyme activities were higher with sawdust/wheat bran than with sawdust/rice bran. The optimal conditions for the extraction of enzyme from SMC required using sodium acetate buffer (pH 5.0, solid/solution ratio 1:5), and extraction over 3 hours.
Conclusion: The removal rates of toxic chemicals by *P. eryngii*, in descending order of magnitude, were SSF > SmF > SMC. The removal rates were BPF > BPA > NP > TBBPA.

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Introduction

Organic toxic chemicals such as bisphenol-A (BPA) and bisphenol-F (BPF) are used as materials for epoxy resins and polycarbonates lining large food containers and water pipes.[1] Nonylphenol polyethoxylates constitute an important group of nonionic surfactants that are used for a variety of industrial, household, and commercial applications.[2] Nonylphenol (NP) in aquatic environments is mostly from the microbial breakdown of NPnEOs.[3] Tetrabromobisphenol-A (TBBPA), a halogenated derivative of BPA, is used as a flame retardant.[4] Because of their widespread use and relatively high water solubility, these compounds are among the most ubiquitous contaminants in the aquatic environment.[1,4,5] These so called "environmental hormone" compounds have also been proposed to be endocrine-disrupting chemicals and may adversely affect endocrine function in humans and wildlife.[6]

Although toxic chemicals may gradually be removed from the environment through sorption, chemical degradation, or photolysis, metabolic breakdown by microorganisms is considered to be one of the major routes of degradation for toxic chemicals. White rot fungi produce three main extracellular enzymes involved in ligninolysis, namely laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP).[7] *Pleurotus eryngii* is a saprophytic mushroom that is taxologically related to *Basidiomycota*, *Agaricomycetes*, *Agaricales*, *Pleurotaceae*, and *Pleurotus*. This fungus can produce ligninolytic enzymes such as laccase, MnP, and the H_2O_2-generating enzyme aryl-alcohol oxidase (AAO).[8]

Enzyme production is an expanding field of biotechnology. Most enzyme manufacturers produce enzymes by submerged fermentation (SmF) techniques. However, in the past decades there has been an increasing trend towards the utilization of the solid-state fermentation (SSF) technique to produce several enzymes.[9] SSF has received new interest not only from researchers but also from industry. Spent mushroom compost (SMC), a waste product of the mushroom industry, still contains many residual enzymes.[10] The use of SMC as a source material for ligninolytic enzymes is a win—win strategy, because it is easily obtained and its use reduces the disposal costs of waste removal.

P. eryngii is one of the most important and widespread white rot fungi in the world. However, few studies have explored the use of *P. eryngii* and its enzymes in the degradation of xenobiotics. Enzyme activities and the removal rates of toxic chemicals are influenced by pH, temperature, substrate concentration, cofactors such as Cu^{2+} and Mn^{2+}, and organic acids in the aqueous environment.[11—13] The choice of the proper mediator substance also plays a key role by affecting the applicability and effectiveness of the laccase mediator systems (LMS). The most commonly used is 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).[13] This research studied the capacity of *P. eryngii* to degrade toxic organic pollutants under both SmF and SSF, and also assessed the removal of toxic chemicals in SMC. The roles of ligninolytic enzymes in the biodegradation in both conditions were also evaluated. The target chemicals were BPA, BPF, NP, and TBBPA.

Materials and methods

Chemicals

BPA and BPF of 99.9% analytical standard were purchased from Sigma—Aldrich (St Louis, MO, USA). NP with 98.0% analytical purity and TBBPA of 97.0% analytical standard were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Solvents were purchased from Mallinckrodt, Inc. (Paris, KY, USA) and all other chemicals were from Sigma—Aldrich.

Microorganisms, SMC, and medium

P. eryngii BCRC 36213 and BCRC 36163 were obtained from the Bioresource Collection and Research Centre (BCRC), Hsinchu, Taiwan. Stock cultures of the fungi were maintained on PDA slants at 4°C. Each fungal inoculum was grown on a PDA plate at 30°C for 12 days. Two agar plugs cut from the PDA culture plate were then transferred into 50 mL of basal medium (BM) in a 250 mL Erlenmeyer flask and incubated at 30°C on a rotary shaker in the dark for 12 days prior to use. SMC of *P. eryngii* was produced in a Baiguzhuang mushroom cultivation factory in Taichung, Taiwan, after the harvest of edible crops, using wheat bran and sawdust-based fermented compost.

