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Selection of Ligninolytic Basidiomycetes Fungi from a Dry Dipterocarp Forest in Thailand

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ABSTRACT

The white-rot fungus secretes a group of extracellular ligninolytic enzymes such as laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP). These enzymes are non-specific and can break down the complex aromatic polymers of lignin, including the organopullutants and synthetic dyes. The high catalytic efficiency of ligninolytic enzymes has led to their utilization in various biotechnological applications and in various industries. Therefore, the aim of this study was to screen and select ligninolytic fungi obtained from natural sources for further potential utilization in various biotechnology applications. A total of 239 mushrooms were collected from different habitats of a natural forest in Phitsanulok Province, Thailand. All samples were screened for the three main ligninolytic enzymes: laccase (Lac), lignin peroxidase (LiP), and manganese peroxidase (MnP). Fresh fruiting body tissues were used to obtain 118 pure mycelia cultures; 70 were isolated from dead wood and 48 from the soil surface. A dye degradation test and liquid culture cultivation assays were performed to identify strains that produced ligninolytic enzymes with varying catalytic efficiencies in liquid culture medium. The fungal isolates were divided into four groups based on the type of ligninolytic enzymes produced as follows: a) the fungal group with strains secreting only one of the three main enzymes, 40 strains; b) the MnP-Lac group, 19 strains; c) the LiP-Lac group, six strains; and d) the LiP-MnP-Lac group, three strains. Among all the fungal strains isolated, *Hygrocybe* sp.NU137, *Dictyophora* sp.NU134, and *Amanita* sp.NU25 were the highest producers of LiP (240.0 ± 4.4 mU/ml), MnP (120.2 ± 3.4 mU/ml), and Lac (24.6 ± 0.6 mU/ml), respectively.

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INTRODUCTION

Basidiomycetes play a crucial role in wood and litter decomposition, soil humidification, and mineralization of soil organic matter. They also mediate the global carbon cycle, especially in forest ecosystems (Casieri *et al*. 2010). Wood-rotting fungi belong to the Basidiomycetes phylum and are known for the production of a variety of oxidative and hydrolytic extracellular enzymes that degrade lignocellulosic material derived from plant cell walls (Casieri *et al*. 2010; Lundell *et al*. 2010). The plant cell walls are primarily composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose can be broken down by hydrolytic enzymes secreted by wood-rot fungi, bacteria, and actinomycetes in the environment (Kuhad *et al*. 2007; Peláez *et al*. 1995). However, lignin is a heteropolymer of phenylpropanoid units of plant cell walls and is one of the most recalcitrant compounds for biodegradation (Elisashvili and Kachlishvili 2009; Pointing *et al*. 2005). Lignin can be degraded by several species of wood-rotting basidiomycetes, including litter-decomposing fungi, brown-rot fungi, and white-rot fungi (Kalmis *et al*. 2008; Saparrat *et al*. 2008). A white-rot fungus is the only known fungus that is reported to efficiently degrade lignin into carbon dioxide and water (Dhouib *et al*. 2005b). The white-rot fungus secretes a group of extracellular ligninolytic enzymes such as laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP) (Solarska *et al*. 2009). These enzymes are non-specific and can break down the complex aromatic polymers of lignin, including the organopullutants and synthetic dyes that have a comparable structure to that of lignin (Premjet *et al*. 2009; Saparrat *et al*. 2008). The high catalytic efficiency of ligninolytic enzymes has led to their utilization in various

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biotechnological applications and in various industries such as the pulp and paper, food, animal feed, laundry, textile, fuel, and chemical industries (Elisashvili *et al*. 2006; Okino *et al*. 2000). Ligninolytic enzymes are obtained from *Phanerochaetechrysosporium*, *Trametesversicolor*, and several other species of white-rot fungi. However, ligninolytic enzymes obtained from most white-rot fungi are produced in a secondary metabolism phase that requires low nitrogen levels (Dhouib *et al*. 2005b; Novotny *et al*. 2004). Elisashvili and Kachlishvili (2005) have highlighted the need to isolate a novel fungal strain that can efficiently produce ligninolytic enzymes by using primary metabolism to reduce cost and for a more convenient production set-up.

Several Thai researchers have surveyed and discovered a high diversity of mushrooms in Thailand. More than 300 species of Basidiomycetes fungi (mushrooms) have been identified, classified, and recorded (Chandrasrikul *et al*. 2011; Chandrasrikul *et al*. 2008; Ruksawong and Flegel 2001). However, databases documenting the ligninolytic enzyme production characteristics of these fungi have not been developed. Therefore, the aim of this study was to screen and select ligninolytic fungi obtained from natural sources for further potential utilization in various biotechnology applications.

