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ENZYME SCREENING OF DIKARYOTIC CULTURES FROM LIGNOCELLULOLITIC BASIDIOMYCETES (FUNGI) COLLECTED IN SOUTHERN BRAZIL

SCREENING DE ENZIMAS DE CULTURAS DICARIÓTICAS DE FUNGOS BASIDIOMYCETES LIGNOCELULOLÍTICOS COLETADOS NO SUL DO BRASIL

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RESUMO

Dezesseis culturas dicarióticas de Basidiomicetes foram caracterizadas de acordo com suas habilidades em produzir enzimas extracelulares por métodos qualitativos, usando meio de cultura semi-sólido, e por método quantitativo, usando meio de cultura líquido. O desenvolvimento miceliano também foi determinado pelo crescimento radial e pela biomassa produzida. O teste oxidativo usando os meios ácido gálico e ácido tânico, mostrou que 15 culturas dicarióticas são causadoras de podridão branca, somente um resultado negativo foi observado para *Antrodia albida,* um fungo causador da podridão castanha, como esperado. O teste enzimático da gota demonstrou tirosinase em *Phellinus flavomarginatus*, *Rigidoporus lineatus* e *Antrodia albida*. A lacase e peroxidases, avaliadas no teste da gota, foram detectadas em 15 culturas, com exceção de *Antrodia albida*. No meio líquido, no entanto, a lacase foi detectada em todas as culturas, bem como a manganês peroxidase, a lignina peroxidase só foi detectada em *Trametes villosa*, *Ganoderma applanatum* e *Ganoderma* sp.

Palavras-chave: Basidiomycetes, culturas fúngicas, enzimas extracelulares

ABSTRACT

Sixteen dikaryotic cultures from Basidiomycetes were characterized according to their ability to produce extracellular enzymes by a qualitative method using

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semi-solid media and a quantitative method using liquid media. Mycelial behaviour through radial growth and biomass production were also tested. The oxidative test with gallic and tannic acid media, showed that 15 dikaryotic cultures were white-rot fungi, while negative results were observed for *Antrodia albida,* a brown-rot fungus, as expected. The drop enzyme tests showed tyrosinase was present in *Phellinus flavomarginatus*, *Rigidoporus lineatus* and *Antrodia albida*. Other enzymes used with this drop test were laccase and peroxidases, which were detected in 15 strains, the exception being *Antrodia albida.* In liquid media, however, laccase was distinguished in all cultures, as was manganese-peroxidase, lignin peroxidase was detected only in *Trametes villosa*, *Ganoderma applanatum* and *Ganoderma* sp.

Key words : Basidiomycetes, fungal culture, extracellular enzymes

INTRODUCTION

Basidiomycetes capable of lignin degradation are collectively referred to as white-rot fungi. They are among the few groups of organisms capable of depolymerizing, degrading, and mineralizing all components of plant cell walls including cellulose, hemicellulose, and the more recalcitrant lignin (Kersten & Cullen 2007).

According to Vicentim & Ferraz (2007), the capacity to degrade lignin stems from extracellular enzymatic action and low molecular mass compounds (LMMCs) as demonstrated in *Ceriporiopsis subvermispora* (Pilát) Gilbn. and Ryv. The major enzymes associated with lignin degradation, referred to as lignin–modifying enzymes or LMEs are two glycosylated heme-containing peroxidases, lignin peroxidase and manganese dependent peroxidase (MnP) (Orth & Tien 1993), and a cooper-containing phenoloxidase, laccase (Pointing *et al.* 2005). Some white rot fungi produce all these enzymes while others produce only one or two of them (Hatakka 1994).

Lignin peroxidase was detected in 1983 from *Phanerochaete chrysosporium* (Glenn *et al.* 1983; Tien & Kirk 1984) and is capable of catalyzing oxidation of nonphenolic aromatic lignin model compounds and releasing small amounts of CO_2 from lignin. Most white-rot fungi produce MnP and laccase. MnP oxidizes Mn^{2+} to Mn^{3+} and is dependent on manganese for its activity, laccase is a copper-containing phenol oxidase that oxidizes phenols to quinones with the concomitant reduction of oxygen to water (Hatakka 1994, Kirk & Farrell 1987).

The nonspecific nature and extraordinary oxidation potential of the peroxidases have attracted considerable interest in the development of bioprocesses such as fiber bleaching and the remediation of organopollutant contaminated soils and effluents (Kersten & Cullen 2007). *Trametes versicolor* is an excellent model for these activities with proven bioremediation capacity.