The BM contained (/L of dH_2O) corn steep liquor 10 g, yeast extract 2 g, KH_2PO_4 0.9 g, Na_2HPO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $CaCl_2 \cdot 7H_2O$ 0.5 g, thiamine HCl 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.0005 g, $MnSO_4 \cdot 4H_2O$ 0.0007 g, $ZnSO_4 \cdot 7H_2O$ 0.043 g, and $CuSO_4 \cdot 5H_2O$ 0.0008 g. The pH of the BM was 5.0 prior to sterilization.

Experimental design

The SmF was performed using 5 mL of *P. eryngii* culture, 45 mL of medium, and 20 mg/L of NP. The following factors were adjusted or added to study their effects on the degradation of NP: pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0); incubation temperature (20°C, 25°C, 30°C, 35°C, 40°C, or 50°C); and addition of $CuSO_4$ (1 mM), $MnSO_4$ (0.5 mM), gallic acid (1 mM), tartaric acid (20 mM), citric acid (20 mM), guaiacol (1 mM), or ABTS (1 mM). The concentrations of the additives were chosen in accordance with previous studies.[14] Prior to sampling, each flask was shaken vigorously to ensure mixing. All cultures were incubated at 30°C on a rotary shaker in the dark for 12 days. The residual NP in each culture was determined periodically. Inoculated control samples were incubated at 30°C and pH 7.0 in the dark. Sterile controls were autoclaved at 121°C for 30 minutes on consecutive days. All experiments were performed in triplicate.

The SSF was performed using 1 g of sawdust, 4 g of wheat bran, and 25 mL of BM. The following factors were adjusted to study their effects on the enzyme extraction: various substrate components (rice bran, wheat straw, and sawdust), sawdust/wheat bran ratios (4:1, 1:1, and 1:4), and moisture content (5 mL/g, 7 mL/g, and 10 mL/g). All samples were incubated at 30°C on a rotary shaker in the dark for 12 days. After 12 days of fungal growth, the extracellular enzymes were extracted twice using a

mechanical extractor with 25 mL of distilled water. The solids were separated by filtration through nylon cloth followed by centrifugation (8000*g*; 15 minutes) at 4°C. The supernatant received after biomass separation was added to 20 mg/L of NP and ABTS (1 mM). The residual NP in each culture was determined periodically.

The extraction of enzyme from SMC was performed using 600 mL of sodium acetate buffer and 120 g of *P. eryngii* SMC. The following factors were adjusted to study their effects on the enzyme extraction: extraction time (3 hours, 1 days, and 4 days), various buffers (phosphate buffer, pH 7.0; phosphate buffer, pH 5.8; sodium acetate buffer, pH 5.0; and sodium acetate buffer, pH 4.5), and solid/solution ratio (1:25, 1:10, and 1:5). The enzyme was used as a crude enzyme. SMC is a potential source of ligninolytic enzymes such as laccase, therefore the crude enzyme measurements represented laccase activity.[15]

In order to compare the removal of toxic chemicals in SmF, SSF, and SMC, the toxic chemicals BPA, BPF, NP, and TBBPA (20 mg/L) were added to 50 mL of crude enzyme, with or without ABTS (1 mM), and incubated for 1 day in the dark.

Analytical methods

The laccase and MnP activity were measured as described in our previous study,[14] BPA, BPF, NP, and TBBPA extraction and analysis were performed as described in our previous study.[4,5,15] The recovery percentages of BPA, BPF, NP, and TBBPA were 96.3%, 97.2%, 91.2%, and 91.5%, respectively, and the detection limits were 100 μg/L, 100 μg/L, 50 μg/L, and 20 μg/L, respectively.