MATERIALS AND METHODS

Collection and isolation of wild mushrooms:

Field experimentation was conducted during two rainy seasons in a dry dipterocarp forest (latitude 16°58′0″N, longitude 101°3′0″E, and at 1614 m mean sea level) located in Amphoe Nakhon Thai, Phitsanulok Province, Thailand. The survey and sample collection were conducted from May to September in both 2011 and 2012. On-site experimentation involved tagging of the fruiting bodies, record keeping, photographing specimens, cleaning, and collecting of samples. All fruiting bodies were individually stored in a paper bag and transported to a cabin where the morphological characteristics of the samples were recorded in detail. In this experiment, the mushroom samples were classified by using a traditional classification method, manual with keys, and with the help of suitable of literature (Alexopoulos *et al*. 1996; Arora 1986; Barnett and Hunter 1998; Bessette *et al*. 1995; Chandrasrikul *et al*. 2011; Chandrasrikul *et al*. 2008; David and Daniel 1977a; David and Daniel 1977b; David *et al*. 2011; Laessoe *et al*. 1996; Miller 1977; Ruksawong and Flegel 2001). Sample measurements, micro-chemical reactions, spore-prints, and microscopic studies were performed for tentative identification of fungi at the genus.

***Fungal Strain Isolation*:**

To obtain a pure culture of wild mushrooms, the external surface of the fruiting body was sterilized with 70% alcohol and the internal tissue was aseptically extracted and immediately cut into small pieces. Two or three cut tissue pieces were inoculated onto potato dextrose agar supplemented with 34 mg/ml chloramphenicol and 0.5% phenol. The inoculated plates were incubated at 25°C and inspected daily for mycelium growth. The active mycelium tips were collected and sub-cultured until a pure mycelium culture was obtained. The pure culture mycelia were transferred onto two slant agar tubes. One slant agar tube was maintained as stock culture. The other tube was used for further experimentation.

***Screening ligninolytic enzyme producers by the dye-decolorizing method*:**

Primary screening for ligninolytic enzyme-producing mushroom strains was performed by using the dye-decolorizing method. Malt extract agar medium supplemented with 0.01% Azure-B was used for determining LiP and MnP activity (Levin *et al*. 2004; Pointing 1999). Lac activity was assessed by using malt extract agar supplemented with 0.04% Remazol Brilliant Blue R (RBBR) (Machado *et al*. 2005). A 0.5-mm disk (Ø) agar plug that was obtained from the growing margins of each test strain colony was inoculated onto the screening medium agar plates and incubated at 25°C for 1–15 days. Screening medium agar plates that were not inoculated were used as negative controls. Three replicate plates were used for assessment of each test culture. The diameters of mycelium colonies and the dye decolorization zones of samples and positive control were compared. *Phanerochaetechrysosporium* NBRC 31249 was purchased from theCulture Collection Division, Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Chiba, Japan and used as a reference strain.

***Ligninolytic Enzyme Production In Liquid Medium*:**

All ligninolytic enzyme-producing fungal isolates identified by using the primary screening method were evaluated for Lac, LiP, and MnP activity. Four disks (Ø 0.5 mm) of each isolate were inoculated into 20 ml of malt extract broth medium in a 150-ml Erlenmeyer flask and incubated at 25°C for 15 days. Triplicate culture flasks were used to assess each test strain. Subsequently, 1 ml of culture medium was centrifuged at 10,000 rpm for 1 min. The supernatant was used to assess enzyme activity by using a method that was previously described by Purnomo *et al*. (2010). Briefly, the veratryl alcohol oxidation method was used to determine LiP activity. The total reaction mixture volume was 1 ml and the mixture contained 50 µl of 20 mMveratryl alcohol,

500 µl of 100 mM sodium succinate buffer (pH 3.0), 250 µl of distilled water, 100 µl of crude enzyme, and 100 µl of 2 mM H_2O_2. The reaction mixture absorbance was determined at the wavelength of 310 nm (ε310 = 9300 $mM^{-1}cm^{-1}$). The MnP activity was determined by using the 2,6dimethoxy phenol (2,6-DMP) oxidation method. The total reaction mixture volume was 1 ml and the mixture contained 50 µl of 20 mM 2,6-DMP, 50 µl of 20 mM $MnSO_4$, 500 µl of 100 mM sodium malonate (pH 4.5), 300 µl of crude enzyme, and 100 µl of 2 mM H_2O_2. The absorbance was measured at the wavelength of 469 nm (ε469 = 9300 $mM^{-1}cm^{-1}$). Lac activity was determined by the 2,6-DMP oxidation method. The total reaction mixture volume was 1 ml and the mixture contained 50 µl of 20 mM 2,6-DMP, 500 µl of 100 mM sodium malonate (pH 4.5), 150 µl of distillated water, and 300 µl of crude enzyme. The absorbance was measured at the wavelength of 469 nm (ε469 = 4960 $mM^{-1}cm^{-1}$). The crude enzyme was heat-inactivated in an autoclave at 121°C for 10 min and used as a control. The unit of enzyme activity was defined as the amount of enzyme that oxidizes 1 mM of substrate in 1 min and was expressed as mU/ml.

