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APLICAÇÕES BIOTECNOLÓGICAS DO FUNGO LIGNINOLÍTICO *PLEUROTUS PULMONARIUS* (FR.) QUÉL.

Maringá
2016

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Tese apresentada à Universidade Estadual de Maringá, como requisito parcial para a obtenção do título de doutor.

Orientadora: Profª. Drª. Rosane Marina Peralta

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No período de 2011 a 2015 lecionou na instituição de ensino profissionalizante SENAI, Serviço Nacional de Aprendizagem Industrial, nos cursos técnicos em Biotecnologia e Química.

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1) Corrêa, R.C.G.; Silva BP Castoldi, R.; Kato CG, Sá-Nakanishi, A. B.; Peralta, R. A.; Souza, C.G. M; Bracht, A. Peralta, R. M. Spent mushrooms substrate of Pleurotus pulmonarius: a source of easily hydrolyzable lignocellulose Folia Microbiologica, March, 2016.v. p. doi:10.1007/s12223-016-0457-8. Fator de impacto 1.335.
2) Peralta, R. M., Da Silva BP, Corrêa RCG, Kato CG, Seixas FAV, Bracht A, Enzymes from basidiomycetes – peculiar and efficient tools for biotechnology, do livro Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications (ELSEVIER), editado por Goutam Brahmachari, Arnold Demain e Jose Adrio, 2016.

Dedico

A Deus

À minha mãe Devanir Silva Polacchine.

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À Deus, por estar sempre ao meu lado com sua infinita misericórdia e grandioso amor.

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RESUMO

Basidiomicetos e Ascomicetos desempenham importante papéis no equilíbrio ambiental. Eles são os maiores decompositores do material lignocelulósico em vários ecossistemas e têm grande importância no ciclo do carbono e outros nutrientes. Existem mais de 10.000 espécies de fungos da podridão branca de madeira, com variadas capacidades em degradar a lignina, celulose e hemicelulose. Entretanto somente algumas dezenas são mais propriamente estudados. Os fungos da podridão branca mais estudados pertencem a seis famílias: *Phanerochaetaceae* (*Phanerochaete chrysosporium*), *Poliporaceae* (*Trametes versicolor* e *Pycnoporus sanguineus*), *Marasmiaceae* (*Lentinula edodes*), *Pleurotaceae* (cogumelos ostra tais como *Pleurotus ostreatus* e *Pleurotus pulmonarius*), *Hymenochaetaceae* (*Inonotus hispidus* e *Phellinus igniarius*), *Ganodermataceae* (*Ganoderma lucidum* e *Ganoderma applanatum*) e *Meruliaceae* (*Bjerkandera adusta*, *Irpex iacteus* e *Phlebia radiate*).

O objetivo do primeiro artigo foi a purificação e a caracterização da enzima peroxidase dependente de manganês (MnP) de Pleurotus pulmonarius bem como a avaliação da sua capacidade em descolorir corantes sintéticos. *P. pulmonarius* foi cultivado por 14 dias sob condições em estado sólido utilizando resíduos de abacaxi como substrato. Nestas condições, MnP foi a principal enzima ligninolítica produzida pelo fungo. A enzima foi purificada até aparente homogeneidade eletroforética através de precipitação com acetona e gel filtração utilizando coluna de Sephadex G-100. Os principais resultados foram: a MnP foi purificada 12,1 vezes, com um rendimento de 28,4% e uma atividade específica de 135,52 U/mg proteína. A proteína monomérica de 42KDa apresentou maior atividade em pH de 4,0 a 6,0 e temperatura de 50°C. A enzima foi estável a temperatura de até 40°C. A -20°C a enzima foi estável por pelo menos 6 meses. Os valores de K_M para Mn^{+2} e H_2O_2 a pH 4,5 e 40°C foram 19,2 e 16,8 □M, respectivamente. A enzima foi totalmente dependente de Mn^{+2} para oxidar compostos fenólicos e não fenólicos. A enzima mostrou alta atividade e estabilidade na presença de solventes orgânicos tais como acetona, etanol, isopropanol e acetonitrila e foi capaz de descolorir o corante antraquinonico Remazol Brilliant Blue R e o corante azo Vermelho do Congo na presença de 1M Na_2SO_4 e NaCl. As propriedades apresentadas pela MnP de *P. pulmonarius* tornam esta enzima um bom agente para o tratamento de efluentes têxteis.

Os objetivos do segundo trabalho foi avaliar a atividade antimicrobiana de extratos miceliais de *Pleurotus pulmonarius* obtidos em cultivos submersos utilizando glicose, amido e bagaço de mandioca como substratos. As maiores atividades antimicrobianas foram obtidas nos extratos miceliais de 6 dias de cultivo utilizando glicose e amido como substrato. Apesar dos esforços, não foi possível identificar quais moléculas são responsáveis pela atividade antimicrobiana nos extratos miceliais de *P. pulmonarius*.

Palavras-chave: *Pleurotus pulmonarius*; fungos da podridão branca; enzima manganês peroxidase; atividade antimicrobiana, atividade antifúngica.

ABSTRACT

Basidiomycetes and Ascomycetes play a crucial role in the balance of ecosystems. They are the major decomposers of lignocellulosic material in several ecosystems and play an essential role in the cycling of carbon and other nutrients. There are about 10,000 species of white rot fungi, with varying capacities to degrade lignin, cellulose and hemicellulose. However, only a few dozen have been properly studied. Most commonly studied species of white rot fungi are subdivided into six families: *Phanerochaetaceae* (*Phanerochaete chrysosporium*), *Poliporaceae* (*Trametes versicolor* and *Pycnoporus sanguineus*), *Marasmiaceae* (*Lentinula edodes*), *Pleurotaceae* (oyster mushrooms such as *Pleurotus ostreatus* and *Pleurotus pulmonarius*) *Hymenochaetaceae* (*Inonotus hispidus* and *Phellinus igniarius*) *Ganodermataceae* (*Ganoderma lucidum* and *Ganoderma applanatum*) and *Meruliaceae* (*Bjerkandera adusta*, *Irpex iacteus* and *Phlebia radiate*).

The objective of the first article was to purify and characterize the enzyme manganese peroxidase (MnP) of *Pleurotus pulmonarius* and evaluate its capability to decolorize synthetic dyes. *P. pulmonarius* was cultured for 14 days under solid state conditions using pineapple wastes as substrate. Under this condition, MnP was the main ligninolytic enzyme found in the culture filtrates. The enzyme was purified to apparent electrophoretic homogeneity through acetone precipitation and gel filtration using Sephadex G-100 column. The main results were: MnP was purified 12.1-folds with a yield of 28.4% and a specific activity of 135.52 U/mg proteins. The 42 KDa-monomeric proteins were active over a large range of pH (4.0 - 6.0) and at a temperature of 50°C. The enzyme was stable at temperatures up to 40°C. At -20°C the enzyme was stable for at least 6 months. The K_M values for Mn^{+2} and H_2O_2 at pH 4.5 and 40°C were 19.2 and 16,8 μM, respectively. The enzyme was strictly dependent of Mn^{+2} for oxidizing phenolic and non-phenolic compounds. It showed high activity and stability in the presence of organic solvents such as acetone, ethanol, acetonitrile and isopropanol, and was able to decolorize the anthraquinonic dye Remazol Brilliant Blue R and the azo dye Congo red in the presence of 1M Na_2SO_4 and NaCl. The properties presented by *P. pulmonarius* manganese peroxidase certainly make this enzyme a good agent for textile dye effluents treatment.