Other enzymes such as tyrosinases are also connected with lignin degradation. Tyrosinases are phenoloxidases that catalyze the transformation of a large num-

ber of phenolic and non-phenolic aromatic compounds. They are used for various applications, including synthetic and analytical purposes, bioremediation, wastewater treatment, and wine stabilization (Durán *et al.* 2002)

Frequently, *in vitro* production of LMEs has been recorded for fungal species using both defined liquid growth (Pointing *et al.* 2005) and agar growth media (Lowe 1992), although different responses have been observed between taxa. Gallic and tannic acid medium is used to show overall phenoloxidase activity: when these enzymes are produced a brown diffusion zone appears in the agar (Pointing, 1999). The drop-test is, in fact, a combination of different methods (Käarik 1965; Harkin & Obst 1973; Taylor 1974; Stalpers 1978) for detection of particular enzymes, like tyrosinase, peroxidases and laccases.

The goal of the present work was to screen 16 strains of Basidiomycetes (including previously untested species), isolated from basidiomata collected in Santa Catarina (southern Brazil), testing them for extracellular enzymes.

MATERIALS AND METHODS

Fungal strains. 16 dicaryotic strains (Basidiomycetes) were studied (table 1). Cultures are preserved on potato dextrose agar (PDA) at the Fungal Culture Collection, Laboratory of Mycology, Department of Botany, Federal University of Santa Catarina, Brazil.

Enzyme test in semi-solid medium. Strains were inoculated on Petri dishes (9.0 cm) with 2 mL malt-extract agar (MEA) at 28 ±1°C for 7 days in the dark. Five Petri dishes for each culture were tested.

Gallic acid and tannic acid tests. These tests were carried out with the addition of tannic acid or gallic acid to extract malt agar media. Macroscopic observation of darkening of the media was interpreted as evidence of a positive oxidative reaction.

Drop-test. A drop of reagent was used for each enzyme tested, which included laccase, peroxidase and tyrosinase, following Käarik (1965), Harkin & Obst (1973), Taylor (1974) and Stalpers (1978). The presence of enzymes was detected by a color change in each reagent. These observations were made immediately, and after 3, 24 and 72 hours.

Enzymatic test in liquid media. After a 7 days incubation period in Petri dishes, some mycelial discs (5 mm diameter) were removed from the apical mat growth zones developed and inoculated in Erlenmeyer flasks (20 mL of potato dextrose broth - PDB). These Erlenmeyer flasks were incubated at 28 ±1°C for 7 days in dark. The cultures were filtered through filter paper (Schleicher and Schuell – 0.45 µm) and assayed to determine enzyme level. These defined the amount of filtered extract that was used as enzyme activity control, after being boiled for 10 min.

Enzyme assays. These assays were performed spectrophotometrically (GBC916-UV visible). Laccase activity was determined by monitoring the oxidation of ABTS (2,2´-azinobis 3-ethylbenzthiazoline-6-sulfonic acid) at 420 nm (ε = 36.000 $M^{-1}.cm^{-1}$) (Buswell *et al.* 1995). Lignin peroxidase (LiP) activity was determined by monitoring the oxidation of veratryl alcohol to veratraldehyde (ε = 9.300 $M^{-1}cm^{-1}$) as indicated by an increase in A_{310} (Tien & Kirk 1984). Mn-dependent peroxidase (MnP) was determined by monitoring the oxidation of phenol red at 610nm (Kuwahara *et al.* 1984). Enzymatic activities were reported in IU per culture instead IU kg^{-1} of substrate.

Radial growth and biomass production. The radius of each strain was measured after two weeks of growth in 20 mL malt-extract agar (MEA) at 28 ±1°C for 7 days in the dark. Biomass was calculated by vacuum filtration of the liquid culture. After filtration, filters with mycelia were dried at 80°C for 48 hours and weighed.

Statistical analysis. The enzyme activity data (except lignin peroxidase) were analyzed by analysis of variance and Tukey test using a statistics computer program (Statistica®). Lignin peroxidase activity, radial growth and biomass production were calculated by media values.