RESULTS AND DISCUSSION

***Isolation of Pure Culture*:**

A total of 239 samples of indigenous mushrooms were collected from different habitats in the forest. The samples were processed and 138 fresh fruit bodies were collected from dead wood and 101 fresh fruit bodies were collected from the soil surface. However, the other 119 collected samples were contaminated and could not be used to isolate fungal strains on agar plates. The fresh fruiting body tissues obtained from dead wood and the soil surface were used to isolate 70 and 48 fungal strains, respectively. A total of 118 fungal strains that belonged to 34 families, 65 genera were isolated and 1 strain was unidentified (Tables 1–3). The majority of the fungal isolates belonged to the family Polyporaceae that includes 11 genera, followed by Tricholomataceae with seven genera, Agaricaceae with four genera, and Clavariaceae with four genera. In addition, three strains belonged to each of the Cortinariaceae, Marasmiaceae, and Boletaceae families. Two strains belonged to each of the Auriculariaceae, Cantharellaceae,Coprinaceae, Fomitopsidaceae, and Russulaceaefamilies. Finally, one strain belonged to each of the Bolbitiaceae,Entolomataceae, Exidiaceae, Ganodermataceae, Geastraceae, Helotiaceae, Hydnangiaceae, Hymenochaetaceae, Lycoperdaceae, Nidulariaceae, Paxillaceae, Phallaceae, Pleurotaceae, Pluteaceae, Pyronemataceae, Sarcoscyphaceae, Sclerodermataceae, Serpulaceae, Stereaceae,and Xylariaceaefamilies. Our data revealed that most fungal isolates were found in previously examined forest habitats of Thailand and that the fungal isolates have been previously examined, classified, recorded, and documented by several researchers (Chandrasrikul *et al*. 2011; Chandrasrikul *et al*. 2008; Kasem 1994; Ruksawong and Flegel 2001).

***The Primary Screening of Peroxidase Activity*:**

A total of 118 pure cultures were tested for their dye decolorization ability on agar plates with two screening media on separate plates supplemented with either Azure B or RBBR dye. The fungal isolates that produced ligninolytic enzymes degraded the dye and the dye decolorization was visible on agar plates of both screening media. Enzyme-mediated dye degradation changed the Azure B color from purple to pink and RBBR dye color was altered from dark green to orange. The results revealed that the decolorization zones of most fungal isolates developed under or around the mycelium growth on the 2^{nd} or 3^{rd} day of incubation. The Azure B and RBBR dyes were not degraded by 50 of the fungal isolates (Table 1). The RBBR dye alone was degraded by 31 test strains (Table 2). Nine strains degraded Azure B dye alone (Table 2). Both Azure B and RBBR dyes were degraded by 25 isolates and by *P. chrysosporium* NBRC 31249 (Table 3). The test strains had a broad range of decolorization capabilities and the ratio of the mycelium growth radius and decolorization zone radius of each strain varied considerably (data not shown). Levin *et al*. (2004) demonstrated that fungal strains show variation in decolorization because they differ in their enzyme activity and production. Pointing (1999) indicated that Azure B dye was not degraded by Lac and that the degradation was strongly correlated with the oxidation reactions of LiP and MnP. Other studies showed that the RBBR dye was degraded by Lac (Borokhov and Rothenburger 2000; Kiiskinen *et al*. 2004). The quantitative screening of ligninolytic enzymes can be performed by using dye decolorization as an indicator of enzyme activity, and this assay is a simple and rapid method for selecting ligninolytic enzyme producers (Levin *et al*. 2004; Pointing 1999). Thus, this method is commonly used for screening wood-rot fungi to identify ligninolytic enzyme producers (Dhouib *et al*. 2005a; Levin *et al*. 2004; Tekere *et al*. 2001). All test strains that tested positive on the agar plates were evaluated by using the liquid culture medium to verify ligninolytic enzyme production.

***Evaluation of Ligninolytic Enzyme Activity*:**

To evaluate the efficiency of ligninolytic enzyme producers, all pure cultures that tested positive for ligninolytic enzyme production were grown in liquid culture medium and production of Lac, LiP, and MnP was evaluated. Kuhar *et al.*(2007) have divided the wood-rot fungi into seven groups based on the type and number of ligninolytic enzymes produced by the strains. However, the fungal isolates obtained in this study could be only

divided into 4 groups, as follows: (A) group of fungal isolates that secreted one of the three main oxidative enzymes, (B) the MnP-Lac group of fungal isolates that secreted two (MnP and Lac) of the three main oxidative enzymes and degraded both indicator dyes, (C) the LiP-Lac group of isolates that secreted two (LiP and Lac) of the three enzymes, and (D) the LiP-MnP-Lac group of isolates that secreted all three oxidative enzymes and degraded both indicator dyes. The isolates of each group are described in detail in the following paragraphs.