The objectives of the second article was to evaluate the antimicrobial activities of mycelial extracts of *Pleurotus pulmonarius* obtained in submerged cultures using glucose, starch and cassava bagasse as carbon source. The highest antimicrobial activities were obtained after 6 days of cultivation using glucose and starch as carbon source. Despite the efforts, it was not possible to identify how molecules are responsible for antimicrobial activity of *P. pulmonarius* mycelia extracts.

Keywords: *Pleurotus pulmonarius*; White-rot fungi; manganese peroxidase; antimicrobial activity, antifungal activity.

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LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2'-azino-bis[3-ethyl-6-benzothiazolinesulfonate] DMP=2,6-dimethoxyphenol
MnP	manganese peroxidase
PDA	potato dextrose agar
RBBR	remazol brilliant blue R
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
WRF	white-rot fungi
MIC	Minimal inhibitory concentration
MBC	Minimal bactericidal concentration
PDA	Potato dextrose agar
ATCC	American type culture collection
NCCLS	Clinical and Laboratory Standards Institute
CFU	Colony forming unit
MFC	Minimum Fungicidal Concentration

SUMÁRIO

Characterization of a Solvent-Tolerant Manganese Peroxidase from *Pleurotus pulmonarius* and its Application in Dye Decolorization

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Abstract:

Background. Manganese peroxidase (MnP) is a common extracellular ligninolytic peroxidase produced by *Pleurotus* spp. It catalyzes the H_2O_2-dependent oxidation of Mn^{2+} into highly reactive Mn^{3+}, which in turn oxidizes phenolic and nonphenolic compounds including a large list of xenobiotics such as synthetic dyes

Objective. To purify and characterize the MnP of *Pleurotus pulmonarius* and to evaluate its capability to decolorize synthetic dyes.

Methods. *P. pulmonarius* was cultured for 14 days under solid state conditions using pineapple waste as substrate. Under this condition, MnP was the main ligninolytic enzyme found in the culture filtrates. The enzyme was purified to apparent electrophoretic homogeneity through acetone precipitation and gel filtration using a Sephadex G-100 column.

Results. MnP was purified 12.1-fold with a yield of 28.4% and a specific activity of 135.52 U/mg protein. The 42 kDa-monomeric protein was active over a large range of pH (4.0-6.0) and at a temperature of 50°C. The enzyme was stable at temperatures up to 40° C. At 4 °C and at -20 °C, it was stable for at least 2 and 6 months, respectively. The K_M for Mn^{2+} and H_2O_2 at pH 4.5 and 40 °C were 19.2 and 16.8 μM, respectively. The enzyme was strictly dependent on Mn^{2+} for oxidizing phenolic and nonphenolic compounds. It showed high activity and stability in the presence of organic solvents such as acetone, ethanol, isopropanol and acetonitrile, and was able to decolorize the anthraquinonic dye remazol brilliant blue R and the azo dye Congo red in the presence 1 M Na_2SO_4 and NaCl.

Conclusion. The properties of the *P. pulmonarius* MnP certainly make this enzyme a good agent for textile dye effluent treatment.

Keywords: azo dye, anthraquinonic dye, biological decolorization, enzyme purification, manganese peroxidase, *Pleurotus pulmonarius*, white-rot fungi.

1. INTRODUCTION

White-rot fungi (WRF) are known for their ability to degrade or modify lignin by enzymatic processes. The major enzymes associated with the lignin-degrading ability of WRF are lignin peroxidases (EC 1.11.1.14), manganese dependent peroxidases (EC 1.11.1.13), versatile peroxidases (EC 1.11.1.16), and laccases (EC 1.10.3.2) [1]. Manganese peroxidases (MnPs) are the most common extracellular ligninolytic peroxidases produced by WRF [2]. They catalyze the H_2O_2-dependent oxidation of Mn^{2+} into Mn^{3+}, which is stabilized via chelation with organic acids such as oxalate, malonate, tartrate and lactate [3]. It is believed that MnP is involved in the initial attack of lignin by Mn^{3+} chelate complexes [4,5]. The enzyme presents capacity to depolymerize synthetic lignin *in vitro* [6], to oxidize phenolic and nonphenolic compounds [7], and to degrade an ample list of xenobiotics such as polycyclic aromatic hydrocarbons, synthetic dyes and mycotoxins [4,8-17]. The enzyme is also useful in the pulp and paper delignification and in the production of high-value chemicals from residual lignin discarded by biorefineries [18]. Furthermore, the enzyme is also used in analytical biochemistry, in the food and beverage industries, in fruit juice clarification, and other sectors [19,20].

The white rot fungus *Pleurotus pulmonarius* is very easy to cultivate and is known for its ability to degrade or modify lignin by enzymatic processes. The major enzyme frequently associated with its lignin-degrading ability is laccase (EC 1.10.3.2), but under certain conditions of solid state cultivation, which include the type of substrate and the initial moisture, the fungus also produces elevated amounts of manganese peroxidase [21,22]. In a recent work, the highest activities of Mn peroxidase (2,200±205 U/L) were produced by *P. pulmonarius* cultured under solid state conditions using pineapple peel as substrate [23]. Taking these previous notions into consideration, the objective of the present work was to purify and characterize a Mn peroxidase produced by *P. pulmonarius* under solid state conditions using pineapple waste as substrate.

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2. MATERIALS AND METHODS

2.1. Microorganism

P. pulmonarius CCB-19 was obtained from the Culture Collection of the Botany Institute of São Paulo. It was cultured on potato dextrose agar (PDA) medium for 2 weeks at 28 °C. When the plates were fully covered with the mycelia, mycelial plugs measuring 10 mm in diameter were made and used as inoculum.

2.2. Culture conditions and enzyme extraction

The cultures were performed in 5 cotton-plugged Erlenmeyer flasks (500 mL) containing each one 10 g of pineapple peel as substrate. A mineral medium [24] was used to adjust the moisture content to 75%. Prior to use, the mixtures were sterilized by autoclaving at 121 °C for 15 min. Each flask received 10 mycelial plugs and was incubated statically under an air atmosphere at 28 °C and in complete darkness. After 14 days of cultivation, a volume of 100 mL of cold water was added to the contents of each flask. The mixtures were stirred for 1 h at 4 °C and centrifuged at 5,000*g* for 20 min. The supernatants obtained from the flasks were pooled and stored at 4 °C and used as crude enzyme extract.

2.3. MnP purification procedure

All procedures were performed at 4 ^{o}C. The crude enzyme extract was concentrated by addition of cold acetone (-20 ^{o}C) to give a 75% saturation and precipitated proteins were collected by centrifugation at 20,000*g* for 50 min. After removing the acetone, the precipitated proteins were re-dissolved in a minimal volume of 50 mM malonate buffer, pH 4.5. The soluble proteins were applied to a Sephadex G-100 column (80 cm x 2.0 cm) previously equilibrated with the same buffer. The fractions (4.0 mL) were eluted at a flow rate of 1 mL/min. Each fraction was assayed for protein ($A_{280\ nm}$), laccase activity and MnP activity. Fractions with high MnP activity and no laccase activity were pooled (fractions 26-32), dialyzed against water, concentrated by freeze-drying and stored at -20 ^{o}C