RESULTS AND DISCUSSION

In the tannic and gallic acid test, 15 strains produced oxidative extracellular enzymes, the exception being *Antrodia albida* as expected. The presence of oxidases demonstrated that those 15 strains were of white-rot fungi, which degrade lignin, cellulose and hemicellulose completely (Griffin 1997; Worrall *et al.* 1997). The absence of a reaction in *A. albida* demonstrated no production of oxidases and confirmed that it is a brown-rot fungus. In brown-rot fungi the enzymes xylanase and cellulase, involved in degradation of holocellulose, are not oxidative, and accordingly they were not detected in this test.

In the drop tests, laccase and peroxidase were not only detected in *A. albida*. Among white-rot fungi tested to date, peroxidase and laccase enzymes are produced (Chung *et al.* 2000). Pointing *et al.* (2005) studying *Ganoderma lucidum*, *Trametes versicolor* and others basidiomycetes reported similar results. In *Ganoderma* species Hseu *et al.* (1989) also detected these enzymes. For *A. albida* a negative reaction is expected, because in brown-rot fungi, these enzymes are not produced or only in quantities undetectable by this test.

The enzyme tyrosinase was recognized in *Phellinus flavomarginatus*, *Rigidoporus lineatus* and *A. albida*. In earlier cultural studies tyrosinase was detected by Stalpers (1978) in *P. flavomarginatus* e *R. lineatus*. The strain of *P. flavomarginatus* has also been studied by Fernandes and Loguercio-Leite (2003) who confirmed those results. This enzyme may be found in white and brown rots (Käarik 1965). Positive tyrosinase reactions are infrequent, but have been observed in other white-rot fungi,

such as *Armillaria gemina*, *Phlebia tremellosa*, *Trametes versicolor* and *Auricularia auricula-judae* (Worrall *et al.* 1997).

Enzymatic test in liquid media. The only enzymes tested in liquid media were laccase, lignin peroxidase and manganese-peroxidase. Lignin peroxidase was detected in *Trametes villosa*, *Ganoderma applanatum* (909, 907 and 125) and *Ganoderma* sp. with low activities (table 2).

Table 2. Activity of LiP in liquid media.

Species and strains	LiP activity UI/L
Trametes villosa- 523	1.074
Ganoderma applantum-125	0.269
Ganoderma applantum- 909	0.430
Ganoderma applantum-907	0.215
Ganoderma sp -885	1.611

This enzyme is commonly produced by *Phanerochaete chrysosporium* and *Trametes versicolor* in significant amounts. Specific growth conditions are generally used for LiP production by *P. chrysosporium* (Pélaez *et al.* 1995), and the use of co-substrate is frequent (Enoki *et al.* 1999). New studies reveal that this enzyme is not essential for lignin degradation. A new lignin peroxidase assay using the dye azure B indicates that secreted lignin peroxidases do not play a role in the *T. versicolor* pulp-bleaching system (Archibald 1992).

Laccase activity levels were highest in *R. lineatus* (58 UI/L); *G. applanatum/907* (30 UI/L), *T. villosa* (19UI/L) and *G. applanatum/*909 (16UI/L). Laccase activity levels were lower in *A. albida* (0.05 UI/L), *S. commune* (0.16 UI/L), *Ganoderma* sp. (0.67 UI/L).

Activities of laccase in this study (graphic 1), for cultures of *Phellinus*, *Trametes* and *Ganoderma* were similar to those obtained by Pélaez *et al.* (1995) and to the results observed by Fernandes and Loguercio-Leite (2003) for *P. flavomarginatus* strains. Pélaez *et al.* (1995) did not detected laccase in their *Schizophyllum communis* strain.

Higher levels of laccase activity are widespread in *Trametes* species, especially in *Trametes versicolor*, when carbon and nitrogen sources are used (Jang *et al.* 2002). Laccase was observed in different *Ganoderma* species, with quantities varying according to strain. For instance *Ganoderma lucidum* produced higher levels of laccase in defined conditions (D'Souza *et al.* 1999).

MnP enzymatic behaviour was detected in all cultures but in different amounts (graphic 2), the *A. albida* strain being at a lower level (126 UI/L). It is nota-

ble that MnP activity appeared frequently among species of *Phellinus* (Pélaez *et al.* 1995). MnP production by *Ganoderma* in this study is similar to that of *Ganoderma australe* (Peláez *et al.* 1995).

Enzymatic levels obtained here for *T. villosa* were higher than those in other *Trametes* studied previously, as for example in *Trametes hirsuta* (Pélaez 1995) and *Trametes trogii* (Levin & Forchiassin, 2001).