Data analysis

The degradation data for toxic chemicals collected for this study fit first-order kinetic equations well: $S = S_0\exp(-k_1 t)$, $t_{1/2} = \ln 2/k_1$, where t is the time, S_0 the initial substrate concentration, S the substrate concentration at time t, and k_1 the degradation rate constant. Toxic chemical remaining percentage [%] = (residual chemical concentration/initial chemical concentration) × 100. Toxic chemical removal rate [%] = [1− (residual chemical concentration/initial chemical concentration)] × 100. Statistical significance was accepted at $p < 0.05$. The data were analyzed by t test with SPSS version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

The concentrations of toxic chemicals in the sterile controls were first examined at the end of the 12 days of incubation. The percentage of toxic chemicals ranged from 91.1% to 97.9%. Therefore, it was concluded that the degradation of the toxic chemicals occurring in all of the following experiments was due to microbial action. A comparison of the effects of two strains of *P. eryngii* on NP degradation and enzyme activity after 12 days of incubation in SmF is shown in Table 1. The remaining percentage of NP for *P. eryngii* BCRC 36213 was lower than that for *P. eryngii* BCRC 36163, and higher levels of laccase and MnP were produced by *P. eryngii* BCRC 36213 than by *P. eryngii* BCRC 36163. Thus, strain BCRC 36213 was used in all of the following experiments.

Table 1 Comparison of the effects of two strains of *Pleurotus eryngii* on nonylphenol degradation and enzyme activity after 12 days of incubation in submerged fermentation[a]

Strain	Laccase (U/mL)	Manganese peroxidase (U/mL)	Nonylphenol remaining (%)
BCRC 36163	24.6 ± 0.4	ND	78.8 ± 1.2
BCRC 36213	53.1 ± 7.8	8.1 ± 5.9	1.1 ± 2.9

[a] All treatment figures were significantly different at $p < 0.05$. Each figure represents the mean of three measurements; in all cases, the coefficient of variation (CV) was <10.0%. CV (%) = [standard deviation (%)/mean value (%)] × 100.

Enzyme production and NP degradation by *P. eryngii* in SmF

The effects of pH and temperature on NP degradation are shown in Fig. 1. At pH 2.0—5.0, the NP remaining percentage ranged from 64% to 4.1%. As the pH was increased from 5.0 to 9.0, the NP remaining percentage increased from 4.1% to 76.1%. At 20—30°C, the NP remaining percentage was 12—3.8%. At 30—50°C, the NP remaining percentage was 3.8—55%. The optimum pH for NP degradation was 5.0 and the optimum temperature was 30°C.

The effects of various additives on enzyme activity and NP degradation are shown in Table 2. In comparison with the inoculated control, adding 1 mM of $CuSO_4$ and 0.5 mM of $MnSO_4$ increased the degradation rate of NP by 30.3% and 75.8%, respectively. Among the four organic acid supplements, gallic acid (1 mM), tartaric acid (20 mM), and citric acid (20 mM) elevated the degradation rate of NP by 18.2%, 24.2%, and 48.5%, respectively. Adding guaiacol (1 mM) and ABTS increased the degradation rate of NP by 51.5% and 87.9%, respectively. The results indicate that the addition of $CuSO_4$, $MnSO_4$, citric acid, gallic acid, tartaric acid, guaiacol, or ABTS enhanced NP degradation, with ABTS yielding a higher NP degradation rate than the other additives. The table also shows that the higher the laccase and MnP activity produced, the greater the increase in NP degradation.

Enzyme production and NP degradation by *P. eryngii* in SSF

The effects of various substrate components, substrate ratios, and moisture contents in SSF with *P. eryngii* on laccase and MnP activity are presented in Fig. 2. After 12 days of incubation, the effects of various substrate components on laccase and MnP activity, in descending order of magnitude, were wheat bran > rice bran > sawdust. The effects of various sawdust/wheat bran ratios on laccase and MnP activity were 1:4 > 1:1 > 4:1. A sawdust/wheat bran ratio of 1:4 is the best substrate ratio

