SCREENING FOR LIGNOCELLULOLYTIC ENZYMES AND METAL TOLERANCE IN ISOLATES OF WOOD-ROT FUNGI FROM CHILE

Yudith Guillén, Götz Palfner and Ángela Machuca

SUMMARY

Wood-rot fungi have been shown to be powerful agents in many biotechnological processes. However, there may be great inter- and intra-specific variability in their performance. Consequently, it is not only important to screen a broad range of species but also different isolates of the same species in order to obtain strains with specific biotechnological profiles. In this study, the presence of wood-rot fungi was monitored in Southern-Central Chile and the biotechnological potential of the isolates was analyzed by determining lignocellulolytic enzymes and tolerance to metal ions (Cu and Cd) in solid medium. Seventy-one strains were isolated from cultures of a total of 144 basidiomes collected from wood substrates and 59 species of 18 different genera were identified, of which four are first records for Chile: Antrodia xantha*,* Gloeophyllum abietinum*,* G. protractum *and* Stereum rameale*. Cellulase and xylanase activity were detected in all strains and 20 strains showed significant ligninolytic activity. The great majority of the strains showed tolerance to 3mM Cu in solid medium, but were inhibited by 1mM Cd. In contrast, some strains of the white-rot fungi* Ganoderma australe*,* Stereum hirsutum *and* Trametes versicolor *presented high lignocellulolytic potential combined with metal tolerance. Possible applications of these strains in biodegradation or bioremediation processes are discussed.*

Introduction

Whereas in the past wood-rot fungi have been mainly considered as an economic threat for causing substantial losses of wood and wood products, they have since been rediscovered as powerful agents in biotechnological processes. The biochemical mechanisms responsible for their lignocellulolytic capacities are the main focus of current studies which aim at their application in solving technological and environmental problems, particularly in the paper industry (Ferraz *et al.*, 2008), and the bioremediation of contaminated soils and industrial wastes (Rodríguez-Couto and Toco-Herrera, 2006; Gao *et al.*, 2010; Magan *et al.*, 2010). When the fungi attack the wood, a range of degradative extracellular, enzymatic and non-enzymatic activities are produced which chemically and morphologically alter the substrate, resulting in three main types of rot: white, brown and soft (Blanchette, 1995). Depending on the type, wood-rot fungi secrete a battery of enzymes and low-molecular weight agents that cause depolymerization of cellulose and hemicelluloses as well as fragmentation of lignin (Kirk and Cullen, 1998). This enzymatic potential, together with tolerance to metals that some wood-rot fungi can develop, has been used in research related to metal biosorption, biopulping, biobleaching and bioremediation of soils, industrial effluents and preserved wood that have been discarded as waste (Pointing, 2001; Baldrian, 2003; Illman and Yang, 2004; Ferraz *et al.*, 2008; Gao *et al.*, 2010; Dashtban *et al.*, 2010).

Chile possesses large areas of forest plantations and is among the main exporting nations of wood products, especially pulp and paper. The international market increasingly demands the use of environmentally friendly techniques, especially in processes like cellulose production. Many studies on the degradative properties of wood-rotting fungi have been performed using model organisms such as the white-rot fungi *Phanerochaete* spp., *Trametes* spp. or *Pleurotus* spp. However, it is also important to study a broad range of fungal species and strains from the same geographical area, where these could be applied in the future, given that locally distributed taxa may show enhanced performance, having adapted to local climate and substrate types (Atagana, 2004; D'Annibale *et al.*, 2006). Moreover, legal restrictions on the use of allochthonous microorganisms in natural environments may impede their biotechnological application in some countries (Matheus *et al.*, 2000). In this context, it must be emphasized that Chilean mycobiota, especially their lignocellulolytic capacities, have not been studied in depth despite their economic importance, and few studies on biotechno-

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DETERMINACIÓN DE ENZIMAS LIGNOCELULOLÍTICAS Y TOLERANCIA A METALES EN CEPAS DE HONGOS PUDRIDORES DE MADERA DE CHILE