Simultaneous production of laccase and MnP was observed for all cultures; demonstrating that these fungi could be useful for studying co-operation between oxidases and peroxidases during lignin degradation.

In this work, although no additional chemicals were used to stimulate enzymatic production, laccase and MnP production was significant in most of the strains studied.

As expected, LiP and MnP enzymes were isolated from ligninolytic cultures of several other white-rot fungi belonging to the *Homobasidiomycetes*, such as *Trametes (Coriolus) versicolor*, *Bjerkandera adusta*, and *Phlebia radiata* (reviewed by Hatakka, 1994). The information accumulated to date indicates that the pre-eminent organisms for use in sustainable papermaking and in biopulping, are strongly ligninolytic basidiomycetes, such as *Ceriporiopsis subvermispora* which degrades wood lignin selectively and expresses multiple extracellular MnPs in combination with laccase-type of phenoloxidases (Canales *et al.* 1998, Hatakka 1994, Lobos *et al.* 1994).

Mycelial growth varied according to species. *R. lineatus*, *L. betulina*, *T. villosa*, *S. commune*, *G. applanatum* (909 and 907), *G. resinaceum* (59, 112, 117) and *G. tornatum* had vigorous mycelial expansion after two weeks. Cultures of *P. flavomarginatus*, *P. umbrinellus*, *P. punctatus*, *A. albida*, G. *applanatum* (125) and *Ganoderma* sp. had lower growth in that period.

Results observed on *R. lineatus*, *T. villosa P. flavomarginatus*, *P. umbrinellus*, *P. punctatus* and *A. albida,* were the same as or similar to those obtained by Fernandes and Loguercio-Leite (2003) and Neves (1998).

Strains of *A. albida, G. applanatum* (125) and *P. punctatus* showed modest biomass production, but G. *resinaceum* (117) produced higher biomass level than other strains of this species. This average growth was not observed for different strains of *G. applanatum.* These variable results for the same species, but different individuals, were observed in strains isolated at different times (Smith & Onions 1994) or perhaps because the individual strain responds to ambient conditions.

There was no correlation between largest radial growth and biomass production.