As mentioned previously, the fungal isolates in group A secreted only one of three main oxidative enzymes (Lac, MnP, and LiP). It has been previously reported that most white-rot fungi commonly secrete only one of the three main ligninolytic enzymes (Kuhar *et al*. 2007). Our data show that Lac was produced by 31 fungal isolates that also degraded RBBR dye. The maximum Lac activity was demonstrated by *Amanita* sp.NU25 (24.6 ± 0.6 mU/ml) and *Coprinus*sp.NU24 (20.4 ± 0.1 mU/ml), whereas the other 10 isolates had Lac activity ranging from 8.3 ± 0.8 mU/ml to 1.1 ± 0.1 mU/ml. The remaining 19 strains showed a lower level of Lac activity, which ranged from 0.9 ± 0.0 mU/ml to 0.1 ± 0.0 mU/ml (Table 2). Several wood-rot fungi, including *Cerrena maxima*, *Coriolopsisgallica*, *Daedaleaflavida*, *Phellinus igniarius*, *Phlebiafascicularia*, *Paralepetopsisfloridensis*, and *Pycnoporuscinnabarinus* were reported to produce Lac but did not secrete LiP or MnP(Kuhar *et al*. 2007). Eggert *et al*. (1997) reported that both LiP and MnP are important for lignin degradation in the natural environment. However, *P. cinnabarinus* did not produce LiP or MnP, but instead rapidly oxidized lignin and degraded lignin as efficiently as *P. chrysosporium*. In addition, it was observed that 3-hydroxyanthranilic acid, an intermediary substrate of non-phenolic lignin oxidation, was produced by *Pycnoporuscinnabarinus* during lignin degradation (Eggert *et al*. 1997). Thus, Lac was produced in considerable amounts for lignin degradation (Kuhar *et al*. 2007).

Only five strains produced MnP alone and degraded Azure B dye (Table 3). The highest MnP activity was produced by *Cyathus*sp.NU254 (66.9 ± 0.4 mU/ml), followed by *Oligoporus* sp.NU255 (20.8 ± 0.5 mU/ml) and *Lactarius*sp.NU23 (20.0 ± 0.2 mU/ml). A lower level of MnP activity was produced by *Trametes* sp.NU264 (2.1 ± 0.0 mU/ml) and *Entoloma* sp.NU10 (0.8 ± 0.0 mU/ml), as shown in Table 2. Manganese peroxide is one of the most commonly produced enzymes by lignin-degrading fungi such as *Perenniporia medulla-panis*, *Phanerochaetesordida*, *Phlebiasubserialis*, *Polyporusversicolor*, and *Trametesvillosa*(Kuhar *et al*. 2007). Several studies have indicated that lignin, synthetic lignin compounds, and chloroginins have diverse chemical structures can be oxidized by both crude and purified forms of MnP(Hofrichter 2002; Kuhar *et al*. 2007; Levin *et al*. 2004; Wariishi *et al*. 1991).

Lastly, only LiP production and Azure B dye degradation was observed in the four fungal isolates. The greatest yield of LiP activity was produced by *Xeromphalina*sp.NU108 (85.2 ± 3.6 mU/ml), followed by *Coprinus*sp.NU244 (68.2 ± 0.4 mU/ml), *Microporus* sp.NU65 (58.2 ± 0.2 mU/ml), and *Crateerellus*sp.NU51 (19.8 ± 0.7 mU/ml) (Table 2). Several wood-rot fungi, including *Coriolopsisoccidentalis*, *Fomesfomentarius*, *Fomeslignosus*, *Ganodermaapplanatum*, *Ganodermaaustrale*, *Lentinusdegener*, *Peniophoragigantea*, *Polyporusosteiformis*, and *Polysporusvarius* were reported to produce only LiP(Kuhar *et al*. 2007). However, some white-rot fungi and litter-decaying fungi, including *Ceriporiopsissubvermispora*, *Dichomitussqualens*, *Panustigrinus*, *Rigidoporuslignosus*, and *Armillaria*spp. rapidly oxidized lignin but did not secrete LiP. Thus, LiP may not be crucial for lignin degradation (Kuhar *et al*. 2007).

The group B, or MnP-Lac group, included fungal strains that degraded both RBBP and Azure B dyes and produced only two (MnP and Lac) of the three ligninolytic enzymes. Interestingly, in this study, we isolated fungi that produced only two ligninolytic enzymes: MnP and Lac (MnP-Lac group) or LiP and Lac (LiP-Lac group). However, fungal strains that produced LiP and MnP (LiP-MnP group) were not isolated. Fungal strains of the MnP-Lac group were obtained from 19 isolates with notably higher production of MnP than Lac. Nine isolates produced MnP with enzyme activity that ranged from 0.1 ± 0.0 mU/ml to 2.6 ± 0.8 mU/ml. The other seven group B fungal strains had MnP activity ranging from 12.4 ± 0.1 mU/ml to 51.2 ± 0.5 mU/ml. The maximum MnP activity was shown by *Dictyophora*sp.NU134 (120.2 ± 3.4 mU/ml), followed by *Scytinopogon* sp.NU156 (100.8 ± 1.7 mU/ml), and *Geastrum*sp.NU249 (82.3 ± 0.1 mU/ml). The maximum Lac activity was shown by *Grifola*sp.NU247 (17.8 ± 0.1 mU/ml) and *Geastrum*sp.NU249 (17.1 ± 0.0 mU/ml). Lower levels of Lac activity (6.9 ± 0.2 - 0.2 ± 0.0 mU/ml) were detected in the other 17 isolates (Table 3). The MnP-Lac group of several wood-rot fungi, including *Ceriporiopsissubvermispora*, *Dichomitussqualens*, *Lentinula edodes*, *Coriolopsispolyzona*, *Coriolopsisrigida*, and *Panustigrinus*, efficiently degraded lignin. Most of these fungi were obtained from hard wood (Kuhar *et al*. 2007; Tuor *et al*. 1995).