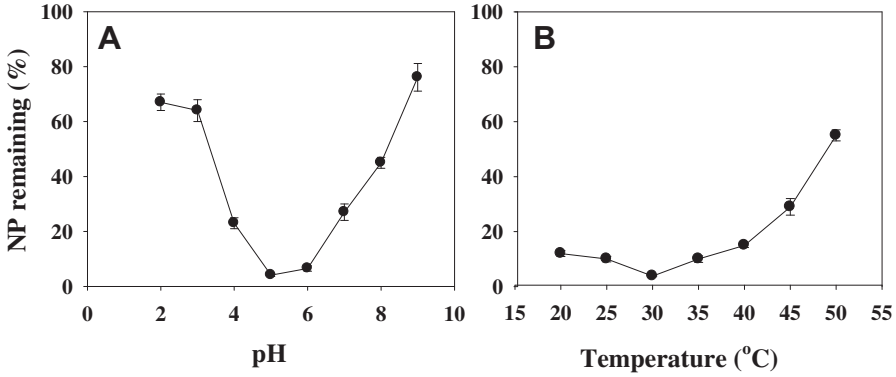

Figure 1. Biodegradation of NP by *Pleurotus eryngii* at various values of (A) pH and (B) temperature in submerged fermentation.

for laccase and MnP activity. A higher wheat bran content in the buffer produced a higher enzyme activity. The effects of moisture content of substrate on laccase and MnP activity, in descending order of magnitude, were 5 mL/g > 7 mL/g > 10 mL/g. A higher moisture content produced lower laccase and MnP activity. It is crucial to provide an optimized moisture content of the fermenting substrate, because the availability of water at higher or lower concentrations may affect microbial activity adversely. Therefore, a sawdust/wheat bran ratio of 1:4 and a moisture content of 5 mL/g were the optimal conditions.

The degradation rate of NP by crude enzyme is shown in Fig. 3. NP was removed completely with and without the addition of ABTS after 3 hours and 24 hours of incubation, respectively. NP degradation was enhanced by adding ABTS. The increased degradation rate of NP produced by *P. eryngii* may be attributed to the increase of enzyme activity.

Table 2 Effects of various additives on enzyme activity and on degradation rate constants (k_1) and half-lives ($t_{1/2}$) of nonylphenol in the cultures of *Pleurotus eryngii* BCRC 36213

Treatment	Laccase[a] (U/mL)	Manganese peroxidase[a] (U/mL)	Nonylphenol[b]		
			k_1 (1/d)	$t_{1/2}$ (d)	r^c
Inoculated control	33.1	8.1	0.33	2.1	0.96
$CuSO_4$ (1 mM)	45.3	9.4	0.43	1.6	0.96
$MnSO_4$ (0.5 mM)	61.5	10.1	0.58	1.2	0.95
Gallic acid (1 mM)	41.5	9.3	0.39	1.8	0.98
Tartaric acid (20 mM)	42.3	10.1	0.41	1.7	0.95
Citric acid (20 mM)	45.5	11.3	0.49	1.4	0.94
ABTS (1 mM)	74.5	12.2	0.62	1.1	0.96
Guaiacol (1 mM)	63.1	10.3	0.50	1.4	0.91

[a] Laccase and manganese peroxidase activity measured on Day 12.
[b] Initial concentration = 20 mg/L.
[c] r = correlation coefficient.
All inoculated control and treatment figures were significantly different at $p < 0.05$.

Enzyme production from SMC

As shown in Fig. 4, the effects of various extraction buffers on laccase activity, in descending order of magnitude, were sodium acetate buffer (pH 5.0) > sodium acetate buffer (pH 4.5) > phosphate buffer (pH 5.8) > phosphate buffer (pH 7.0). The effects of various extraction times on the laccase activity, in descending order of magnitude, were 3hours > 1 day >4 days. These results indicate that sodium acetate buffer (pH 5.0) and an extraction time of 3 hours are the best running conditions for measuring laccase activity. The effects of various solid/solution ratios on laccase activity are presented in Fig. 5. After 3 hours of extraction, the effects on laccase activity of extraction buffers with various solid/solution ratios, in descending order of magnitude, were 1:5 > 1:10 > 1:25. These results reveal that a solid/solution ratio of 1:5 is the best extraction ratio for crude laccase activity.