2.4. MnP and laccase assays and protein determination

The MnP activity was assayed by the oxidation of 1.0 mM $MnSO_4$ in 50 mM sodium malonate buffer, pH 4.5, in the presence of 0.05 mM H_2O_2 at 30 ^{o}C. Manganic ions (Mn^{3+}) form a complex with malonate, which absorbs at 270 nm (ε_{240}=7,800$M^{-1}cm^{-1}$) [17]. Additionally, the MnP activity was assayed by the oxidation of 1.0 mM 2,6-dimethoxyphenol (DMP) (ε_{469}=27,500 $M^{-1}cm^{-1}$) or 1.0 mM ABTS (2,2'-azino-bis[3-ethyl-6-benzothiazolinesulfonate]) (ε_{420}=36,000 $M^{-1}cm^{-1}$). The laccase activity was measured with 2 mM ABTS in 50 mM sodium acetate buffer, pH 5.0 [21]. The enzymatic activities were expressed as international units (U), defined as the amount of enzyme required to produce 1 µmol product min^{-1} at 30 ^{o}C. Protein contents were determined by the method of Bradford [25] using bovine serum albumin as the standard

2.5. Electrophoretic analysis and determination of molecular weight

The molecular weight of the purified MnP was estimated under denaturing electrophoresis. SDS-PAGE was performed on a 10% polyacrilamide gel [26]. The following Mw standards (MW-SDS-70 kit-Sigma) were used: bovine serum albumin (66 kDa), ovoalbumin (45 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), and β-lactoglobulin (18.4 kDa). Protein bands were visualized by Coomassie Blue R-250. The MnP molecular weight was also estimated by gel filtration chromatography using Sephadex G-100 equilibrated with 50 mM malonate buffer, pH 4.5 plus 0.1 M NaCl. The column was equilibrated with standard proteins (MWGF-70 kit Sigma).

2.6. Characterization of purified MnP

For determining the pH optimum, the enzyme was assayed over a pH range of 3.5-7.0 at 30 ^{o}C. To evaluate the pH stability, the purified MnP was incubated at different pH's (3.5-7.0) for 6 h. The residual MnP activity was measured under standard conditions. The effect of temperature on the MnP activity was measured incubating the enzyme at temperatures ranging from 30 to 60 ^{o}C under standard conditions. Thermal stability was investigated by incubating the enzyme at different temperatures for up to 2 h. The remaining activities were measured under standard conditions.

$CaCl_2$, $HgCl_2$, $CuSO_4$, $NiCl_2$ and $FeCl_3$ (at concentrations of 1.0 and 5.0 mM) were used to evaluate the influence of metal ions on the activity of MnP. Methanol, ethanol, isopropanol, and acetonitrile (at concentrations up to 20%) were used to evaluate the influence of organic solvents on the activity of MnP. NaCl and Na_2SO_4 (at 0.5 and 1.0 M) were used to investigate the action of high salt concentrations on the enzyme activity. In all cases, the residual activity was calculated based on the control (set as 100%).

2.7. Dye decolorization by purified manganese peroxidase

A volume of 0.5 mL of remazol brilliant blue R (RBBR, CI 61200) or Congo red (CI 22120) solutions, to give final concentrations of 100 ppm, and 0.5 mL of purified MnP (0.25 U) were added to 4.0 mL of 50 mmol/L malonate buffer, pH 4.5, containing 1 mM $MnSO_4$ and 0.1 mM H_2O_2. The mixtures were incubated in a rotary shaker at 40 °C for 2 h. Dye disappearance was determined in a spectro-photometer by monitoring the absorbance at the wavelength of maximum absorbance for each dye. Boiled enzyme was used as negative control. To evaluate the effect of metal ions, organic solvents and high salt concentrations on the capability of MnP to decolorize RBBR and Congo red, the experiments were carried out using the concentrations described in item 2.6.

3. RESULTS

3.1. Enzyme purification

MnP was concentrated and purified in a rapid purification scheme described in the material and methods section. Shortly, after concentration by acetone precipitation, the crude enzymes were applied to a Sephadex G-100 column (Fig. 1). Laccase was eluted first, followed by Mn peroxidase. The fraction 26 to 32, where no laccase activity was detected, were pooled, dialyzed against water and lyophilized. The decision of pooling only 8 fractions with Mn peroxidase resulted in a high loss of MnP activity, but this strategy was necessary to avoid contamination by other proteins, especially laccase. Table 1 summarizes the steps used in the purification process. At the end of the process, MnP was purified 12.13-fold with a yield of 28.4%. Specific activity of purified Mn peroxidase was 135.52 U/mg protein.

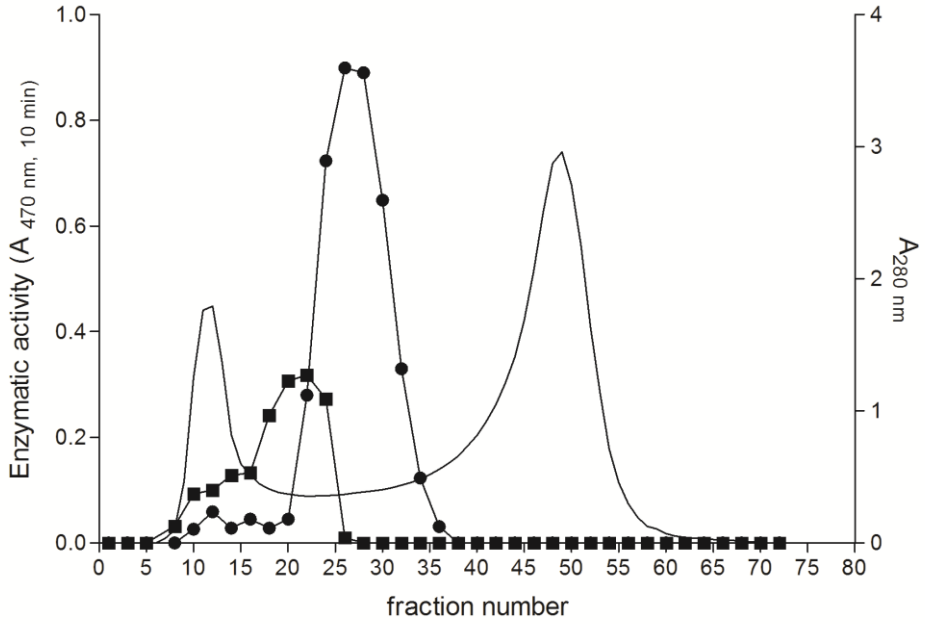

Fig. 1. Elution profile of MnP activity on Sephadex G-100. (-) A_{280nm}; (●) Mn peroxidase activity; (■) laccase activity.

Table 1. Purification of Mn peroxidase from *Pleurotus pulmonarius.*

Procedure	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	yield (%)	Purification factor
Crude extract	300	619.94	55.5	11.17	100.0	1.00
Acetone precipitation	2	499.46	10.8	46.25	80.6	4.14
Gel filtration Sephadex G-100 (fractions 26-32)	26	176.18	1.3	135.52	28.4	12.13

3.2. Physico-chemical properties of purified MnP and substrate specificity

The purified MnP yielded a single band on denaturing SDS-PAGE, with a molecular mass of 42 kDa (Fig. 2). This molecular mass was confirmed by Sephadex G-100 gel filtration. These results indicate that the enzyme is a monomeric protein.

The purified MnP showed high activity and stability over a broad pH range (4.0-6.0) (Fig. 3). The optimum temperature was found to be 50 ^{o}C, and in the absence of substrate the enzyme was stable at temperatures up to 40 ^{o}C (Fig. 4) At 4 and 8 ^{o}C, the enzyme was stable for at least 2 months. At -20 and at -80 ^{o}C, the enzyme was stable for at least 6 months.