The group C, or LiP-Lac group, included five fungal isolates that belonged to the LiP-Lac group that produced higher levels of LiP than Lac. The highest LiP activity was observed in *Hygrocybe*sp.NU137 (240.0 ± 4.4 mU/ml), followed by *Megacollybia*sp.NU16 (188.7 ± 9.9 mU/ml) and *Lentinus*sp.NU242 (153.3 ± 3.7 mU/ml). A lower

level of LiP activity was detected in *Ganoderma* sp.NU47 (40.3 ± 1.3 mU/ml), *Psilocybe*sp.NU201 (36.2 ± 1.1 mU/ml), and *Leucocoprinus*sp.NU260 (17.9 ± 0.7 mU/ml). The reference strain*P. chrysosporium* NBRC 31249 was used as a control and produced lower levels of Lac (0.2 ± 0.0 mU/ml) and MnP activity (0.1 ± 0.0 mU/ml) than the majority of the test strains (Table 3).Among the fungal isolates, the Lac with highest enzyme activity (18.2 ± 0.1 mU/ml) was produced by*Megacollybia*sp.NU16, whereas Lac with lower activity levels (0.2 ± 0.0 - 6.8 ± 0.7 mU/ml) were produced by the four other fungal strains (Table 3). However, the LiP-Lac group of fungal strains, including *Junghuhniaseparabilima*, *Oudemansiellaradicata*, *Phlebiaochraceofulva*, *Pleurotusflorida*, *Polyporusbrumalis*, *Polyporuspinsitus*, and *Polyporusplatensis*, degraded lignin less efficiently than the strains from the LiP-MnP and MnP-Lac groups (Kuhar *et al*. 2007). Notably, the fungi from the LiP-MnP and MnP-Lac groups are commonly found on hard wood in the natural environment (Tuor *et al*. 1995).

The isolates from fungi from group D degraded both RBBP and Azure B dyes and produced all three types of ligninolytic enzymes. Only three fungal isolates secreted all three enzymes. *Clavicorona*sp.NU227 produced the highest LiP levels (153.0 ± 2.0 mU/ml) and MnP levels (87.2 ± 0.1 mU/ml), followed by *Microporus*sp.NU104 (LiP, 151.4 ± 3.4; MnP, 30.0 ± 0.1mU/ml). By contrast, *Lenzites* sp.NU251 produced lower levels of LiP (21.0 ± 0.5 mU/ml) and MnP (2.0 ± 0 mU/ml). The highest Lac-producing strain in group D was *Microporus*sp.NU104 (21.4 ± 0.1 mU/ml), followed by*Lenzites*sp.NU251 (2.8 ± 0.0 mU/ml) and the lowest Lac-producing strains was *Calvicorona*sp.NU227 (0.4 ± 0.0 mU/ml). The MnP-Lac-LiP group of several white-rot fungi, including *Cyathusbulleri*, *Ganodermalucidum*, *Irpexlacteus*, *Naematolomafrowardii*, *P. chrysosporium*, *Phlebiabrevispora*, *Phlebiaradiata*, *Pleurotusostreatus*, *Pleurotussajor-caju*, *Pycnoporussanguineus*, *Trametesgibbosa*, *Trametes hirsute*, *Trametestrogii*, and *T. versicolor*, rapidly degraded all wood fractions and completely oxidized lignin to carbon dioxide and water (Kuhar *et al*. 2007; Ward *et al*. 2004). The fungi in this group are commonly found on deciduous trees, with the exception of *P. radiate*, which grows on coniferous substrates (Isroi *et al*. 2011; Tuor *et al*. 1995). However, *P. chrysosporium* differed from the other fungal strains because it produced both LiP and MnP under the experimental conditions used in this study, whereas previous reports indicate that Lac is produced by these strains under different culture conditions (Isroi *et al*. 2011; Kuhar *et al*. 2007; Tuor *et al*. 1995). Our data showed that the *P. chrysosporium* NBRC 31249 control strain showed Lac (0.2 ± 0.0 mU/ml) and MnP activity (0.1 ± 0.0 mU/ml) that was at a lower level than observed with most test strains, as shown in Table 3.