Comparison of toxic chemicals removal by *P. eryngii* in SmF, SSF, and SMC

As shown in Table 3, the toxic chemicals BPA, BPF, NP, and TBBPA could be removed by *P. eryngii*; the removal was enhanced when ABTS was added in SmF, SSF, and SMC. The removal rates of the three chemicals by *P. eryngii* in SmF, SSF, and SMC, in descending order of magnitude, were BPF > BPA > NP > TBBPA. Comparing the degradation of the three chemicals by *P. eryngii* in SmF, SSF, and SMC, the removal rates of toxic chemicals, in descending order of magnitude, were SSF > SmF > SMC.

Discussion

In this study, the optimum pH and temperature for NP degradation were 5.0 and 30°C, respectively, in SmF. *P. eryngii* was cultivated and the enzyme produced was very stable at pH 4.0–6.0; the optimum conditions for enzyme activity were a pH of 5.0.[16] Laccase production by *Pleurotus ostreatus* D1 peaked on Day 6 or Day 7 at 25°C and the optimum pH for enzyme activity was 4.5.[13] Laccase activity from *P. eryngii* in the BM had an optimum pH of 4.5.[17] The optimum temperature for the production of enzyme from *P. eryngii* was 30°C, but when the enzyme was exposed to

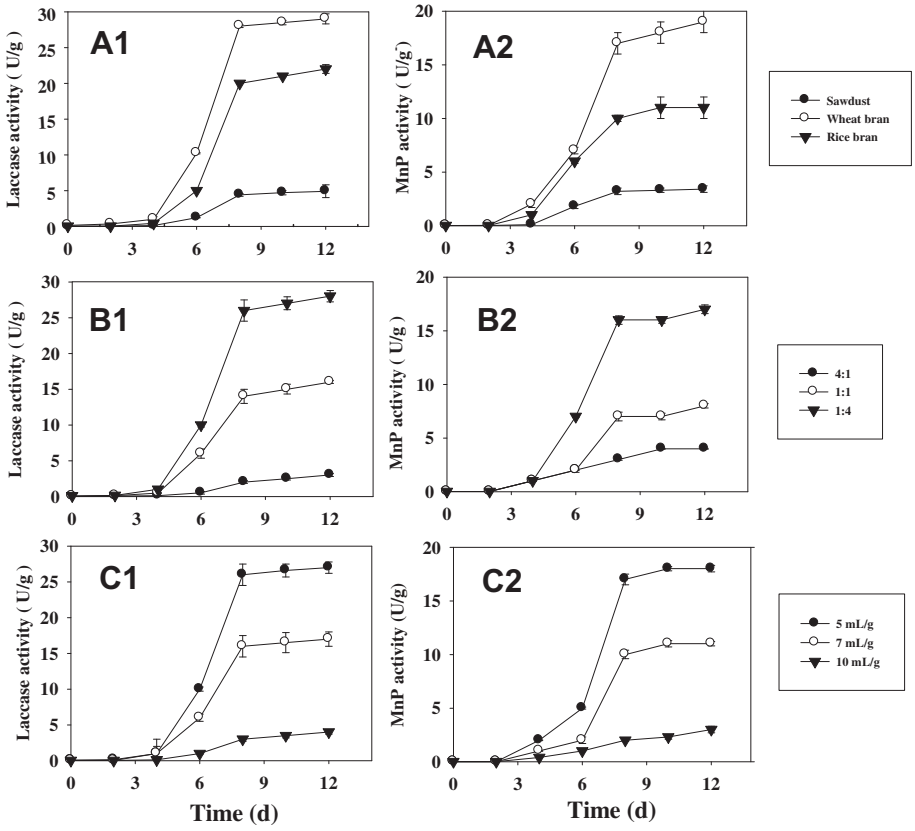

Figure 2. Effects on (1) laccase and (2) manganese peroxidase (MnP) activity of (A) the addition of various substrate components, (B) sawdust/wheat bran ratios, and (C) moisture content by *Pleurotus eryngii* in solid-state fermentation.

temperatures of over 50°C, the activity of the enzyme decreased dramatically.[16] The optimum NP removal by *Trametes versicolor* was also at 30°C.[18]

Copper is a cofactor of laccase. $CuSO_4$ not only induced laccase production, but also increased enzyme activity.[11] Manganese is an essential part of the catalytic cycle of MnP. Mn^{2+} has also been shown to increase MnP activity in the cultures of *Bjerkandera* sp.[12] Organic acids have been shown to influence the activities of degrading enzymes such as laccases and MnP. Chelating Mn^{2+} with α-hydroxyl acids, such as citrate and oxalate, could enhance the dissociation of the MnP—Mn complex and help to complete the catalytic