The catalytic parameters of MnP with respect to substrates Mn^{2+} and H_2O_2 *Pleurotus* were determined on the basis of typical Michaelis-Menten behaviour. The K_M values of MnP for Mn^{2+} and H_2O_2 were 19.2 and 16.8 µM, respectively, at pH 4.5.

Data shown in Table 2 reveal that the enzyme was strictly dependent on Mn^{2+} for oxidizing organic substrates such as DMP and ABTS. *P. pulmonarius* MnP oxidized more efficiently ABTS than DMP. Figure 5 shows the effect of H_2O_2 on the activity and stability of purified MnP. Maximal activity was obtained in the presence of 0.5-1.0 mM H_2O_2. The highest concentrations of H_2O_2 inhibited the enzyme. In the presence of 5.0 mM H_2O_2, the enzyme presented 60% of its maximal activity (Fig. 5A). Experiments in which the enzyme was maintained in buffer containing different concentrations of hydrogen peroxide for 2 h reveal that it was stable in the presence of H_2O_2 up to 1.0 mM. In the presence of 2.0 mM H_2O_2, the enzyme presented 50% of its initial activity after 100 min (Fig. 5B).

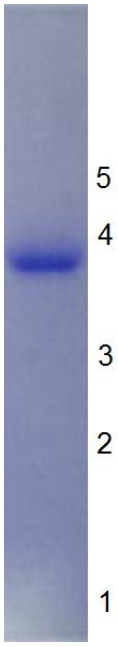

Fig. 2. SDS-PAGE analysis of the purified MnP from *Pleurotus pulmonarius* (Coomassie Blue R-250 staining). Standard proteins: 1- β-lactoglobulin (18.4 kDa); 2- trypsinogen (24 kDa); 3- pepsin (34.7 kDa); 4- ovoalbumin (45 kDa); 5- bovine serum albumin (66 kDa).

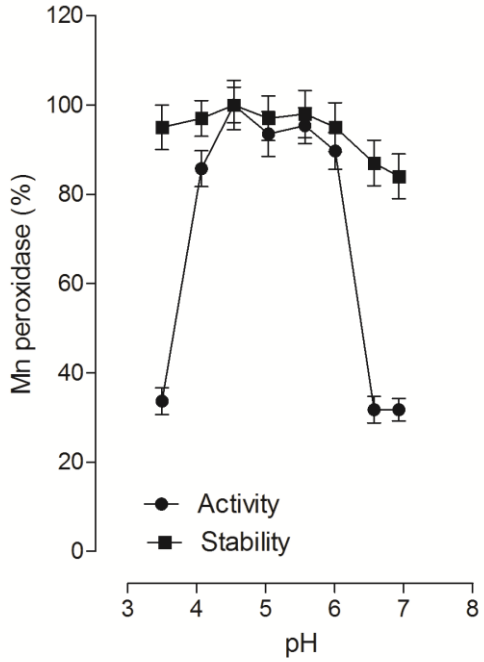

Fig. 3. Effect of pH on the activity and stability of purified MnP from *P. pulmonarius*

Table 2. Comparison of oxidation of DMP and ABTS by purified Mn peroxidase of *Pleurotus pulmonarius*.

Substrate at 1.0 mM	ε_{max} ($M^{-1}cm^{-1}$)	λ (nm)	Mn peroxidase (U)	
			with Mn^{2+}	without Mn^{2+}
ABTS	36000	420	846.92	50.2 (5.9%)
DMP	27500	469	549.83	8.6 (1.5%)

3.3. Effects of cations, high salt concentration and organic solvents on the MnP activity and its capability to decolorize synthetic dyes

Low concentrations (1.0 mM) of Ca^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+} and Fe^{3+} had little or no effect on the MnP activity (Table 3). At 5 mM, the cations inhibited around 50% of the enzyme activity. The enzyme was not inhibited by high salt concentration (1.0 M NaCl or Na_2SO_4). The presence of organic solvents up to 10% did not cause any inhibition. The addition of organic solvents at 20%, caused a reduction in the activity.

MnP was able to decolorize 87% of RBBR and 63% of Congo red under standard conditions, (pH 4.5 and 40 ^oC in the presence of 1 mM $MnSO_4$ and 0.1 mM H_2O_2 (Table 3). In the absence of $MnSO_4$ or H_2O_2 the enzyme was not able to decolorize the dyes. The addition of H_2O_2 alone caused a reduction inferior to 10% in both dyes. Table 3 shows also the effects of salts and organic solvents on the capability of MnP to decolorize both dyes. The enzyme was not affected by the presence of 1.0 M NaCl and Na_2SO_4 and 1.0 mM Ca^{2+}, Hg^{2+}, Cu^{2+} , Ni^{2+} and Fe^{3+}. In the presence of 5.0 mM of these cations and 20% of the organic solvents ethanol, acetone, acetonitrile and isopropanol, the decolorization of RBBR and Congo red was inhibited around 50% (Table 3).

5. DISCUSSION

The broad potential industrial applications of Mn peroxidase have stimulated investigations for finding convenient substrates to obtain large amounts of low-cost enzymes and finding robust enzymes able to act over a wide range of conditions (extreme pH, temperature, osmolarity, pressure, organic solvents, etc). In this context, solid state cultivation appears as an interesting option for fungal culturing thanks the high enzyme productivity, low cost and recent improvements in reactor designs [27,28]. In this work, the Mn peroxidase produced by *P. pulmonarius* cultured on pineapple waste under solid state conditions was ourified for the first time to apparent eletrophoretic homogeneity and its physico-chemical properties compared to other well-known enzymes.

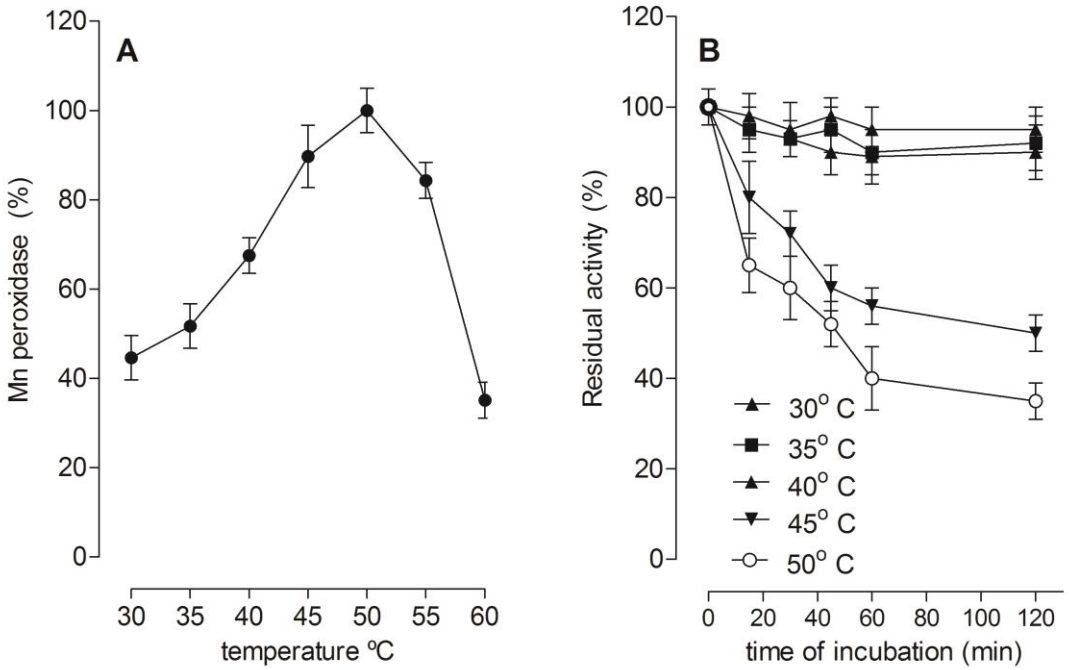

Fig.. 4. Effect of temperature on the activity and stability of purified MnP from *P. pulmonarius*