Overall, the data revealed the diversity in ligninolytic enzyme production of fungal isolates obtained from thedry dipterocarp forest. Our results corroborate previously published reports of variability in the number of ligninolytic enzymes produced by the fungal isolates (Dhouib *et al*. 2005b; Dritsa *et al*. 2007; Levin *et al*. 2004; Okino *et al*. 2000; Tekere *et al*. 2001). In addition, most fungal isolates obtained in this study secreted Lac, which has been previously reported to be produced by most wood-rot fungi (Casieri *et al*. 2010; Kuhar *et al*. 2007). Thus, some strains of wood-rot fungi produced only one or two of the three enzymes and some other strains produced all three lignin-degrading enzymes. These data indicate that some strains of wood rot fungi that mediate delignification in nature do not use all three lignin-degrading enzymes (Arora and Gill 2001; Dhouib *et al*. 2005b; Kalmis *et al*. 2008; Levin *et al*. 2004; Risna and Suhirman 2002). Various wood-rot fungi have distinct ligninolytic systems (Isikhuemhen and Nerud 1999). A recent study by Xu *et al*. (2013) showed that mushroom mycelium growth was directly correlated with the amount of excreted LiP and MnP; thus, the extended cultivation period increased LiP and/or MnP production. In addition, a similar correlation was reported for Lac production by edible mushrooms with sporophores and mycelia (Chen *et al*. 2004; Chen *et al*. 2003; Ohga and Royse 2001; Xiao *et al*. 2013). The requirement for varied substrates for growth and enzyme production may be indicative of the substrates available in the natural environment (Christine and Hedger 2001). Previous studies have shown that the enzyme catalytic efficiency depends on the fungal strain used and is influenced by the experimental conditions used. For example, the enzyme catalytic efficiency can be influenced by the substrate concentration, carbon source, nitrogen level, aeration, temperature, and pH level used (Elisashvili and Kachlishvili 2009; Elisashvili *et al*. 2006; Giardina *et al*. 2000; Saparrat and Guillén 2005; Stajić *et al*. 2006). Therefore, further studies are required to determine the optimum conditions for obtaining maximum enzyme production from the six candidate fungal isolates that produced the highest enzyme activity in each group.

Table 1: Fungal isolates with no ligninolytic activity on plates test

Family	Scientific name	Family	Scientific name
Agaricaceae	*Agaricus*sp.NU13	Sclerodermataceae	*Scleroderma* sp.NU152
	*Agaricus*sp.NU20	Helotiaceae	*Chlorociboria*sp.NU210
	Macrolepiota sp.NU153	Hymenochaetaceae	*Phellinus* sp.NU212
	Macrolepiota sp.NU119	Pyronemataceae	*Scutellinia*sp.NU241

Bolbitiaceae	*Bolbitius*sp. NU132	Geastraceae	*Geastrum*sp.NU129
Clavariaceae	*Clavulinopsis*sp.NU234		*Geastrum*sp.NU130
	*Deflexula*lsp.NU117	Fomitopsidaceae	*Fomitopsis*sp.NU224
Coprinaceae	*Psathyrella*sp.NU205		*Fomitopsis*sp.NU125
	*Psathyrella*sp.NU214		*Fomitopsis*sp.NU59
Cortinariaceae	*Crepidotus*sp.NU26		*Ischnoderma*sp.NU257
	*Gymnopilus*sp.NU28	Ganodermataceae	*Ganoderma*sp.NU8
Entolomataceae	*Entoloma*sp.NU207	Polyporaceae	*Daedaleopsis*sp.NU229
Hydnangiaceae	*Laccaria*sp.NU197		*Polyporus* sp.NU258
Marasmiaceae	*Chaetocalathus*sp.NU219		*Polyporus*sp.NU226
	*Marasmius*sp. NU203	Polyporaceae	*Trametes*sp.NU158
	*Marrasmius*sp.NU64		*Trametes*sp.NU182
Pleurotaceae	*Hohenbueheli*asp.NU61		*Trametes*sp.NU15
Pluteaceae	*Amanita* sp.NU14		*Trametes*sp.NU9
Tricholomataceae	*Arrhenia*sp.NU231		*Trichaptum*sp. NU211
	*Collybia*sp.NU35	Russulaceae	*Lactarius*sp.NU253
	*Termitomyces*sp.NU4		*Russula*sp.NU250
	*Termitomyces*sp.NU27		*Russula*sp.NU60
Auriculariaceae	*Auricularia*sp.NU173		*Russula*sp.NU41
Boletaceae	*Boletus* sp.NU11	Stereaceae	*Stereum*sp.NU237
	*Strobilomyces*sp. NU195	Exidiaceae	*Pseudohydnum*sp.NU106

NU was isolated code.