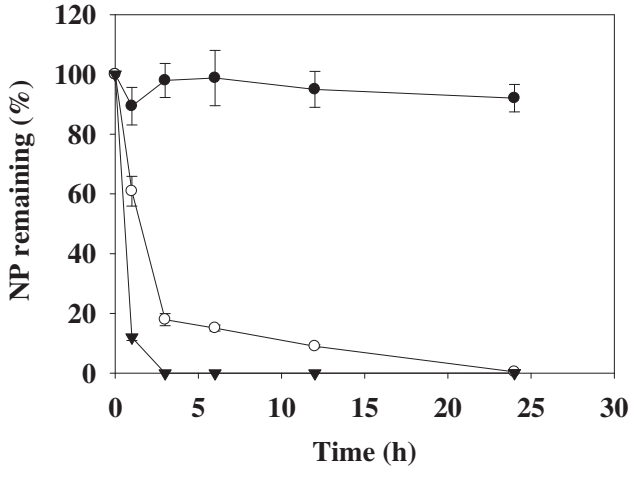

Figure 3. Nonylphenol (NP) degradation by *Pleurotus eryngii* in solid state fermentation. Symbols: ●, sterile control; ○, without 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); ▼, with 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid).

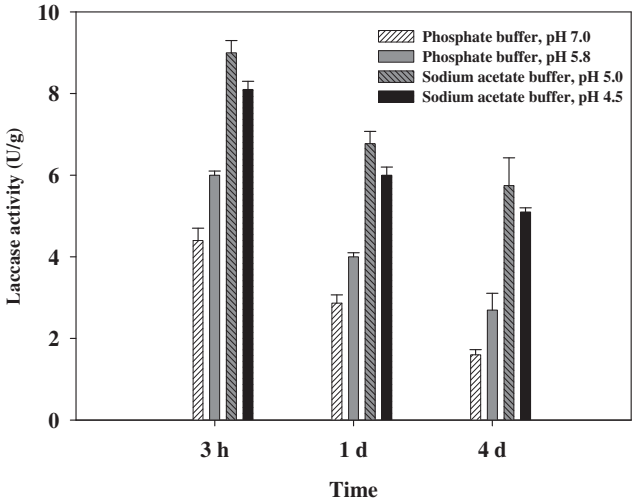

Figure 4. The effects of various extraction times and buffers on laccase activity from spent mushroom compost.

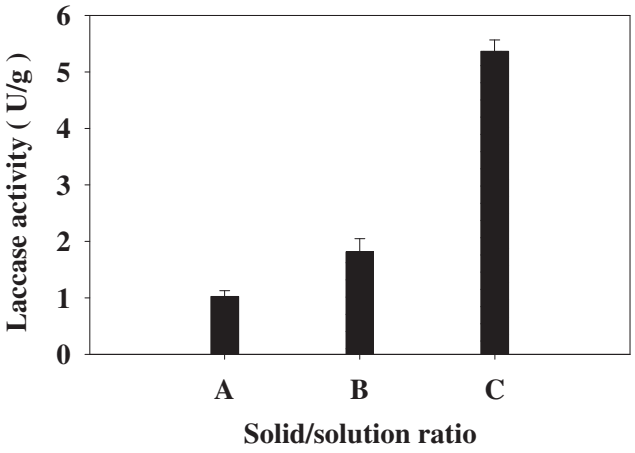

Figure 5. The effects of various solid/solution ratios on laccase activity from spent mushroom compost: (A) 1:25; (B) 1:10; and (C) 1:5.

cycle of the enzyme.[12] Guaiacol, a lignin monomer, closely resembles natural substrates of ligninolytic enzymes. It has been shown to be a strong inducer of laccase and MnP. Adding guaiacol to the culture of *Trametes versicolor* increased laccase activity.[19]

