Enzymatic, Antioxidant, Antimicrobial, and Insecticidal Activities of *Pleurotus pulmonarius* and *Pycnoporus cinnabarinus* Grown Separately in an Airlift Reactor

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Crude extract samples of *Pleurotus pulmonarius* and *Pycnoporus cinnabarinus* were taken during growth in liquid broth in an airlift reactor. Growth was monitored indirectly by sugar consumption and pH profile. During growth *Pleurotus pulmonarius* consumed glucose more slowly than *Pycnoporus cinnabarinus*, reaching a final pH of 8.0. In contrast, *Pycnoporus cinnabarinus* started consuming glucose faster from the beginning to the end with a pH of 3.6, suggesting the production of different metabolites while they grow in the same culture broth. Additionally, antioxidant activity, polyphenol and flavonoid contents, as well as laccase and hydrolase activities were quantified in the culture extracts during the fermentation. *Pleurotus pulmonarius* showed higher antioxidant activity than *Pycnoporus cinnabarinus.* Both fungi have a very low polyphenol and flavonoid content. Values of amylase and pectinase activities were similar in crude extracts of both fungi; however, cellulase, xylanase, invertase, and laccase activities showed higher levels in crude extract of *Pleurotus pulmonarius*. Antimicrobial and insecticidal activities were also evaluated in each crude extract. In fact, *Pycnoporus cinnabarinus* presented a very strong bacteriostatic and bactericidal effect against *Escherichia coli* and *Staphylococcus aureus* and reliably killed *Diatraea magnifactella* larvae, while *Pleurotus pulmonarius* did not showed any negative effect on the growth of these bacteria or larvae.

Keywords: Antimicrobial activity; Antioxidant activity; Enzymes; Insecticidal activity; Pleurotus pulmonarius; Pycnoporus cinnabarinus

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INTRODUCTION

Fungi produce bioactive compounds that directly or indirectly show health benefits. There have been many efforts to extract, identify, and evaluate the effects of fungal bioactive compounds either *in vivo* or *in vitro*. The search for new fungi strains that produce antimicrobial and insecticidal compounds, as well as the conditions to increase their production, is very important. Moreover, there is an urgent need to develop low-cost, safe control alternatives that are environmentally friendly.

Antioxidant, antitumor, antiviral, antihypercholesterolemic, antihyperlipidemic, and antihyperglycemic activities, as well as antiallergic effects have been observed in fungi

(Díaz-Godínez 2015). *Pleurotus* and *Pycnoporus* are classified as white-rot fungi; these species grow on many lignocellulosic materials such as rotten wood, wood waste, and agricultural waste, as they efficiently degrade lignins besides cellulose and hemicellulose, due to their secretion of enzymes including hydrolases and phenoloxidases such as laccases. Proposed functions for laccases during mushroom development include cross-linking of cell walls of hyphae for structure stabilization, tissue and spore pigment synthesis, and gill browning (Kües and Rühl 2011).

There are also bioactive compounds in these fungi with antimicrobial activity; among them α and β linkages of glucan compounds, phenols (p-benzoic acid, p-phenyl acetic acid, o-coumaric acid, ferulic acid, and chrysin), polyphenols, alkaloids, flavonoids, citric acid, cinnabarinc acid, pigments, and enzymes (Eggert 1997, Rames and Pattar 2010, Adebayo *et al.* 2012).

Jeena *et al*. (2014) investigated the antioxidant activity and the total polyphenols in methanol extracts of the mycelium and fruiting bodies of *Pleurotus sajor-caju*, *Pleurotus ostreatus*, and *Pleurotus sapidus*. Results suggested that fruiting bodies of *Pleurotus sajor-caju* contains the highest phenol content (1.53 mg/g) and flavonoids (1.88 mg/g). Chirinang and Intarapichet (2009) pointed out that *Pleurotus ostreatus* possessed more antioxidant than *Pleurotus sajor-caju*. The EC50 of *Pleurotus ostreatus* and *Pleurotus sajor-caju* water extracts were 11.56 and 13.38 mg/mL, respectively, while those of the ethanol extracts were 31.75 and 58.44 mg/mL, respectively. On the other hand, poliporin isolated from *Pycnoporus sanguineus* shows antimicrobial activity against Gram-positive and Gram-negative bacteria and without toxicity in experimental animals (Bose 1946).

Smânia *et al*. (1995) showed that *Pycnoporus sanguineus* produces cinnabarine, an orange pigment active against *Bacillus cereus*, *Staphylococcus aureus*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, and several *Streptococcus* spp. *Pleurotus ostreatus* and *Meripilus giganteus* show broad-spectrum antimicrobial activity, with a maximum effect shown by the ethanolic extracts of *Pleurotus ostreatus* against *Sarcina lutea* (Kalyoncu *et al*. 2010). The ether extract of *Pleurotus sajor-caju* has shown high antibacterial activity against *Staphylococcus aureus*, whereas *Staphylococcus epidermidis* is sensitive to the ethanol extract (Tambekar *et al*. 2006). Aqueous extracts from the fruiting bodies of *Pleurotus ostreatus* inhibit the growth of *Candida albicans*, *Cryptococcus humicola*, *Trichosporon cutaneum*, *Staphylococcus aureus*, and *Escherichia coli* (Younis *et al*. 2015).