Table 2: Fungal isolates that produced one of the three ligninolytic enzymes

Family	Scientific name	Plate Test		Enzyme activity (mU/ml)		
		A[1]	R[2]	MnP[3]	Lac[4]	LiP[5]
Pluteaceae	*Amanita* sp.NU25	-	+	-	24.6 ± 0.6	-
Coprinaceae	*Coprinus*sp.NU24	-	+	-	20.4 ± 0.1	-
Coprinaceae	*Psathyrella*sp.NU5	-	+	-	8.3 ± 0.8	-
Polyporaceae	*Trichaptum*sp.NU233	-	+	-	8.4 ± 0.0	-
Agaricaceae	*Agaricus*sp.NU1	-	+	-	7.8 ± 0.2	-
Agaricaceae	*Leucocoprinus*sp.NU18	-	+	-	7.1 ± 0.2	-
Polyporaceae	*Cryptoporus*sp.NU261	-	+	-	7.0 ± 0.1	-
Lycoperdaceae	*Calvatia*sp.NU76	-	+	-	3.6 ± 0.8	-
Stereaceae	*Stereum*sp.NU6	-	+	-	3.6 ± 0.0	-
Tricholomataceae	*Filoboletus*sp.NU50	-	+	-	3.4 ± 0.1	-
Entolomataceae	*Entoloma*sp.NU3	-	+	-	2.5 ± 0.1	-
Clavariaceae	*Ramaria*sp.NU56	-	+	-	1.1 ± 0.1	-
Cortinariaceae	*Inocybe*sp.NU204	-	+	-	0.9 ± 0.0	-
Cortinariaceae	*Crepidotus*sp. NU186	-	+	-	0.5 ± 0.0	-
Clavariaceae	*Scytinopogon*sp.NU57	-	+	-	0.3 ± 0.0	-
Marasmiaceae	*Marasmiellus*sp.NU193	-	+	-	0.3 ± 0.0	-
Polyporaceae	*Pycnoporus*sp.NU220	-	+	-	0.3 ± 0.0	-
Stereaceae	*Stereum*sp.NU111	-	+	-	0.3 ± 0.0	-
Xylariaceae	*Daldinia*sp.NU116	-	+	-	0.3 ± 0.0	-
Agaricaceae	*Agaricus*sp.NU171	-	+	-	0.2 ± 0.0	-
Pluteaceae	*Amanita* sp.NU48	-	+	-	0.1 ± 0.1	-
Pluteaceae	*Amanita* sp.NU19	-	+	-	0.2 ± 0.0	-
Russulaceae	*Russula*sp.NU222	-	+	-	0.2 ± 0.0	-
Sarcoscyphaceae	*Cookeina*sp.NU190	-	+	-	0.2 ± 0.0	-
Ganodermataceae	*Ganoderma*sp.NU2	-	+	-	0.2 ± 0.0	-
Polyporaceae	*Microporus*sp.NU31	-	+	-	0.2 ± 0.0	-
Boletaceae	Unidentified NU239	-	+	-	0.1 ± 0.0	-
Tricholomataceae	*Arrhenia*sp. NU231	-	+	-	0.1 ± 0.0	-
Cantharellaceae	*Cantharellus*sp.NU262	-	+	-	0.1 ± 0.0	-
Fomitopsidaceae	*Fomitopsis*sp.NU44	-	+	-	0.1 ± 0.0	-
Russulaceae	*Lactarius*sp.NU252	-	+	-	0.1 ± 0.0	-
Nidulariaceae	*Cyathus*sp.NU254	+	-	66.9 ± 0.4	-	-
Polyporaceae	*Oligoporus*sp.NU255	+	-	20.8 ± 0.5	-	-
Russulaceae	*Lactarius*sp.NU23	+	-	20.0 ± 0.2	-	-
Polyporaceae	*Trametes*sp.NU264	+	-	2.1 ± 0.0	-	-
Entolomataceae	*Entoloma*sp.NU10	+	-	0.8 ± 0.0	-	-
Tricholomataceae	*Xeromphalina*sp.NU108	+	-	-	-	85.2 ± 3.6
Coprinaceae	*Coprinus*sp.NU244	+	-	-	-	68.2 ± 0.4
Polyporaceae	*Microporus*sp.NU65	+	-	-	-	58.2 ± 0.2
Cantharellaceae	*Craterellus*sp.NU51	+	-	-	-	19.8 ± 0.7

A[1]= Azure B dye, R[2]=RBBR dye, MnP[3]= manganese peroxidase, Lac[4]= laccase, LiP[5]= lignin peroxidase. NU was isolated code.
Note: - and + symbol was invisible and visible decolorization, respectively. Values present in the mean, n =3, ± SD.

Table 3: Fungal isolates that produced (manganese peroxidase and laccase), (lignin peroxidase and laccase) and (lignin peroxidase, manganese peroxidase, and laccase)