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RESUMEN

El enorme potencial de los hongos pudridores de madera en muchos procesos biotecnológicos ha sido demostrado. Sin embargo, éstos pueden presentar grandes variaciones inter- e intraespecíficas en su desempeño. Así, cuando se desea obtener especies fúngicas para usos biotecnológicos específicos, es necesario analizar no sólo una amplia variedad de especies, sino también diferentes cepas de una misma especie. En este estudio fue investigada la presencia de hongos pudridores de madera en una región del centro-sur de Chile, y su potencial biotecnológico fue evaluado a través de la detección de enzimas lignocelulolíticas y de la tolerancia a iones metálicos como Cu y Cd, en medio de cultivo sólido. De los 144 basidiomas recolectados a partir de sustratos leñosos fueron obtenidos 71 cultivos puros y de éstos, 59 especies de 18 géneros diferentes fueron identificadas, de las cuales cuatro son informadas por primera vez en Chile: Antrodia xantha, Gloeophyllum abietinum, G. protractum *y* Stereum rameale. *Se detectó actividad de celulasa y xilanasa en todas las cepas fúngicas y sólo 20 mostraron una significativa actividad ligninolítica. La mayoría de las cepas fue tolerante a 3mM Cu, pero fueron inhibidas por 1mM Cd. Algunas cepas de los hongos de pudrición blanca* Ganoderma australe, Stereum hirsutum *y* Trametes versicolor *presentaron un eficiente potencial lignocelulolítico combinado con una alta tolerancia a metales. Se discuten posibles aplicaciones de estas cepas en procesos de biodegradación o bioremediación.*

DETERMINAÇÃO DE ENZIMAS LIGNOCELULOLÍTICAS E TOLERÂNCIA A METAIS EM ISOLADOS DE FUNGOS DA DECOMPOSIÇÃO DA MADEIRA DE CHILE

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RESUMO

O enorme potencial dos fungos da decomposição da madeira em uma variedade de processos biotecnológicos tem sido demonstrado. Contudo, tais fungos podem apresentar uma grande variabilidade inter- e intraespecífica no seu desempenho. Assim, quando se deseja obter especies fúngicas com fins biotecnológicos específicos, é necessario analisar não somente uma ampla variedade de especies, senão também diferentes isolados de uma mesma especie. No presente estudo, a presença de fungos da decomposição da madeira foi pesquisada numa região do centro-sul do Chile, e seu potencial biotecnológico foi avaliado mediante a detecção das enzimas lignocelulolíticas e da tolerância a íons metálicos tais como Cu e Cd, em meio de cultura sólido. Dos 144 basidiocarpos recoletados a partir de sustratos lenhosos, 71 cultivos puros foram obtidos e desses, 59 especies de 18 géneros diferentes foram identificadas, das quais quatro são descritas pela primeira vez no Chile: Antrodia xantha, Gloeophyllum abietinum, G. protractum *e* Stereum rameale. *As atividades de celulase e xilanase foram detectadas em todos os isolados fúngicos e somente 20 mostraram uma significativa atividade ligninolítica. A maioría dos isolados foi tolerante a 3mM Cu, mas foi inibida por 1mM de Cd. Alguns isolados dos fungos da decomposição branca* Ganoderma australe, Stereum hirsutum *e* Trametes versicolor *apresentaram um eficiente potencial lignocelulolítico junto com uma elevada tolerancia a metais. Discute-se a respeito das possíveis aplicações desses isolados em processos de biodegradação ou biorremediação.*

logical potential of native fungi exist (Donoso *et al*., 2008; Mendonça *et al*., 2008; Tortella *et al*., 2008; Rubilar *et al*., 2011; Acevedo *et al*., 2010, 2011). For this reason, the aim of the present study was to isolate and identify wood-rot fungi (Basidiomycota) from Southern-Central Chile and determine their lignocellulolytic capacity. To this end, pure mycelial cultures of the basidiomes collected in the field were subjected to different qualitative enzyme assays in solid medium to detect hydrolytic and ligninolytic enzymes (Gramms *et al*., 1998; Pointing, 1999; Kjoller *et al*., 2000). These assays also enable corroboration of the type of wood rot caused by some species of xylophagous fungi, mainly the traditional Bavendamm test (with tannic or gallic acid), used for many years to distinguish between white- and brown-rot fungi based on the presence or absence of phenoloxidase production, respectively (Käärik, 1965). In addition, the effect of Cu and Cd ions on mycelial growth rates in solid medium was analyzed in order to select strains with biotechnological potential to be used in the bioremediation of substrates and/or environments contaminated with metals.

Materials and Methods

Study area

Basidiomes from wood-rot fungi were collected between latitudes 36º and 38ºS in Southern Central Chile, in different locations in the Provinces of Concepción, Bío-Bío and Ñuble (Figure 1). The climate of the region is Mediterranean with a dry, hot summer that can last up to four months, contrasted by a rainy and cool winter (Hoffmann, 1998). Most fungal species tested were collected in native forests dominated by southern beech (*Nothofagus* spp.) and sclerophyllous trees of the Myrtales and Laurales orders (Table I). Other species were collected from commercial plantations, on living or dead wood of *Pinus radiata* D. Don, *Eucalyptus globulus* Labill and *E. nitens* Maiden. Samples were collected from substrates including standing or uprooted stems and/or wood structures at different stages of decomposition, between autumn and spring 2004 and 2005.