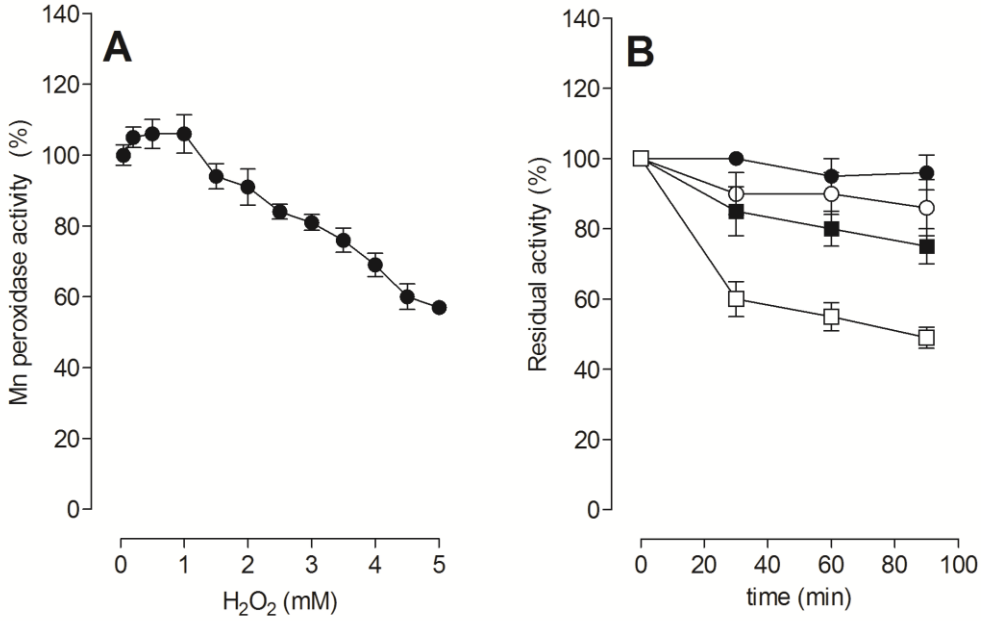

Fig. 5. Effect of hydrogen peroxide in the activity (A) and in the stability (B) of purified *P. pulmonarius* MnP. In B, the enzyme was maintained in the presence of H_2O_2 at 0.25 mM (●), 0.5 mM (○), 1.0 mM (■) and 2.0 mM (□) and assayed in standard condition.

Table 3. Effect of salts and organic solvents on *P. pulmonarius* Mn peroxidase activity and its capability to decolorize synthetic dyes

Chemical		Relative activity (%)	Decolorization (%)	
			RBBR	Congo red
Control		100±7	87±7	63±5
$CaCl_2$	1 mM	96±6	86±9	66±7
	5 mM	74±5*	65±8*	46±5*
$HgCl_2$	1 mM	91±7	83±6	61±8
	5 mM	53±5*	56±7*	33±4*
$CuSO_4$	1 mM	102±6	87±6	62±8
	5 mM	48±3*	33±5*	23±3*
$NiCl_2$	1 mM	95±5	85±7	63±4
	5 mM	44±3*	32±5*	23±5*
$FeCl_3$	1 mM	94±6	81±9	60±7
	5 mM	42±3*	32±4*	33±5*
NaCl	0.5 M	98±6	85±9	61±6
	1.0 M	97±8	87±8	63±8
Na_2SO_4	0.5 M	98±8	87±6	63±7
	1.0 M	99±5	87±8	67±4
Acetone	1%	97±6	83±6	67±4
	5%	95±4	82±5	65±5
	10%	87±6	73±6	60±3
	20%	61±4*	57±3*	32±3*
Isopropanol	1%	119±10	80±5	65±4
	5%	108±8	80±5	60±2
	10%	86±5	86±3	63±2
	20%	67±2*	51±3*	32±1*
Ethanol	1%	103±8	84±5	60±6
	5%	97±2	84±4	65±2
	10%	74±3	50±4	60±3
	20%	61±2*	42±4*	40±4*
Acetonitrile	1%	100±3	85±5	65±3
	5%	98±5	84±6	60±2
	10%	88±7	79±2	58±2
	20%	52±3*	45±2*	34±2*

*differ significantly from control ($p \leq 0.05$), *t* test

The molecular mass of *P. pulmonarius* MnP fell within the range reported for the MnP family (38-50 kDa) and it was similar to the molecular mass of the MnP from *Pleurotus ostreatus* and *Pleurotus eryngii* (Table 4).

Concerning the effect of pH and temperature on the activity and stability of MnP, the enzyme of *P. pulmonarius* did not differ from the MnP of other white-rot fungi: MnP are described as active between pH 2.0 and 6.0, with an optimum between 4.0 and 4.5 [4,9], and they are maximally active at 35-45 ^{o}C and stable at temperatures up to 40 ^{o}C [9,12,14]. An exception of this behaviour is the MnP of *Irpex lacteus*, which presents high activity at 70 ^{o}C [30].

The Michaelis-Menten constants (K_M) of *P. pulmonarius* MnP found in this work for H_2O_2 and Mn^{2+} of 19.2 and 16.8 μM, respectively, are in agreement with values found for other *Pleurotus* spp (Table 4).

The genus possesses both Mn dependent peroxidase and versatile peroxidase [31]. The MnP gene family (mnps) of *P. ostreatus*, for example, comprises five Mn^{2+} dependent peroxidases and four versatile peroxidases [2]. The *P. pulmonarius* MnP purified in this work can be classified as a true Mn^{2+} dependent peroxidase, because in the absence of Mn^{2+}, it was unable of oxidizing organic substrates.

P. pulmonarius MnP was able to decolorize efficiently the synthetic dyes RBBR and Congo red. This enzyme has four important properties to indicate its practical use in biological decolorization of dye effluents. Firstly, the enzyme is relatively stable and active in the presence of increasing amounts of hydrogen peroxide. In general, the rate and efficiency of peroxidase-catalysed reactions are affected by the progressive inactivation of the enzyme by the substrate H_2O_2. This process, referred to as mechanism-based inhibition or suicide inactivation, common for peroxidases in general, limits the potential use of these enzymes in industrial-scale conversion processes [9,30,32]

Table 4. Comparison of molecular weight and K_M of Mn peroxidases from *Pleurotus* spp

Microorganism	MW (kDa)	K_M (μM)		Ref.
		Mn^{2+}	H_2O_2	
Pleurotus pulmonarius	42.0	16.8	19.2	This work
Pleurotus pulmonarius	45.0	10.0	7.0	[22]
Pleurotus eryngii – MP-1	43.0	6.0	20.0	[29]
Pleurotus eryngii MP-2	43.0	10.0	20.0	[29]
Pleurotus ostreatus MnP-2	41.0	83.8	29.3	[4]
Pleurotus ostreatus MnP-3	47.0	14.0	29.4	[4]

The inactivation of MnP by high amounts of hydrogen peroxide is a well-known phenomenon [33] and has also been considered as the explanation for the ephemeral peak of MnP activity during cultivation of white rot fungi, including *P. pulmonarius* [34]. Secondly, P. *pulmonarius* is tolerant to the presence of organic solvents such as ethanol, acetone,

methanol, and acetonitrile, even at high concentrations of 20%. The stability of MnP in the presence of different organic solvents was reported for a few peroxidases, such as the enzymes of *Lentinula edodes* [9] *Irpex lacteus* [30] and *Ganoderma lucidum* [12]. Thirdly, the enzyme was tolerant to a series of different metals, such as Ca^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+} and Fe^{3+}. Finally, the enzyme and its capability to decolorize synthetic dyes were not affect by 1.0 M NaCl and Na_2SO_4. These salts are usually found in textile wastewaters. Besides peroxidases, laccases from white-rot fungi have been extensively studied in the biological decolorization of synthetic dyes [35]. However, it is well-known that laccases are inhibited by chloride ions, making it difficult to be used in the treatment of dye effluents [36-37]. In this context, MnPs active and stable at high salt concentration, such as that one described in this paper, can be more properly useful for this purpose.