The activity and number of isoenzymes of fungal laccases are influenced by environmental factors such as temperature, pH, inductors, culture conditions, and medium composition (Téllez-Téllez *et al*. 2008; Piscitelli *et al*. 2011; Pezzella *et al*. 2012). Laccase isoenzyme expression can be constitutive or inducible (Palmieri *et al*. 2000; Téllez-Téllez *et al*. 2012). There are many reports on the production, characterization, and uses of phenoloxidases in white-rot fungal species (Díaz-Godínez 2012).

However, there is very little information on the production of insecticidal activity and hydrolytic enzymes in *Pleurotus* and *Pycnoporus* species, and these enzymes have applications in various sectors. On the other hand, antibacterial and antioxidant activities from natural sources are currently studied. In this study, the enzymatic, antioxidant, antimicrobial and insecticidal activities were evaluated in the culture medium of *Pleurotus pulmonarius* and *Pycnoporus cinnabarinus* grown in an Airlift reactor. In this reactor type, the aeration conditions ensure enough oxygen and agitation to encourage the fungal growth allowing determine basal levels of production of the biological activities mentioned.

EXPERIMENTAL

Materials and Methods

Microorganisms

Pleurotus pulmonarius (HEMIM-129) and *Pycnoporus cinnabarinus* (HEMIM-79) were acquired from the Center for Biological Research at the Autonomous University of Morelos State (UAEM), Cuernavaca, Morelos, México. The strains were kept on potato dextrose agar under refrigeration (4 °C) until their use.

Escherichia coli (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) bacteria were used for antimicrobial assays. They were purchased directly from the American Type Culture Collection (ATCC) through The Global Bioresource Center.

Diatraea magnifactella larvae colonies for insecticidal assays were obtained from the Biotechnology Research Center of UAEM.

Culture conditions

Each fungus was grown in submerged fermentation (SmF) using a 5.5 L AirLift bioreactor at 75% of its capacity. The culture medium was prepared at pH 6.5 and contained the following (in g/L): glucose, 10; yeast extract, 5; KH_2PO_4, 0.6; $MgSO_4$-$7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4$-$5H_2O$, 0.25; $FeSO_4$-$7H_2O$, 0.05; $MnSO_4$, 0.05; and $ZnSO_4$-$7H_2O$, 0.001 (Téllez-Téllez *et al*. 2008). The reactor temperature was 25 °C with an air flow of 1 vvm (vessel volume per minute). Culture broth samples were taken at 72 h after inoculation and then every 24 h. In each crude extract (CE) sample, the activity of five hydrolases and laccases as well as antioxidant, antimicrobial, and insecticidal activities were evaluated. The pH and residual glucose of each CE were measured by potentiometry and refractometry, respectively. The submerged fermentation and all analysis were carried out in triplicates.

Enzymatic assays

Five hydrolase activities in CEs were assayed by quantifying reducing sugars using the DNS (3,5-dinitrosalicylic acid) method (Miller 1959). The assay mixture contained 475 µL of substrate (0.5% polygalacturonic acid, 1% sucrose in 0.1 M phosphate buffer at pH 6.5, 0.5% starch in 0.1 M phosphate buffer at pH 6.5, 0.5% birch xylan in 0.1 M acetate buffer at pH 5.3, and 1.0% carboxymethylcellulose in 0.1 M acetate buffer at pH 5.0, for amylase, pectinase, cellulase, xylanase, and invertase, activities, respectively) and 25 µL of CE. The reaction temperatures were 45, 35, 35, 50, and 50 °C, respectively. One unit of hydrolase activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per minute under assay conditions. Laccase activity was determined in each CE by changes in the absorbance at 468 nm, using 2,6-dimethoxyphenol (DMP) as the substrate. The assay mixture contained 950 µL substrate (2 mM DMP in phosphate buffer pH 6.5) and 50 µL CE, which was incubated at 40 °C for 1 min (Téllez-Téllez *et al*. 2005). One enzymatic unit (U) of laccase activity was defined as the amount of enzyme that gives an increase of 1 unit of absorbance per min in the reaction mixture. The activity was expressed in U/L of CE.

Total phenolic content assay

The total phenolic content was measured according to the method of Singleton *et al*. (1999). Slowly, 0.5 mL of sample was added to 4.5 mL of distilled water and then mixed with 0.2 mL of Folin-Ciocalteu phenol reagent and 0.5 mL saturated Na_2CO_3 solution.

Finally, 4.3 mL of distilled water were added to the solution. The reaction mixtures were incubated for 60 min in darkness at room temperature, and the absorbance values were measured at 725 nm. Total phenolic content was expressed as mg of gallic acid equivalents (GAE) per mL of culture medium (mg GAE/mL).

Flavonoid content

Flavonoid content was determined by a colorimetric method with aluminum chloride, as previously described (Chang *et al*. 2002). A total of 0.5 mL of sample was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate, and 2.8 mL of distilled water. This mixture was incubated at room temperature for 30 min in darkness, and absorbance at 415 nm was read. Flavonoid content was calculated in mg quercetin equivalents per g per mL of culture medium (mg QE/mL).