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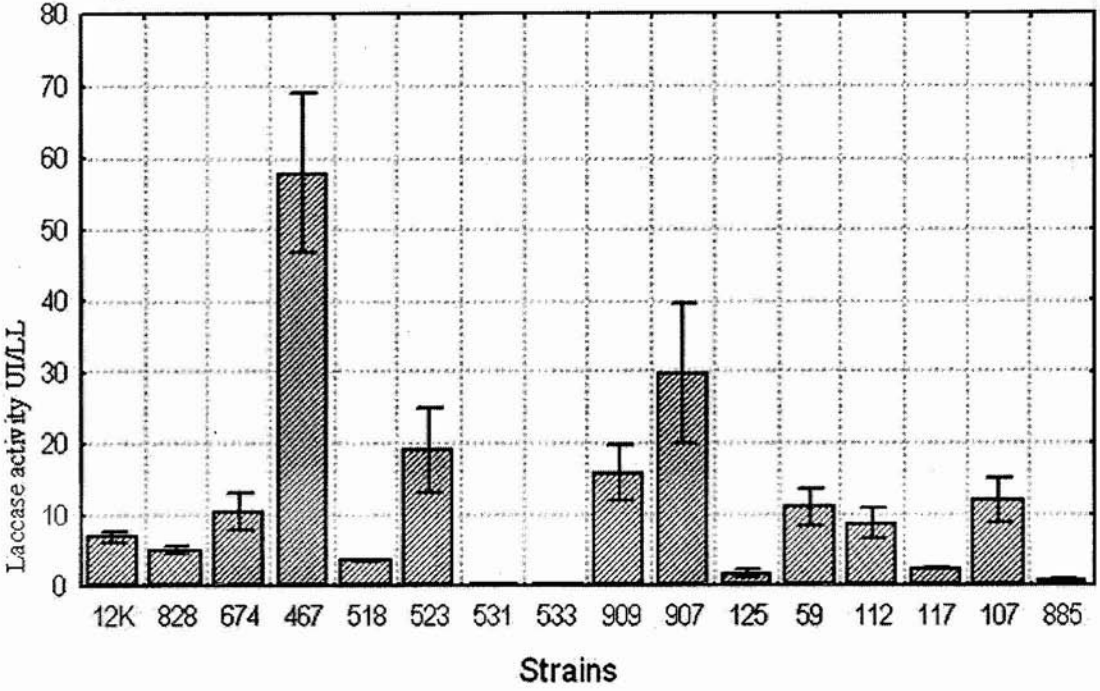
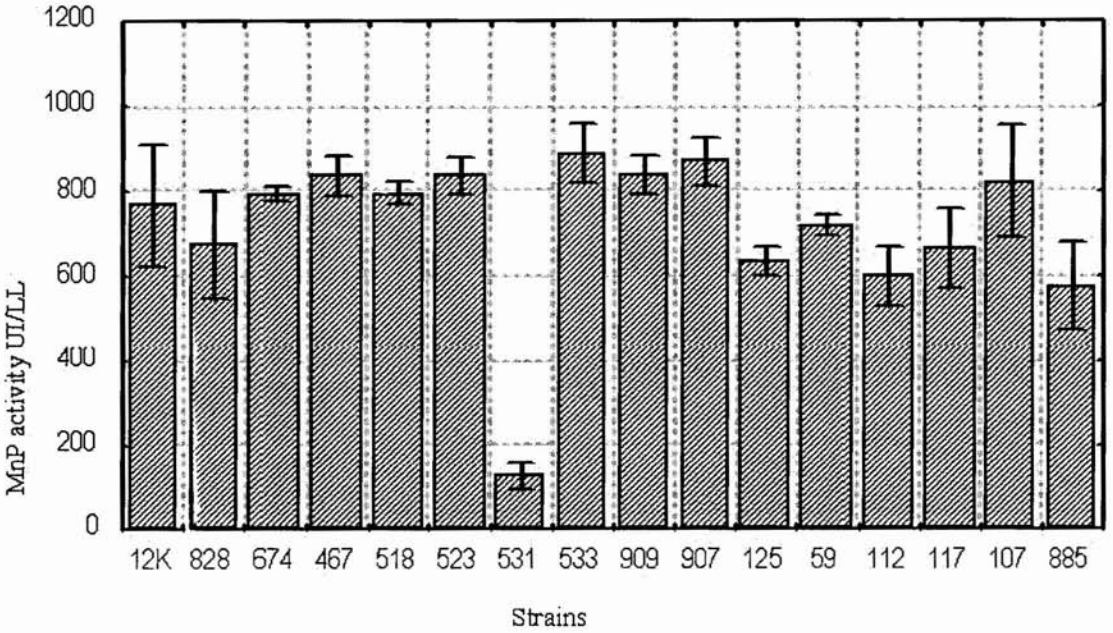
Table 1. Systematic arrangement of species. Species, strains and Herbaria number and, origin within Santa Catarina state. * without basidiomata

Order	Family	Species	Strains	FLOR	Locality
Agaricale	Schizophyllaceae	*Schizophyllum commune* (L.) Fr.	533	-*	Lagoa do Peri, Florianópolis
Hymenochaetales	Hymenochaetaceae	*Phellinus flavomarginatus* (Murr.) Ryv.	12K	10.923	Morro da Lagoa, Florianópolis
		Phellinus punctatus (Karst.) Pil.	674	11.188	
		Phellinus umbrinellus (Bres.) Herrera and Bond.	828	11.379	
Polyporales	Ganodermataceae	*Ganoderma applanatum* (Pers.) Pat.	909	11.909	Morro da Lagoa, Florianópolis
				11.907	Rincão
		Ganoderma applanatum (Pers.) Pat.	907	11.250	Rincão
		Ganoderma applanatum (Pers.) Pat.	125	11.109	Campus UFSC, Florianópolis
		Ganoderma resinaceum Boudier	59	11.348	Campus UFSC, Florianópolis
		Ganoderma resinaceum Boudier	112	11.206	Campus UFSC, Florianópolis
		Ganoderma resinaceum Boudier	117	11.119	Morro da Lagoa, Florianópolis
		Ganoderma tornatum (Pers.) Bres.	107	--*	S. Antônio Lisboa, Florianópolis
		Ganoderma sp.	885		
	Meripiliaceae	*Antrodia albida* (Fr.) Donk.	531	11.158	Lagoa do Peri, Florianópolis
		Rigidoporus lineatus (Pers.) Ryv.	467	10.998	Morro da Lagoa, Florianópolis
	Polyporaceae	*Lenzites betulina* (Fries) Fries	518	11.086	Gramados, Rio das Antas
		Trametes villosa (Fr.) Kreisel	523	11.084	



Graphic1. Laccase activity



Graphic 2. MnP production