Family	Scientific name	Plate test		Enzyme activity (mU/ml)		
		A[1]	R[2]	MnP[3]	Lac[4]	LiP[5]
Phallaceae	*Dictyophora*sp.NU134	+	+	120.2 ± 3.4	0.8 ± 0.0	-
Clavariaceae	*Scytinopogon*sp.NU156	+	+	100.8 ± 1.7	4.9 ± 0.1	-
Geastraceae	*Geastrum*sp.NU249	+	+	82.3 ± 0.1	17.1 ± 0.0	-
Polyporaceae	*Polyporus*sp.NU2	+	+	51.2 ± 0.5	0.4 ± 0.0	-
Marasmiaceae	*Marasmius*sp.NU63	+	+	50.4 ± 0.6	0.8 ± 0.0	-
Meripilaceae	*Grifola*sp.NU247	+	+	25.0 ± 1.5	17.8 ± 0.1	-
Sclerodermataceae	*Scleroderma* sp.NU248	+	+	19.6 ± 0.2	1.9 ± 0.1	-
Russulaceae	*Lactarius*sp.NU223	+	+	19.0 ± 0.1	1.1 ± 0.0	-
Marasmiaceae	*Marasmius*sp.NU215	+	+	13.9 ± 0.9	6.9 ± 0.2	-
Nidulariaceae	*Cyathus* sp.NU256	+	+	12.4 ± 0.1	0.6 ± 0.1	-
Polyporaceae	*Fomes*sp.NU213	+	+	2.6 ± 0.8	0.5 ± 0.1	-
Polyporaceae	*Daedaleopsis*sp.NU42	+	+	1.1 ± 0.0	0.3 ± 0.0	-
Ganodermataceae	*Ganoderma*sp.NU265	+	+	0.4 ± 0.0	0.3 ± 0.0	-
Tricholomataceae	*Hygrocybe*sp.NU192	+	+	0.4 ± 0.0	0.1 ± 0.0	-
Paxillaceae	*Gyrodon*sp.NU245	+	+	0.4 ± 0.0	0.1 ± 0.0	-
Agaricaceae	*Lepiota*sp.NU246	+	+	0.2 ± 0.0	0.2 ± 0.1	-
Serpulaceae	*Serpula*sp.NU266	+	+	0.2 ± 0.0	0.1 ± 0.0	-
Polyporaceae	*Lentinus*sp.NU259	+	+	0.2 ± 0.0	0.1 ± 0.0	-
Polyporaceae	*Polyporus*sp.NU263	+	+	0.1 ± 0.0	0.2 ± 0.0	-
Tricholomataceae	*Hygrocybe*sp.NU137	+	+	-	0.2 ± 0.0	240.0 ± 4.4
Tricholomataceae	*Megacollybia*sp.NU16	+	+	-	18.2 ± 0.1	188.7 ± 9.9
Polyporaceae	*Lentinus*sp.NU242	+	+	-	0.6 ± 0.0	153.3 ± 3.7
Ganodermataceae	*Ganoderma*sp.NU47	+	+	-	2.8 ± 0.1	40.3 ± 1.3
Strophariaceae	*Psilocybe*sp.NU201	+	+	-	0.3 ± 0.0	36.2 ± 1.1
Agaricaceae	*Leucocoprinus*sp.NU260	+	+	-	6.8 ± 0.7	17.9 ± 0.7
Auriscalpiaceae	*Clavicorona*sp.NU227	+	+	87.2 ± 0.1	0.4 ± 0.0	153.0 ± 2.0
Polyporaceae	*Microporus*sp.NU104	+	+	30.0 ± 0.1	21.4 ± 0.1	151.4 ± 3.4
Polyporaceae	*Lenzites*sp.NU251	+	+	2.0 ± 0.1	2.8 ± 0.0	21.0 ± 0.5
P. chrysosporium NBRC 31249		+	+	0.1 ± 0.0	0.2 ± 0.0	-

A[1]= Azure B dye, R[2]=RBBR dye, MnP[3]= manganese peroxidase, Lac[4]= laccase, LiP[5]= lignin peroxidase. NU was isolated code.
Note: - and + symbol was invisible and visible decolorization, respectively. Values present in the mean, n =3, ± SD.

Conclusion:

Overall, a total of 120 Basidiomycetes fungi that belong to 34 families, 65 genera, and one unidentified strain were isolated. Only 68 fungal isolates could degrade either RBBR or Azure B dye in agar plates. Our data showed that 31 fungal isolates produced Lac and degraded only RBBR. Azure B was degraded by four isolates that produced LiP and by five isolates that produced MnP. The fungal isolates in group A secreted only one of the three main oxidative enzymes. The highest Lac, LiP, and MnP activity was shown by *Amanita* sp.NU25(24.6 ± 0.6 mU/ml), *Xeromphalina*sp.NU108 (85.2 ± 3.6 mU/ml), and *Cyathus*sp.NU254 (66.9 ± 0.4 mU/ml), respectively. In addition, only two of the three enzymes were produced by the 25 isolates that degraded both Azure B and RBBR dyes. Of the isolated strains, 19 strains belonged to the MnP-Lac group and 6 strains belonged to the LiP-Lac group. The maximum MnP and LiP activity was shown by *Dictyophora*sp.NU134 (120.2 ± 3.4 mU/ml) and *Hygrocybe*sp.NU137 (240.0 ± 4.4 mU/ml), respectively. Finally, three fungal strains belonged to the LiP-MnP-Lac group, and these strains secreted all three main classes of ligninolytic enzymes. The highest LiP and MnP production was obtained from *Clavicorona*sp.NU227 (153.0 ± 2.0 and 87.2 ± 0.1 mU/ml, respectively), followed by *Microporus*sp.NU104 (151.4 ± 3.4 and 30.0 ± 0.1mU/ml, respectively). Notably, the 6 isolated strains with high enzyme production were novel fungal strains that have not been previously reported.

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