ABTS is a substrate of laccase. It is also a mediator that can enhance the activity of this enzyme. A previous study reported that ABTS increased the oxidation of anthracene by blue laccase from *P. ostreatus*.[13] *Pleurotus* species secrete versatile peroxidase, laccase, and AAO. Versatile peroxidase is a new type of peroxidase that oxidizes phenolic and nonphenolic aromatic compounds, as well as oxidizing Mn^{2+} to Mn^{3+}, and it acts as a diffusible oxidizing agent; it has been reported in *P. eryngii*.[20] Laccase can oxidize phenolic compounds and aromatic amines using molecular oxygen as an electron acceptor.[17] AAO oxidizes aromatic and aliphatic polyunsaturated primary alcohols, and it is involved, together with intracellular dehydrogenases, in extracellular H_2O_2 production through a redox cycle.[21]

Among the several factors that are important for microbial growth and enzyme production using a particular substrate, particle size and moisture level/water activity are the most critical in SSF.[22] Wheat bran is one of the most abundant and cheapest crop residues in many countries and was therefore selected as a potential substrate for laccase production by *P. eryngii* in solid-state conditions. By the selection of the appropriate growth substrate and mode of fungus cultivation, it is possible to increase greatly the secretion of both laccase and MnP.[23] Many species of the genera *Pleurotus* have been described as laccase and MnP producers.[24,25]

BPA, BPF, NP, and TBBPA are organic toxic chemicals commonly present in the environment.[26] The removal rates of chemicals by *P. eryngii* in SmF, SSF, and SMC, in descending order of magnitude, were BPF > BPA > NP > TBBPA. These results are similar to those of Nicolucci et al.[1] who reported that BPF had the highest biodegradation capacity. Comparison with this finding shows that compounds with higher molecular weights and structural complexity are more resistant to biotransformation than those with lower molecular weights.[1,27]

SSF holds tremendous potential for the production of enzymes. It may be of special interest in those processes where the crude fermented product may be used directly as the enzyme source. SSF processes are distinct from SmF, because microbial growth and product formation occur at or near the surface of the solid substrate particle. SSF is favorable for laccase and MnP secretion by the majority of *Pleurotus* strains, whereas SmF provides better production of hydrolytic enzymes.[17] Thus, the degradation rates of toxic chemicals in SSF was higher than those in SmF.

In conclusion, the cultures of *P. eryngii* in SmF, SSF, and SMC tested in this study were all capable of degrading the toxic chemicals NP, BPA, BPF, and TBBPA. The addition of ABTS yielded a higher degradation rate of these chemicals. The removal rates of toxic chemicals with SMC were lower than those with SmF and SSF. However, SMC is a source material for extracting ligninolytic enzymes, and this green technology should be feasible as well as economically profitable. We believe that these findings could provide useful information for improving the efficiency of the removal of organic toxic chemicals in the environment.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Table 3 The removal rates of toxic chemicals in submerged fermentation (SmF), solid-state fermentation (SSF), and spent mushroom compost (SMC) by *Pleurotus eryngii* after 1 day of incubation[a]

Treatment	Removal rates (%)					
	SmF		SSF		SMC	
	A	B	A	B	A	B
BPA	95.9 ± 1.4	98.6 ± 3.4	98.8 ± 2.1	99.8 ± 2.9	94.9 ± 2.7	97.1 ± 3.1
BPF	96.1 ± 3.2	98.9 ± 2.9	99.1 ± 4.1	99.9 ± 3.2	95.9 ± 2.1	97.8 ± 2.8
NP	93.7 ± 2.3	96.9 ± 3.2	98.6 ± 2.8	99.5 ± 3.4	92.1 ± 1.2	95.1 ± 3.2
TBBPA	92.3 ± 2.1	96.1 ± 2.4	98.3 ± 3.1	99.3 ± 2.2	90.1 ± 1.7	94.3 ± 1.1

[a] Concentration of toxic chemicals = 20 mg/L. Each figure represents the mean of three measurements; in all cases, the coefficient of variation (CV) was <10.0%. CV (%) = [standard deviation (%)/mean value (%)] × 100.
A = without ABTS; B = with ABTS; BPA = bisphenol A; BFP = bisphenol F; NP = nonylphenol; TBBPA = tetrabromobisphenol A.

Acknowledgments

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