Scavenging activity of DPPH radical

DPPH radical scavenging activity was determined according to Moraes-de-Souza *et al*. (2008) with some modifications. The reaction mixture consisted of 0.5 mL of extract, 3 mL of methanol, and 0.3 mL of 0.5 mM DPPH radical solution in methanol. After incubation for 45 min, absorbance was determined in spectrophotometer at 517 nm. The antioxidant activity (% Inhibition) was calculated by Eq. 1,

$$\% \text{ Inhibition} = [(A_{control} - A_{sample}) / A_{control}] \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of the negative control at the moment of solution preparation and A_{sample} is the absorbance of the sample after 45 min.

Scavenging activity of ABTS radical

ABTS radical scavenging activity was determined according to Re *et al*. (1999) with some modifications. ABTS was dissolved in water to a 7 mM concentration. The ABTS radical cation (ABTS•+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubating the mixture in the dark at room temperature for 12 to 16 hours before use. The ABTS•+ solution was diluted with water to an absorbance of 0.70 (± 0.02) at 734 nm. The reaction mixture consisted of 0.07 mL of extract and 3 mL of the ABTS radical. After incubation for 6 min, absorbance was determined in a spectrophotometer at 734 nm. The antioxidant activity was calculated by Eq. 1, where $A_{control}$ is the absorbance of the negative control at the moment of solution preparation A_{sample} was the absorbance of the sample after 6 min.

Antimicrobial assays

The antimicrobial activity of CE from cultures of *Pleurotus pulmonarius* and *Pycnoporus cinnabarinus* was evaluated by the broth microdilution assay (Rodríguez *et al*. 2014) with minor modifications. A total of 100 µL of fungal crude extract was added to 100 µL of the bacterial suspension (1×10^5 CFU/mL) in Müeller Hinton Broth. Each mixture was transferred into a well of a 96-well microtiter plate, and bacterial growth was evaluated by measuring absorbance every 2 h for a total of 12 h at 37 °C. The optical density (OD) of each well was measured at 600 nm in an ELISA reader (BioRad, model 450, Hercules, CA, USA). The positive control contained only the bacterial suspension and 100 µL of the culture media used for the growth of the fungal cultures, and the negative control contained a mixture of the sterile culture media. Antimicrobial effects (bactericidal

or bacteriostatic) of CE against *Escherichia coli* and *Staphylococcus aureus* was observed from changes in bacterial growth kinetics.

Insecticidal assay

Insecticidal activities of CEs were evaluated by the larvae microinjection technique (Corzo *et al.* 2000). *Diatraea magnifactella* larvae were injected in the pronotum with a volume of CE equivalent to 10% of their weight using glass capillary pipettes, and they were placed in plastic 55-mm Petri dishes with artificial Meridic diet. Paralytic and lethal effects were observed at 24, 48, 72, and 96 h after injection, using a group of three larvae for each CE. A group of three larvae were injected with sterile water as control.

RESULTS AND DISCUSSION

The morphology of both fungi during growth within the reactor was irregular, and biomass accumulations of different shapes and sizes were observed. Thus, it was impossible to directly quantify fungal growth because when broth samples were taken, traces of biomass were obtained. To indirectly measure fungal growth, glucose disappearance from the culture broth was quantified, and, in both cases, fermentation was stopped when the glucose concentration was close to zero. Figure 1 shows the glucose consumption and pH pattern of cultures.

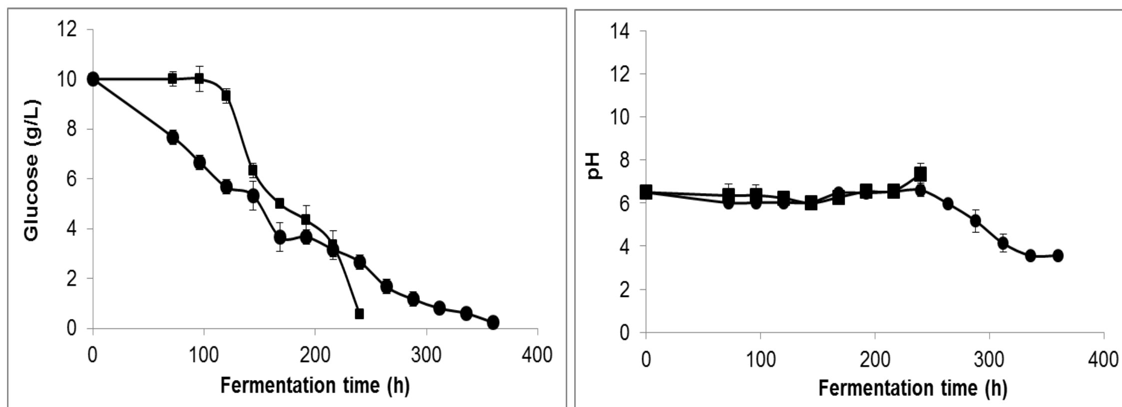

Fig. 1. Glucose concentration (a) and pH (b) of the culture broth of *Pleurotus pulmonarius* (■) and *Pycnoporus cinnabarinus* (●) grown in an Airlift reactor