Isolation and identification of wood-rot fungi

Pure mycelial cultures were obtained under aseptic conditions, placing small fragments or spores of the basidiomes collected on malt extract agar medium prepared with 2% (w/v) malt extract (Fluka) and 2% (w/v) agar (Merck). Petri dishes were incubated at 24 ±1ºC in the dark and after variable incubation periods the axenic cultures were transferred to test tubes with 2% MEA and kept constantly at 3ºC in the Laboratory of Fungi Biotechnology at the Universidad de Concepción, Campus Los Ángeles, Chile. Furthermore, the respective basidiomes were oven-dried at 40-50ºC for 48h and deposited as voucher material in the Fungi Collection of the Herbarium of the Universidad de Concepción (CONCF). Classification of genera and species was performed according to the techniques suggested by Ryvarden (1987) and Rajchenberg (2006). For microscopy, hand-cut sections of specimens were mounted in 3-5% KOH and stained with Melzer's reagent and 1% phloxine. Sources of identification keys and species descriptions were the publications by Wright and Deschamps (1972), Horak (1979), Breitenbach and Kränzlin (1986), Ryvarden (1987, 1991), Bernicchia (2005) and Rajchenberg (2006). Some of the basidiomes were compared to material deposited in the herbarium at the Patagonian Andes Forest Research and Extension Center (CIEFAP), Esquel, Argentina.

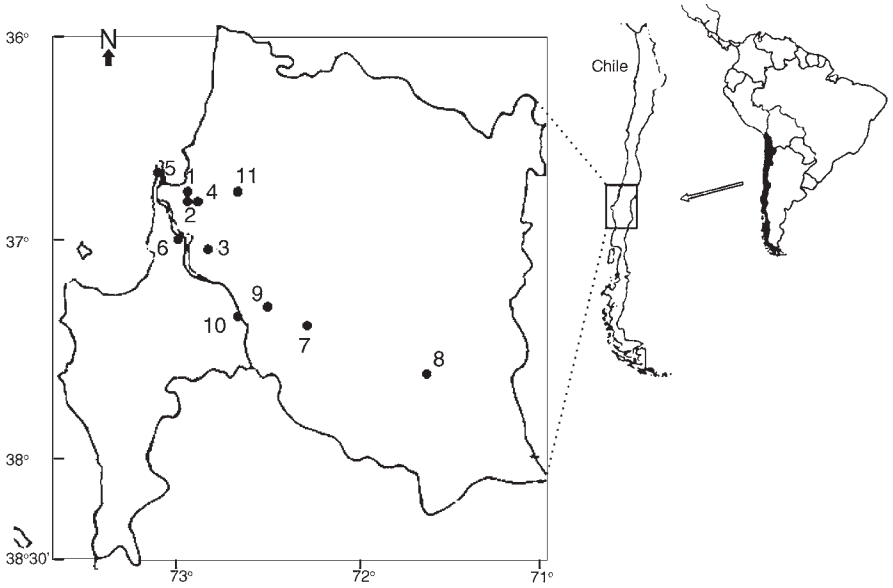

Figure 1. Map of VIII[th] Region (southern-central) Chile, showing the location of the collection sites. 1: Concepción, UDEC university campus (36º49'), 2: La Cantera and el Guindo Forest (36º52'), 3: San Ignacio Estate, Quilacoya, Hualqui (37º03'), 4: Concepción center (36º47'), 5: Tumbes Park, Talcahuano (36º38'), 6: Jorge Alessandri Park, route to Coronel (37º02'), 7: Los Ángeles, UDEC university campus (37º27'), 8: San Lorenzo Estate, Santa Bárbara (37º29'), 9: Saltos del Laja Park, Laja (37º09'), 10: Los Patos Sector, Nacimiento (37º26'), 11: Collanmahuida Park, Florida (36º33').

Detection of lignocellulolytic enzymes

The qualitative assays for detection of lignocellulolytic enzymatic activity in mycelial fungi culture were performed in solid medium. The substrates were added to 0.1% MEA (pH 4.5) after sterilization, in different concentrations (w/v): 2% carboxymethylcellulose (CMC), 2% xylan from birchwood, 0.01% guaiacol, 0.04% Remazol Brilliant Blue R (R-BBR), 0.02% Poly R-478 and 0.5% tannic acid, following Pointing's methodology (1999). Two 7mm-diameter disks obtained from the active growth zone of each fungal strain culture were placed equidistantly at opposing edges of a Petri dish. The dishes were incubated in the dark at 24 ±1ºC for 10 days, a period in which the enzymatic activity could be seen by the formation of coloration or discoloration halos in the media containing the different enzymatic indicators. The brown-rot fungi *Wolfiporia cocos* ATCC 62778 and *Gloeophyllum trabeum* ATCC 11539 and white-rot fungi *Trametes versicolor* DEBIQ and *Ganoderma australe* A464, cultivated under conditions similar to those of the native strains, were used as references in the lignocellulolytic enzyme detection assays.

