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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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A B S T R A C T

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 shows that the mycelial extracts of *P. pulmonarius* are promising natural source of antimicrobial agents.

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, anti-neoplastic, antitumor, immunomodulatory and anti-inflammatory activities and are responsible by the functional and medicinal properties of *Pleurotus* 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* (Schillacietal, 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 and Swaminathan, 2003; Elisashvili, 2012). The aim of the present study was to evaluate the antimicrobial activities of *Pleurotus pulmonarius* intra- and extracellular extracts, obtainedby submerged cultures using different carbon sources (glucose, cassava starch and cassava bagasse) cultivation times of up to 8 days. Cassava bagasses an abundant and easy to obtain agricultural residue, reducing the costs for culturing *P. pulmonarius*.

Materials and Methods

Experimental

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 of frozen fungal biomass, corresponding to both tested carbon sources were macerated with glass beads, centrifuged at 10,000 rpm, for 15 min at 4°C, and the supernatants were subsequently used as the intracellular extracts. Both intra- and extracellular extracts were lyophilized and stored in a freezer (-20°C) until use.

Antimicrobial activity

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 pneumoniae* (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. With the purpose of standardization, 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. 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 as controls. The halos were measured after 24 hours of incubation.

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 (final concentrations varying 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. With the purpose of standardization 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, 10 µL of inoculum was added. In the determination of the antimicrobial activity against yeast, inoculum was added to obtain final concentrations ranging from 0.5 x $10^3$to 2.5 x 10^3 cells/mL. Negative controls without extracts were conducted in the same way. The antibiotics gentamicin and streptomycin and the 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 corresponded to 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 □ L 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 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.

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.

Results and Discussion

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 and the results are presented in 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 show that highest antimicrobial activities are found in 6 d-mycelial extracts obtained in submerged cultures using glucose and starch as carbon sources.

Antifungal test

Antifungal test of the extracts were performed against *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (Table 3). The extracts were not effective against *Saccharomyces cerevisiae* but for *Candida albicans* the best result (disc diffusion) is the 6d-intracellular extract obtained in cultures using starch as substrate. Extracts able to inhibit 50% of *Candida albicans* were the 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 was no fungicidal effect on the maximum concentration tested.

Infectious diseases are the second cause of death worldwide and the research for active compounds for treatment of these diseases is required (WHO, 2012. 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). However, 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 on antimicrobial activity was detected against *B. cereus.* In the same work, a *P. ostreatus* chloroform extract was effective against *S. paratyphi* (16.32±0.6 mm) and *E. 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.

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 extracellular and intracellular activity 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	2days	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	--	--	--	--	--	--	--	--	--	--
	4days	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	--	--
	6days	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	--	--
	8days	Glucose	--	--	--	--	--	--	--	--	--	--
		Starch	--	--	--	--	6.12	12.5	6.25	100.0	--	--
		Bagasse	--	--	--	--	1.56	3.25	3.12	UN	--	--
Intracellular Extracts	2days	Glucose	--	--	--	--	--	--	--	--	--	--
		Starch	--	--	--	--	--	--	--	--	--	--
		Bagasse	--	--	100.0	UN	6.25	UN	12.5	25.0	--	--
	4days	Glucose	--	--	--	--	--	--	--	--	--	--
		Starch	--	--	--	--	--	--	--	--	--	--
		Bagasse	--	--	--	--	6.25	12.5	--	--	--	--
	6days	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	--	--
	8days	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
2days	Glucose	15.0±0.5	--	75.0	--
	Starch	12.0±0.7	14.0±0.7	UN	UN
	Bagasse	--	--	--	--
4days	Glucose	--	--	--	--
	Starch	--	--	--	--
	Bagasse	--	--	--	--
6days	Glucose	--	17.0±0.5	--	100.0
	Starch	--	19.0±1.4	--	75.0
	Bagasse	--	--	--	--
8days	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.

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* ssp. 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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