CONCLUSION

Manganese peroxidases have promising potential uses in industrial-scale catalytic conversion due to the combination of their catalytic efficiency and capability to oxidizing several phenolic and non phenolic xenobiotics. In this report, a Mn peroxidase from *P. pulmonarius* produced in an inexpensive medium using pineapple peel as substrate was for the first time isolated, characterized and applied in the decolorization of azo and anthraquinonic dyes. The enzyme showed high activity and stability in the presence of organic solvents such as acetone, ethanol, isopropanol and acetonitrile, and was able to completely decolorize the anthraquinonic dye remazol brilliant blue R and the azo dye Congo red in the presence of Na_2SO_4 and NaCl. Considering that the textile effluents possess elevated amounts of salts, the properties of *P. pulmonarius* MnP certainly make the enzyme a good agent for textile dye effluent treatment

LIST OF ABBREVIATIONS

ABTS=2,2'-azino-bis[3-ethyl-6-benzothiazolinesulfonate]
DMP=2,6-dimethoxyphenol
MnP= manganese peroxidase
PDA=potato dextrose agar
RBBR=remazol brilliant blue R
SDS-PAGE=sodium dodecyl sulfate polyacrylamide gel electrophoresis
WRF=white-rot fungi

CONFLICT OF INTEREST

The authors declare no conflict of interest. The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant 477825/2012-5 for funding this study. A. Bracht and R.M. Peralta research grant recipients of CNPq.

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Effects of carbon sources and time of cultivation on the antimicrobial activities of intra and extracellular extracts of *Pleurotus pulmonarius* cultured in submerged conditions.

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ABSTRACT

Pleurotus spp are edible mushrooms that have also high nutritional and medicinal value. The aim of the present study was to evaluate the antimicrobial activities of *Pleurotus pulmonarius* intra- and extracellular extracts, obtained in submerged cultures using different carbon sources (glucose, cassava starch and cassava bagasse) and cultivation times of up to 8 days. The antimicrobial activities were characterized by evaluating the zone of inhibition, the minimal inhibitory concentration and the bactericidal concentration (MIC and MBC, respectively). The results showed that highest antimicrobial activities were found in 6 day mycelial extracts obtained in cultures using glucose and starch as carbon source. These extracts presented considerable antimicrobial activity against *Escherichia coli, Salmonella enterica, Staphylococcus aureus, Bacillus subtilis and Bacillus cereus.* The same extracts were also able to inhibit growth of *Candida albicans.* These results show that the mycelial extracts of *P. pulmonarius* are a promising natural source of antimicrobial agents.

Keywords: *Pleurotus pulmonarius*, medicinal mushroom, oyster mushroom, antimicrobial activity, antifungal activity.

1 – INTRODUCTION

Pleurotus spp are famous for owning all three properties expected from a food, nutrition, taste, and physiological functions, being thus appreciated for both their sensory characteristics and outstanding nutritional profile (Corrêa et al., 2016). Both basidiomata and mycelium of mushrooms contain different groups of compounds such as terpenoids, lactones, organic acids, steroids, polyphenols, tocopherols, flavonoids, phenolics,

alkaloids, polysaccharides and dietary fibers. These compounds possess antioxidant, antineoplastic, antitumor, immunomodulatory and anti-inflammatory activities and are responsible by the functional and medicinal properties of *Pleurotu*s spp (Corrêa et al., 2016).

Furthermore, an antimicrobial activity was also reported for both the basidioma and the mycelium of various species of the genus such as *P. nebrodensis* and *P. eryngii* (Schillaci et al., 2013), *P. djamor* (Dharmaraj et al., 2014) *P. ostreatus* (Younis et al., 2015), *P. ostreatoroseus* (Corrêa et al., 2015) and *P. pulmonarius* (Adebayo et al., 2012).

The production of basidiomata by *Pleurotus* spp may take months. An alternative for obtaining bioactive compounds in shorter periods could be to explore the mycelial biomass obtained from submerged cultures (Ragunathan & Swaminathan, 2003; Elisashvili, 2012). The aim of the present study was to evaluate the antimicrobial activities of *Pleurotus pulmonarius* intra- and extracellular extracts, obtained in submerged cultures using different carbon sources (glucose, cassava starch and cassava bagasse) and cultivation times of up to 8 days.

2- EXPERIMENTAL

2.1. Cultivation of *P. pulmonarius* in submerged conditions and obtainment of intra- and extracellular extracts

P. pulmonarius CCB 19 was obtained from the Culture Collection of the Botany Institute of São Paulo, Brazil. It was cultured on potato dextrose agar (PDA) medium for 1 week at 28 °C. Stock cultures were maintained on agar-potato-dextrose plates (PDA) and stored at 4 °C for up to 2 months. The cassava bagasse was dried on oven at 40 °C under air circulating and subsequently milled in a knife mill. Three mycelia discs (Ø 10mm) were inoculated in 150 mL of liquid medium containing potato extract (200 g/L), glucose (15g/L) and Vogel mineral solution (Vogel, 1956) and maintained under agitation of 120 rpm at 28^{o} C. After 5 days, around 5.7 g of the moist mycelium (± 0.254 g mycelia dry weight) were transferred to a 1000 mL Erlenmeyer flask containing 300 mL of a medium consisting in 3.4% carbon source (cassava bagasse, cassava starch or glucose), 0.2% of NH_4NO_3 and Vogel salts without nitrogen source. The cultures were maintained for up to 8 days at 28 ^{o}C under agitation of 120 rpm. Fungal biomass and extracellular material were

separated by centrifugation at 5000 rpm for 10 min at 4 °C. The fungal biomass was washed twice with cold distilled water, pressed between sheets of paper and frozen. Equal amounts frozen fungal biomass of each cultivation was macerated with glass beads, centrifuged at 10,000 rpm, for 15 min at 4 °C, and the supernatant was subsequently used as the intracellular extract. Both intra- and extracellular extracts were lyophilized and stored in a freezer (-20 °C) until use.

2.2. Antimicrobial activity

2.2.1. Determination of antimicrobial activity by the disk diffusion test

The bacteria were grown in Mueller Hinton Agar (Merck) and Mueller Hinton Broth (Merck) and the yeasts were grown in PDA and Sabauround Broth. The following Gram-negative bacteria were used: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 7966), *Salmonella enterica* (ATCC 13076), *Klebsiela pneumoneae* (ATCC 700603), *Aeromonas hydrophila* (ATCC 7966). The following Gram-positive bacteria were used: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6051), *Bacillus cereus* (INCQS 00003). Two fungi were used: *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae.* All microorganisms were obtained from the Microbiology Laboratory of Water, Environment and Food of the State University of Maringá.