As shown, *Pleurotus pulmonarius* started to incorporate glucose at 96 h, after which consumption was accelerated until glucose depletion at 240 h; *Pycnoporus cinnabarinus* started to incorporate glucose from the beginning of culture, with a steady but slower rate leading to glucose depletion at 336 h (Fig. 1a). Notably, the reactor conditions allowed airflow in both cases, and the resulting growth rates were lower than those reported for these fungi in SmF or solid-state fermentation (SSF) (Téllez-Téllez *et al.* 2008). Figure 1b shows the pH patterns in fungal cultures. During the first 216 h of growth, the pH values were near 6.5. After that point, *Pleurotus pulmonarius* showed a slight increase in pH, while *Pycnoporus cinnabarinus* showed a decrease in broth pH. This result suggested that each fungus produces different metabolites, although both are white-rot fungi grown in similar conditions. Díaz *et al*. (2013) examined the effect of pH on the activity and

production of laccases as well as the differences in isoenzyme number produced by *Pleurotus ostreatus*. In this previous report, pH 4.5 and 6.5 were the best for culture and enzymatic activity, respectively, while the highest number of isoenzymes was observed at pH 8.8. Lu *et al*. (2007) reported that *Pycnoporus sanguineus* showed constitutive laccase activity and purified one isoenzyme, reporting optimum pH and temperature of 3.0 and 65 °C, respectively. The enzyme was stable up to 40 °C, and high laccase activity was maintained at pH 2.0 to 5.0.

Figure 2 shows enzyme activity of CE obtained in the airlift reactor. Amylases were the most active enzymes in both fungi, reaching approximately 2500 U/L at 200 h of fermentation (Fig. 2a). *Pleurotus pulmonarius* showed pectinase activity of about 700 U/L at 96 h, which afterwards declined to a steady 500 U/L. For *Pycnoporus cinnabarinus*, the pectinase activity values were 660 and 850 U/L at 168 and 264 h, respectively, and at other time points, the values were between 200 and 400 U/L (Fig 2b). Cellulase activity reached in *Pleurotus ostreatus* and *Pycnoporus cinnabarinus* 1000 and 100 U/L (Fig. 2c), respectively. Throughout fermentation, *Pleurotus pulmonarius* showed xylanase activity values between 400 and 560 U/L, and *Pycnoporus cinnabarinus* exhibited activity values from 50 to 100 U/L (Fig. 2d). Invertase activity was observed only in the *Pleurotus pulmonarius* culture with maximum value near to 280 U/L (Fig. 2e). In general, both fungi showed low enzymatic activities. However, the presence of hydrolases activity suggests that these enzymes are constitutive (except for the invertases in *Pycnoporus cinnabarinus*) and might have enhanced activity in the presence of inducers. Álvarez-Cervantes *et al*. (2013) reported approximately 12,000 U/L of xylanases in *Sporisorium reilianum* grown in SmF with birch xylan. In a recent study, *Stenocarpella maydis* produced almost 8000 and 9500 U/L of cellulase activity in SmF and SSF, respectively (Hernández-Domínguez *et al*. 2014).

Ko *et al*. (2005) evaluated the activity of α-amylase, cellulase, β-glucosidase, xylanase, and laccase in the spent compost of four species of edible fungus, including *Pleurotus ostreatus*. The highest activities of α-amylase (229 nkat/g), cellulose (759 nkat/g), and β-glucosidase (767 nkat/g) were observed in the compost of *Lentinula edodes*, and in the compost of *Pleurotus ostreatus*, the highest laccase activity (1452 nkat/g) was found. The highest xylanase activity (119 nkat/g) was found in the compost of *Flammulina velutipes*. In another study, a commercial strain of *Pleurotus ostreatus* was grown on rice straw and sawdust in plastic bags. Endoglucanases, carboxymethylcellulases, and pectinases activities produced on sawdust (6.0, 13.5, and 22 U/g, respectively) were higher than those observed in rice straw (4.0, 5.0, and 14.0, respectively). Xylanase activity produced in the sawdust showed higher activity in the mycelium (21 U/g) than in the fruiting stage (11 U/g). The exoglucanase activity was about the same in both residues (Sherief *et al*. 2010). Cellulolytic activities were compared in two white-rot fungi, *Bjerkandera adusta* and *Pycnoporus sanguineus*, that were cultivated on wheat straw agar. *Bjerkandera adusta* showed 1.6 times higher carboxymethylcellulase activity than *Pycnoporus sanguineus*. However, enzymes from *Pycnoporus sanguineus* were more robust, resisting one hour of incubation at high temperatures (up to 80 °C) and exhibiting activity and stability from pH 2 to 8. Zymograms of cellulolytic isoenzymes from both species showed molecular masses ranging from 25 to 90 kDa (Quiroz-Castañeda *et al*. 2009).

Falkoski *et al*. (2012) grew *Pycnoporus sanguineus* in SmF using corn cobs as a carbon source, and the resulting polygalacturonase, xylanase, FPase, endoglucanase, β-glucosidase, mannanase, α-galactosidase, and α-arabinofuranosidase activities were

evaluated. Endoglucanase activity was higher at 60 °C, in a pH range of 3.5 to 4.0, and β-glucosidase and FPase activities were higher at 55 °C, pH 4.5. All cellulase activities were stable at 40 and 50 °C through 48 h of pre-incubation.

Iandolo *et al*. (2011) reported the activity of three enzymes from *Pleurotus ostreatus* and *Trametes versicolor* grown on tomato pomace using sorghum stalks as support. Laccases, proteases, and xylanases activities were 36, 34000, and 50 U/g dry matter, respectively, while *Pleurotus ostreatus* produced approximately half of those values.