The disk diffusion test was performed on Muller-Hinton agar for bacteria and Muller-Hinton agar medium with 2% dextrose and 0.5 μg/mL methylene blue for yeasts, as recommended by NCCLS M2-A8 and NCCLS M44-A2, respectively. The disks containing extracts of *P. pulmonarius* were produced using 6 mm filter paper discs containing 10 μL of a solution with 100 mg/mL extract. In order to standardize of the inoculum samples were collected from three to five colonies of each organism and suspended in 3.0 mL of a physiological saline solutions (0.85%). The turbid suspension was then visually compared with the standard 0.5 of the McFarland scale, equivalent to 10^8 CFU/mL for bacteria and 10^6 CFU/mL for yeasts. Subsequently, the suspensions were sown and the discs placed on the agar. The plates were incubated at 37°C for bacteria and 35°C for yeasts for 24 hours. After this period the halos were measured. The experiment was performed in duplicate in

three independent events. Disks containing gentamicin (10 μg) (Laborclin), vancomycin (30 μg) (Laborclin), chloramphenicol (30 μg) (Laborclin), for bacteria and fluconazole (25 μg/ml) for yeasts, were used ass controls. The halos were measured after 24 hours of incubation.

2.2.2. Determination of antimicrobial activity by the micro dilution method

The antimicrobial activity was determined as the minimum inhibitory concentration (MIC), which was determined using the micro dilution methodology as described in the NCCLS M7-A6 with slight modifications. Different concentrations of intra- and extracellular extracts of *P. pulmonarius* CCB19 (extract final concentrations from 0.39 to 100 mg/mL) were prepared by serial dilution in broth media specific for each microorganism, Muller Hilton broth (Merck) for bacteria and Sabauround broth for yeasts. In order to standardize of the inoculum, samples were collected from three to five colonies of each organism and suspended in 3.0 mL of a physiological saline solution (0.85%). The turbid suspension was then visually compared with the standard 0.5 of the McFarland scale, equivalent to 10^8 CFU/mL for bacteria and 10^6 CFU/mL for yeasts. In the micro dilution test in 96-well plates, bacteria samples containing 10^8 CFU/mL were removed and placed in 0.1 mL of 0.85% physiological saline solution. To each well containing 190 μL of extract, it was added 10 μL of inoculum. Then, each well contained 10^5 CFU/mL. Sample 10^6 CFU/mL of yeast several dilutions were made in culture medium in order to obtain the inoculum used 2 times more concentrated in the test. In each well containing 100 μL of sample, it was added 100 μL of inoculum coming up to the desired final concentration of 0.5×10^3 at 2.5×10^3 cells/ml. They also performed the control of the medium, control of yeast and bacterial growth, and a negative control for each extract. The antibiotics gentamicin and streptomycin and antifungal fluconazole were used as positive controls. The 96 well plates were incubated for 24 hours in an oven 37 °C for bacteria and 35 °C for yeasts and optical performed in a spectrophotometer at 630 nm. The MIC was given the lowest concentration of extract that inhibited growth of the microorganism.

The Minimum Bactericidal Concentration (MBC) was determined based on the Santurio methodology (2007), whereas the wells in which no visible bacterial growth was removed from an aliquot of 10 μL and plated on agar surface Muller Hilton incubated at 37 °C for

24 hours, the MBC was defined as the lowest concentration of the extract able to cause the death of inoculum. To determine the Minimum Fungicidal Concentration (MFC), an aliquot of 10 μL of each micro plate well was plated on plates containing Sabouraud dextrose agar. After incubation then for 48 h at 37 °C, the MFC was defined as the lowest concentration of drug capable of causing the death to 100% of fungal cells. This was demonstrated by the absence of colonies in plate.

2.3. Statistical analysis

All analyses were performed in triplicate. The data were expressed as means ± standard deviations. Data were analyzed using GraphPad Prism 6.0 program.

3. RESULTS

3.1. Antimicrobial activity by diffusion test and micro dilution method

The intra- and extracellular extracts of *P. pulmonarius* were tested against several Gram-positive and Gram-negative bacteria on agar disc diffusion method (Table 1). Extracts producing inhibition zones greater than 10 mm were considered as highly active. Both intra- and extracellular extracts can be classified as highly active, especially against the Gram negative bacteria *E. coli* and *S. enteric*, and the Gram positive bacteria, *S. aureus*, *B. subtilis* and *B. cereus.*

Considering the results above, the minimal inhibition concentration (MIC) and the minimal bactericidal concentration (MBC) were determined for the five most sensitive bacteria. The results are presented in Table 2. The results showed that highest antimicrobial activities were found in 6 d-mycelial extracts obtained in submerged cultures using glucose and starch as carbon sources.

3.2. Antifungal test

Antifungal test of the extracts were performed against *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (Table 3). The extracts were not effective for *Saccharomyces cerevisiae* but for *Candida albicans* the best result (disc diffusion) was the

6d-intracellular extract obtained in cultures using starch as substrate. The extract able to inhibit 50% of *Candida albicans* was 2d-extracellular extract obtained using glucose as substrate (75.0 mg/mL) and the 6d-intracellular extract obtained in cultures using starch as substrate (75.0 mg/mL). There were no fungicidal effects on the maximum concentration tested.

4. DISCUSSION

Infectious diseases are the second cause of death worldwide and the research for active compounds for treatment of these diseases is required (WHO, 2012). Since antibiotic resistance among bacteria has been increasing drastically, new antibacterial drugs are needed and the mushrooms could be a natural source of antibiotics. Among *Pleurotus* spp, the majority of studies of antimicrobial activity was conducted using both mushroom and mycelia of *P. ostreatus* (Barak and Sadik, 2014, Sala et al., 2015, Vamanu, 2012, Younis et al., 2015). The number of investigations on the antimicrobial activity of *P. pulmonarius* is still small (Adebayo et al., 2012). In the present study 100 mg/disc of an aqueous 6d-*P. pulmonarius* extract, obtained using different substrates, were effective against the Gram-positive bacteria *B. subtilis* (26.0 ± 1.2 mm), *S. aureus* (33.0 ±1.4 mm), and *B. cereus* (8.0±0 mm) and against the Gram-negative bacteria *Salmonella enteric* (22.5±3.5 mm) and *E. coli* (16.5 ± 2.1 mm). For comparative purposes, Sala et al. (2015) evaluated the antimicrobial activity of hexane, chloroform and ethyl acetate extracts of *P. ostreatus* at a concentration of 500 mg/disc and found that the hexane extract was active against the Gram-positive bacteria *B. subtilis* (12.80 ± 0.3 mm) and *S. aureus* (20.21 ± 0.7 mm), while no antimicrobial activity was detected against *B. cereus.* In the same work, a *P. ostreatus* chloroform extract was effective against *Salmonella paratyphi* (16.32 ± 0.6 mm) and *Escherichia coli* (17.00 ± 0.1 mm). These data allow concluding that *P. pulmonarius* extracts can be as effective as *P. ostreatus* extracts as antimicrobial agents.

It is well known that Gram-negative bacteria are highly resistant to many antibiotics and our findings are consistent with this observation. When antimicrobial activity against Gram-negative bacteria was evaluated, two out of five Gram-negative bacteria tested were sensitive to the extract, and only for *E. coli* it was possible to determine a MBC value. On

the other hand, all Gram-positive bacteria were sensitive to the aqueous *P. pulmonarius* extract.