In this study, laccase activity of *Pleurotus pulmonarius*, reached 550 U/L at 96 h of culture and then leveled off at 450 U/L. *Pycnoporus cinnabarinus* showed very low laccase activity throughout fermentation, with values about half of those in *Pleurotus pulmonarius* (Fig. 2f). These values were low compared with those reported for *Pleurotus ostreatus* grown in the same culture medium in shake flasks and SSF using polyurethane foam as an inert support (Téllez-Téllez *et al*. 2008). In the previous study, almost 3 times higher laccase activity was reported SSF, suggesting that the amount of oxygen available to the fungus during the bioprocess is important, at high concentrations biomass is favored, but in limited oxygen, laccase increased its production. This effect may be related to the stress response element found in the promoter of laccase genes (Téllez-Téllez *et al*. 2012). Dantán-González *et al*. (2008) reported a 68-kDa protein purified from submerged cultures of *Pycnoporus sanguineus*; this protein showed laccase activity towards 2,2´-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), guaiacol, syringaldazine, and o-dianisidine. Valeriano *et al*. (2009) reported an increase in *Pycnoporus sanguineus* laccase activity in the presence of xylidine and ethanol as inducers and observed the maximum laccase activity of 2019 U/L and 1035 U/L, with 200 mg/L of 2,5-xylidine and 50 g/L of ethanol, respectively. Laccases from *Pycnoporus cinnabarinus* are involved in cinnabarinic acid production with antibacterial activity (Eggert 1997). In this work, low laccase activity and high antimicrobial activities were found in CEs of *Pycnoporus cinnabarinus*; on the other hand, antimicrobial activity was not observed in CEs of *Pleurotus pulmonarius*. even though high laccase activities were observed. These results suggest that *Pleurotus pulmonarius* is not able to produce cinnabarinic acid.

Figure 3 shows the antioxidant activity and content of polyphenols and flavonoids of all CE. The percentage inhibition of the radical ABTS (Fig. 3a) with samples of *Pleurotus pulmonarius* was approximately 30 to 50%. In *Pycnoporus cinnabarinus*, low values were observed during the first 14 h and, subsequently, achieved about 50% inhibition. DPPH radical reduction (Fig. 3b) was between 20 and 30% with samples of *Pleurotus pulmonarius*, and the samples of *Pycnoporus cinnabarinus* had values around 30% (just one sample reached 50% inhibition). The observed values of the total polyphenol content in samples of *Pleurotus pulmonarius* were about 0.4 mg GAE/mL, which was four times higher than those observed in samples of *Pycnoporus cinnabarinus* (0.1 mg GAE/mL). The total flavonoid content of the *Pleurotus pulmonarius* or *Pycnoporus cinnabarinus* broth was between 0.03 and 0.06 mg QE/mL or 0.03 to 0.05 mg QE/mL, respectively, being generally higher earlier in the culture.

The total polyphenol and flavonoid contents were very low in both fungi, suggesting that different compounds provided the antioxidant activity. Recently, antioxidant activity in the wild medicinal mushrooms *Lenzites betulina*, *Lentinus polychrous*, *Trametes versicolor*, *Pycnoporus cinnabarinus*, *Pycnoporus* sp., *Inonotus radiatus*, and *Microporus xanthopus* was evaluated. *Inonotus radiatus* had the highest flavonoid content (13.50 ± 1.33 mg/g) and total phenolic content (3.5 ± 0.052 mg/g). The

carotenoid content of *Pycnoporus* sp. (23.93 ± 0.87 mg/g) and *Pycnoporus cinnabarinus* (20.84 ± 1.56 mg/gm) was much higher than in *Inonotus radiatus* (4.61 ± 1.23 mg/g) or *Lentinus polychrous* (1.31 ± 0.1 mg/g) (Tripathy *et al*. 2014). In another study, antioxidant activity in *Pycnoporus sanguineus* broth was evaluated *via* the DPPH method. The highest antioxidant activity (around 80% of radical inhibition) was registered during the stationary phase, with a similar potential as the synthetic antioxidant BHT, after 30 days of cultivation (Borderes *et al.* 2011).

When antimicrobial activity in CE was evaluated, *Pleurotus pulmonarius* did not inhibit the growth of either bacteria evaluated (Fig. 4a, b), while *Pycnoporus cinnabarinus* showed antimicrobial activity on both (Fig. 4c, d). The CE obtained at 240 h of culture showed a bacteriostatic effect; at 264 and 288 h of fermentation, a bactericidal effect was observed. While the responsible metabolite was not investigated, it could have been phenoxazine type-1 compounds. Cinnabarine and cinnabarinic acid possess antimicrobial activity, and both compounds are produced by this fungi. Eggert (1997) reported that laccase secreted by *Pycnoporus cinnabarinus* oxidizes the precursor 3-hydroxyanthranilic acid to cinnabarinic acid, and this last compound has the antibacterial activity, mainly against Gram-positive bacteria of the genus *Streptococcus*.