In this work we found that the antimicrobial activity of the extracts of *P. pulmonarius* varied according to the source of carbon and the cultivation time. A similar dependence on the type of nitrogen source of the antimicrobial activity was reported previously for *Pleurotus ostreatus* cultured in submerged conditions (Vamanu, 2012). These results suggest that the production of antimicrobial molecules by mushrooms can be regulated by the carbon source, the nitrogen source, or both.

Both high molecular weight metabolites, such as proteins and polysaccharides and low molecular weight compounds, such as the sesquiterpenes and other terpenes, steroids, anthraquinones, benzoic acid derivates, and quinolones, can be responsible by antimicrobial activity of mushrooms and could be used for the development of new antimicrobial drugs (Alves et al., 2012). In fact, numerous bioactive compounds with antimicrobial activity have been identified from the fruiting bodies, cultured mycelia and culture filtrates of *Pleurotus* sp and include polysaccharides (Llauradó et al., 2015), fatty acid esters (Suseem and Saral, 2013) and an organic acid identified as 3-(2-aminophenylthio)-3-hydroxypropanoic (Younis et al., 2015).

In conclusion, aqueous mycelial extracts of *P. pulmonarius* seems to be a promising natural source of antimicrobial agents. Experiments must be conducted to identify the molecules responsible for this activity.

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Table 1. Preliminary antimicrobial testing of *Pleurotus pulmonarius* extracts through determination of zone of inhibition (mm ± SD)*

Extracts		Extracellular extracts					Intracellular extracts				
		Gram-negative		Gram-positive			Gram-negative		Gram-positive		
		E.coli 25922	*S. enteric* 13076	*S. aureus* 25923	*B. subtilis* 6051	*B. cereus* 00003	*E.coli* 25922	*S. enteric* 13076	*S. aureus* 25923	*B. subtilis* 6051	*B. cereus* 00003
2 days	Glucose	--	7.0±1.4	20.5±0.7	16.0±1.4	--	--	--	--	--	--
	Starch	7.5±0.7	16.5±0.7	26.5±0.7	26.5±0.7	--	--	--	--	--	--
	Bagasse	--	--	--	--	--	--	16.5±2.1	28.0±1.4	20.5±0.7	--
4 days	Glucose	--	--	--	--	--	--	--	--	--	--
	Starch	10.5±0.7	16.5±2.1	24.5±0.7	22.0±1.4	--	--	--	--	--	--
	Bagasse	11.0±1.4	18.0±1.4	26.5±0.7	25.5±0.7	--	--	--	10.5±2.1	--	--
6 days	Glucose	--	10.5±0.7	20.5±3.5	24.5±0.7	--	15.5±0.7	22.5±0.7	31.0±1.4	26.0±4.2	8.0±0
	Starch	--	9.5±0.7	25.5±0.7	22.5±0.7	--	16.5±2.1	22.5±3.5	33.0±1.4	17.0±1.4	7.0±0
	Bagasse	--	10.0±1.4	25.5±0.7	22.0±1.4	--	--	16.5±2.1	25.5±0.7	24.0±0	--
8 days	Glucose	--	--	--	--	--	12.0±1.4	17.0±1.4	28.5±2.1	28.5±0.7	--
	Starch	--	--	12.5±0.7	20.5±0.7	--	--	8.0±1.4	21.5±2.1	23.5±0.7	--
	Bagasse	--	--	17.5±0.7	23.5±2.1	--	--	7.5±0.7	19.0±1.4	21.5±2.1	--
Chloramphenicol		33.5±2.1	32.0±1.4	29.5±0.7	32.5±1.5	20.0±0.5					
gentamicin		25.0±0	14.0±0	24.5±2.1	29.0±1.0	29.0±1.0					
Vancomicin		9.5±2.1	7.5±0.7	20.0±1.4	23.0±2.0	18.5±0.5					

* The diameters of zone of inhibition were expressed in millimeter (mm) as mean ± stand deviation (SD).

Table 2. Antimicrobial activity extracellular and intracellular extracts obtained by using three different carbon sources and days of cultivation. Values are of 3 separate determinations, each in triplicate.

			Gram-negative				Gram-positive					
			E. coli 25922		*S. enteric* 13076		*S. aureus* 25923		*B. subtilis* 6051		*B. cereus* 00003	
			MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml
Extracellular Extracts	2 days	Glucose	--	--	100.0	UN	12.5	25.0	6.25	25.0	--	--
		Starch	25.0	UN	100.0	UN	12.5	UN	6.25	25.0	--	--
		Bagasse	--	--	--	--	--	--	--	--	--	--
	4 days	Glucose	--	--	--	--	--	--	--	--	--	--
		Starch	12.5	100.0	UN	UN	3.12	UN	12.5	UN	--	--
		Bagasse	6.25	100.0	100.0	UN	6.25	100.0	0.39	0.78	--	--
	6 days	Glucose	--	--	100.0	UN	25.0	100.0	3.12	25.0	--	--
		Starch	--	--	100.0	UN	12.5	UN	3.12	25.0	--	--
		Bagasse	--	--	100.0	UN	6.25	25.0	6.25	25.0	--	--
	8 days	Glucose	--	--	--	--	--	--	--	--	--	--
		Starch	--	--	--	--	6.12	12.5	6.25	100.0	--	--
		Bagasse	--	--	--	--	1.56	3.25	3.12	UN	--	--
Intracellular Extracts	2 days	Glucose	--	--	--	--	--	--	--	--	--	--
		Starch	--	--	--	--	--	--	--	--	--	--
		Bagasse	--	--	100.0	UN	6.25	UN	12.5	25.0	--	--
	4 days	Glucose	--	--	--	--	--	--	--	--	--	--
		Starch	--	--	--	--	--	--	--	--	--	--
		Bagasse	--	--	--	--	6.25	12.5	--	--	--	--
	6 days	Glucose	6.25	12.5	100.0	UN	12.5	12.5	3.12	25.0	12.5	25.0
		Starch	6.25	6.25	50.0	UN	50.0	100.0	3.12	6.25	25.0	50.0
		Bagasse	--	--	100.0	UN	12.5	100.0	3.12	25.0	--	--
	8 days	Glucose	12.5	50.0	100.0	UN	3.12	12.5	3.12	100.0	--	--
		Starch	--	--	100.0	UN	3.12	UN	6.25	100.0	--	--
		Bagasse	--	--	100.0	UN	1.56	100.0	0.78	100.0	--	--

UN: undetermined, above 100 mg/mL

Table 3: Preliminary antifungal testing of *Pleurotus pulmonarius* extracts through determination of zone of inhibition (mm ± SD)* for *Candida albicans* ATCC 10231

Extracts		Antibiograma (IDZ mm)		MIC (mg/ml)	
		Extracellular	Intracellular	Extracellular	Intracellular
2 days	Glucose	15.0±0.5	--	75.0	--
	Starch	12.0±0.7	14.0±0.7	UN	UN
	Bagasse	--	--	--	--
4 days	Glucose	--	--	--	--
	Starch	--	--	--	--
	Bagasse	--	--	--	--
6 days	Glucose	--	17.0±0.5	--	100.0
	Starch	--	19.0±1.4	--	75.0
	Bagasse	--	--	--	--
8 days	Glucose	--	--	--	--
	Starch	--	--	--	--
	Bagasse	--	--	--	--
Fluconazole (0.025mg/ml)		21.0±0.7			0.02

* The diameters of zone of inhibition (IDZ) were expressed in millimeter (mm) as mean ± stand deviation (SD). Each disc containing 10 µl of 100 mg/ml of fungi extracts.