Adebayo *et al*. (2012) reported the antioxidant and antimicrobial activities of *Pleurotus pulmonarius*-LAU09 (JF736658). The evaluated mushroom extracts gave positive results with free radical scavenging activity. The highest zone of inhibition was obtained against *Staphylococcus aureus* (30 mm), while the lowest zone size obtained was against *Escherichia coli* (7 mm). In this work *Pycnoporus cinnabarinus* showed higher free radical scavenging activity than *Pleurotus pulmonarius.*

The effect of inorganic and organic nitrogen sources on the antioxidant and antimicrobial potential of mycelium ethanolic extract from *Pleurotus ostreatus* PQMZ91109 has been evaluated (Vamanu 2011). Of the organic nitrogen sources, ammonium sulfate increased the production and accumulation of bioactive compounds, showing low values of EC_{50} and minimum inhibitory concentration (MICs). Among the organic sources, peptone, followed by corn extract, led to increased radical-scavenging activity (DPPH).

The extracts selectively inhibited two *Candida* strains at an MIC value of 1.25 mg/mL. In contrast, total phenol, antioxidant, and antimicrobial properties of extracts obtained from *Pleurotus ostreatus* were evaluated on three different tropical woody substrates: *Cananium* sp., *Pycnanthus ongoleubis*, and *Ceiba pentandra*. The mushroom cultivated on *Pycnanthus ongoleubis* had the highest total phenol content (2.63 µg/ml), and the scavenging activity against DPPH radicals ranged from 73.75 to 90.75%. It inhibited all the test organisms with zones of inhibition ranging from 5.33 mm to 20.33 mm (Oyetayo and Ariyo 2013).

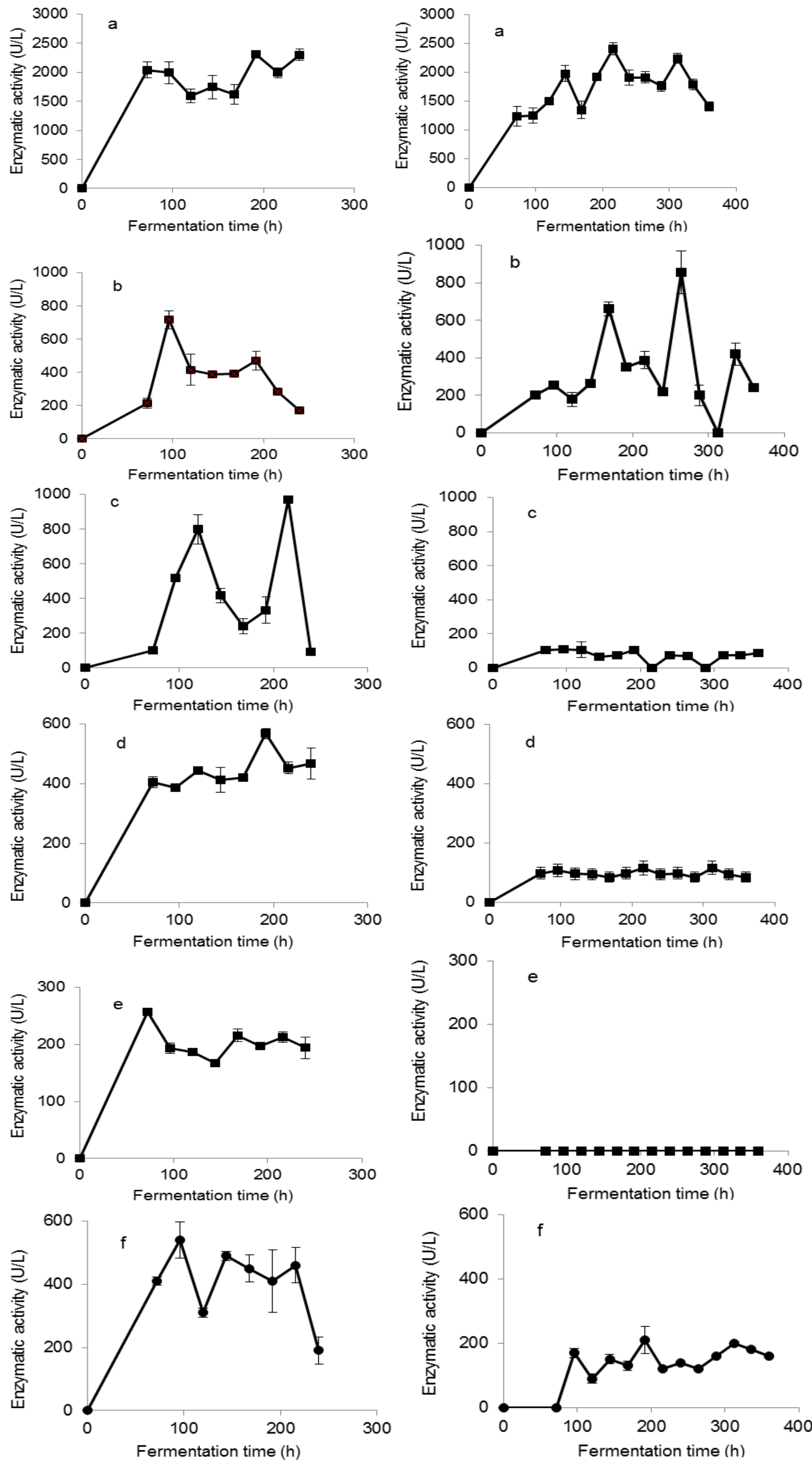

Fig. 2. Enzymatic activity produced by *Pleurotus pulmonarius* (left) and *Pycnoporus cinnabarinus* (right) grown in an AirLift reactor; Amylases (a), pectinases (b), cellulases (c), xylanases (d), invertases (e), laccases (f)

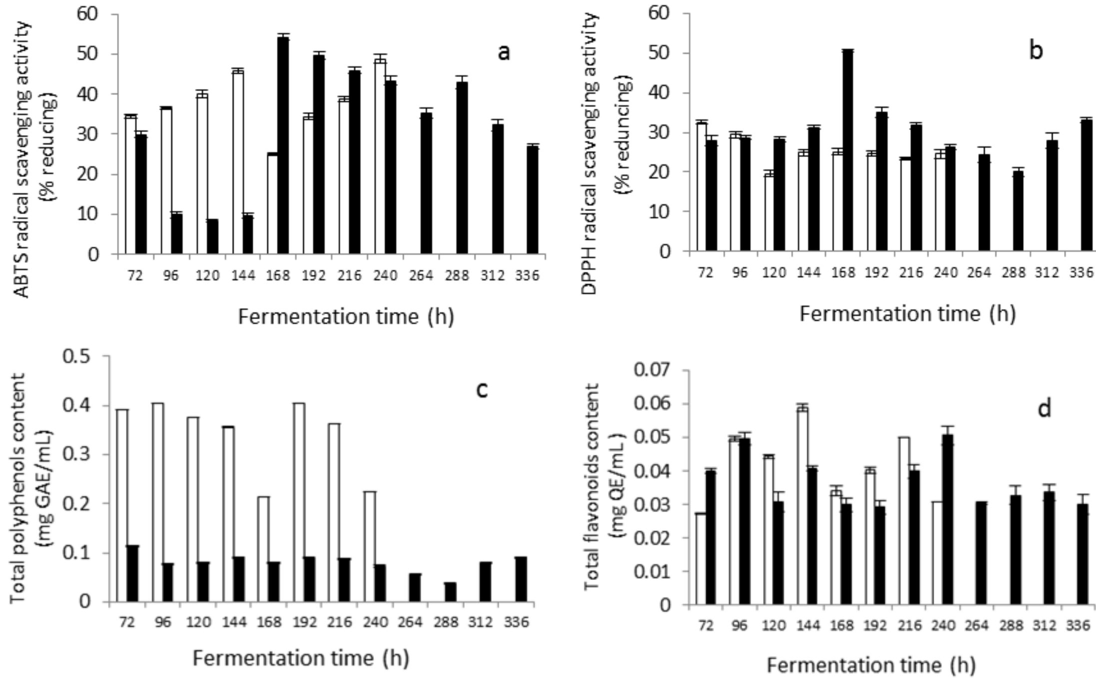

Fig. 3. Percentage of ABTS (a) and DPPH (b) radical inhibition, total polyphenols content (c) and total flavonoids content (d) of culture media where grown in AirLift reactor *Pleurotus pulmonarius* (open bars) and *Pycnoporus cinnabarinus* (solid bars)

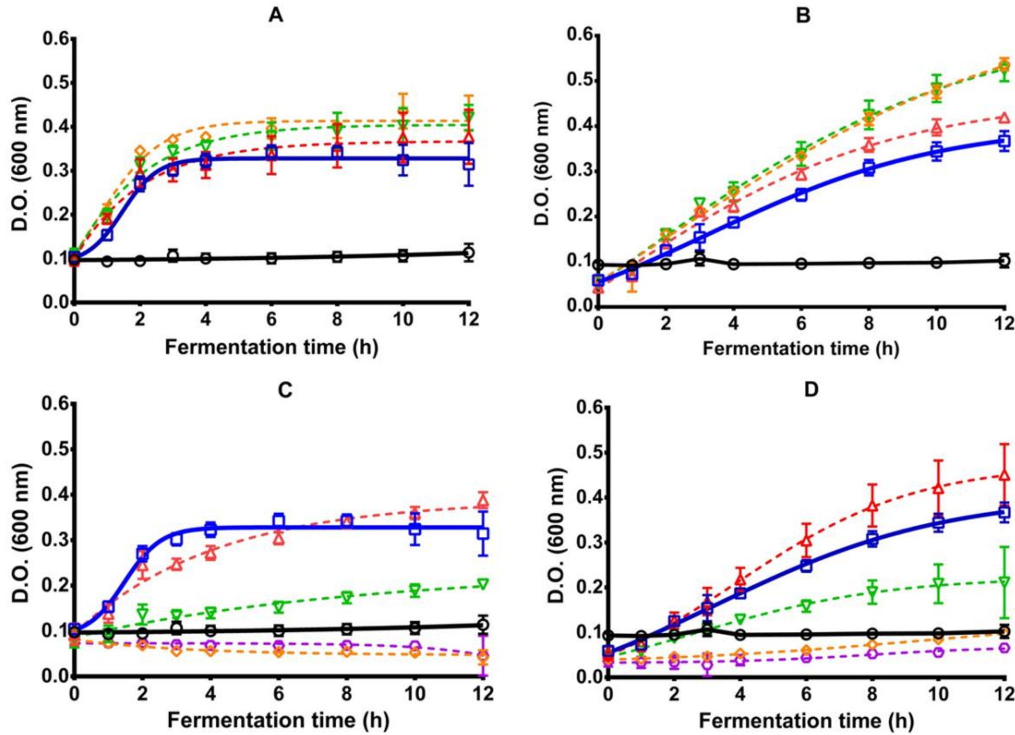

Fig. 4. Antimicrobial activity of CE of *Pleurotus pulmonarius* (A and B) and *Pycnoporus cinnabarinus* (C and D), against *Escherichia coli* (A and C) and *Staphylococcus aureus* (B and D). Culture media (○), bacteria growth (□), CE of *Pleurotus pulmonarius* at 120 h and of *Pycnoporus cinnabarinus* at 216 h (∆),CE of *Pleurotus pulmonarius* at 144 h and of *Pycnoporus cinnabarinus* at 240 h (▽), CE of *Pleurotus pulmonarius* at 168 h and of *Pycnoporus cinnabarinus* at 264 h (◊), CE of *Pycnoporus cinnabarinus* at 288 h (○)

To determine the antimicrobial activity of *Pleurotus ostreatus*, a disk diffusion technique was used. Ethyl acetate, hexane, and chloroform extracts were examined with ten organisms; hexane extract showed highest antibacterial activity for both Gram-positive and Gram-negative bacteria. The average zone of inhibition for hexane extract was from 11 to 24 mm. The total phenolic content ranged from 3.69 mg GAE/g to 8.49 mg GAE/g (Sala *et al.* 2015). The antimicrobial activity of *Pleurotus eryngii* var. *ferulae* grown on various agro-wastes was investigated. Extracts of this fungus were evaluated for antimicrobial activity by the disk diffusion method using *Bacillus megaterium* DSM 32, *Staphylococcus aureus* COWAN 1, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* FMC 5, *Candida albicans* FMC 17, *Candida glabrata* ATCC 66032, *Trichophyton* spp., and *Epidermophyton* spp. (Akyuz and Kirbag 2009). Methyl alcohol extracts inhibited microbial growth to different degrees (7.7 to 10.3 mm). Surekha *et al.* (2011) evaluated the antimicrobial activity of 10% ethyl acetate extracts of the fruiting bodies of *Pleurotus sajor-caju*, *Volvariella volvaceae*, *Agaricus bisporus*, and *Pleurotus ostreatus*. *Agaricus bisporus* and *Pleurotus ostreatus* showed important inhibitory activity against *Staphylococcus aureus*, which is a commensal inhabitant of humans. However in this work non antimicrobial activity was observed in CEs of *Pleurotus pulmonarius.*

Table 1. Insecticidal Activity in *Pleurotus pulmonarius* and *Pycnoporus cinnabarinus* CEs

Strain	Time of Sampling (h)											
	72	96	120	144	168	192	216	240	264	288	312	336
Pleurotus pulmonarius	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Pycnoporus cinnabarinus	RP	RP	D	D	Nd	D	Nd	D	Nd	Nd	RP	RP

Nd = not detected; RP = rigid paralysis observed after 96 h injection; D = Dead larvae observed after 72 h injection. A different group of larvae was used for each time point.

The insecticidal activity of CEs obtained from *Pleurotus pulmonarius* and *Pycnoporus cinnabarinus* was evaluated in *Diatraea magnifactella* larvae, a sugar cane borer (Table 1). The larvae of the control group showed no effect. The CEs of strain *Pleurotus pulmonarius* did not have any insecticidal activity or effect at 24, 48, 72, or 96 h post-injection. Noshad *et al*. (2015) evaluated insecticidal activity against *Macrosiphum rosae* (rose aphids) of the ethyl acetate extracts from fruiting bodies, mycelia, and fermentation filtrate obtained from *Pleurotus ostreatus.* The fermentation filtrate showed a LC_{50} value of 25.03 μg/mL. Interestingly, CE from *Pycnoporus cinnabarinus* taken at 120, 144, 192, and 240 h of culture did kill larvae after 72 h injection, and the rest of the samples showed rigid paralysis at 96 h post-injection.

CONCLUSIONS

1. Both fungi considered in this work produced hydrolase enzymes and laccases in a medium with glucose in the AirLift reactor. However, all enzymes except amylase and pectinase showed higher values in CE of *Pleurotus pulmonarius* than in CE of *Pycnoporus cinnabarinus*. These results suggest that these enzymes are constitutive

(except for invertase in CE of *Pycnoporus cinnabarinus*) and that production levels would be increased if inducers are added.

2. Even though $CuSO_4$-$5H_2O$ was used as a laccase inducer, *Pycnoporus cinnabarinus* presented very low laccase activity. It is suggested to increase laccase production by optimization of the fermentation parameters involving low aeration, type, and concentration of carbon source, temperature, and pH of culture medium among others.
3. *Pleurotus pulmonarius* had higher antioxidant activity than *Pycnoporus cinnabarinus*, and in both cases the observed antioxidant activity was due mainly to different polyphenols. Although radical inhibition was low, the culture conditions could be optimized to obtain higher antioxidant activities.
4. *Pycnoporus cinnabarinus* presented very strong bacteriostatic and bactericidal effects against *Escherichia coli* and *Staphylococcus aureus,* that may be due to the presence of phenoxazine type-1 compounds, such as cinnabarine or cinnabarinic acid in the CEs. *Pleurotus pulmonarius* did not show any negative effect on bacterial growth.
5. When testing insecticidal activity towards *Diatraea magnifactella*, *Pleurotus pulmonarius* was negative, but *Pycnoporus cinnabarinus* killed *Diatraea magnifactella* larvae at 72 h post-injection, showing interesting results that could be used to control this insect pest.

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