Effect of Cu and Cd on growth rate

In order to evaluate the effect of metal ions on the growth of fungal strains, Cu or Cd was added to the 2% MEA (pH 4.5) medium as solid salts ($CuSO_4.5H_2O$ or $CdSO_4.8H_2O$) after sterilization when the medium had reached an appropriate temperature, to final concentrations of 3mM and 1mM, respectively. Petri dishes (9cm diameter) containing MEA medium with or without metal ions were inoculated in the center with a 7mm diameter disk obtained from the stock cultures and incubated in the dark at 24 ±1ºC for a maximum of two months. Growth was determined by measuring the radius of the mycelial colonies at various time intervals. Growth rates for each fungal strain were calculated from the respective growth curves and expressed in mm/day (Guillén and Machuca, 2008). The assay was performed with three replicates for each isolated strain with its respective control (MEA medium without metal).

Results and Discussion

Many studies related to wood-rot fungi have been conducted with different biotechnological goals, but the vast majority of these studies have been performed with a limited number of well-known fungal species and strains, mainly white-rot. While this concept of model organisms makes sense for basic research, studies of specific properties for future use in defined geographical areas may require the use of local taxa adapted to site-specific climates, substrates and other variables. In Chile, a country with abundant fungal diversity, extensive areas of native forests and forest plantations (Conaf-Conama-Birf, 1999a, b; Mujica *et al.*, 1980), few studies on native mycobiota exist. Within this context, the aim of the present work was to study the enzymatic activity and heavy metal tolerance of endemic taxa like *Anthracophyllum discolor* and *Bondarzewia guaitecasensis* (Wright and Deschamps, 1972; Lazo, 2001), as well as to test different strains of cosmopolitan species like *Trametes versicolor* or *Stereum hirsutum* for intraspecific variability of properties that are of particular interest for the biodegradation or

TABLE I
COLLECTIONS OF BASIDIOMES OF WOOD-ROTTING FUNGI FROM DIFFERENT SUBSTRATES AND SITES IN SOUTHERN-CENTRAL CHILE

Species	Strain Nº	Herbary Code	Site	Substrate
Agrocybe sp.	97	CONC-F0489	7	Stem of *Acer* sp.
Anthracophyllum discolor (Mont.) Singer	18	CONC-F0455	2	Unidentified log
Antrodia xantha (Fr.) Ryvarden *	139	CONC-F0509	11	Stump of *Pinus radiata*
Bjerkandera adusta (Willd. ex Fr.) Karst	30	CONC-F0463	2	Unidentified log
B. adusta	62	CONC-F0470	2	Log of *Eucalyptus nitens*
B. adusta	119	CONC-F0499	1	Stem of *Liquidambar styraciflua*
B. adusta	122	CONC-F0500	1	Stem of *Fraxinus excelsior*
Bondarzewia guaitecasensis (Henn.) J.E. Wright apud Singer	140	CONC-F0510	11	Stem of *Nothofagus obliqua*
Clitocybula dusenii (Bresadola) Singer	144	CONC-F0514	10	Log of *Nothofagus obliqua*
Flammulina velutipes (Curtis) Singer	95	CONC-F0488	7	Stem of *Acer* sp.
F. velutipes	98	CONC-F0490	7	Unidentified log
F. velutipes	117	CONC-F0498	7	Stem of *Acer* sp.
Ganoderma australe (Fr.) Pat.	28	CONC-F0462	2	Unidentified wood
G. australe	64	CONC-F0471	2	Log of *Eucalyptus nitens*
G. australe	86	CONC-F0480	4	Stem of *Prunus armeniaca*
G. australe	87	CONC-F0481	2	Stem of *Robinia pseudoacacia*
G. australe	100	CONC-F0492	5	Unidentified stump
G. australe	105	CONC-F0493	5	Unidentified stump
G. australe	142	CONC-F0512	8	Log of *Nothofagus obliqua*
Gloeophyllum abietinum (Fr.) P. Karst*	132	CONC-F0504	8	Post of *Cupressus* sp.
G. protractum (Fr.) Imaz.*	31	CONC-F0464	6	Log of *Pinus radiata*
Gloeophyllum sp.	8	CONC-F0447	1	Stem of *Prunus cerasifera*
Gymnopilus spectabilis (Fr.) Smith	137	CONC-F0507	6	Stump of *Pinus radiata*
G. spectabilis	138	CONC-F0508	6	Stump of *Pinus radiata*
Lenzites betulina (Fr.) Fr.	20	CONC-F0457	2	Branch of *Acacia melanoxylon*
L. betulina	22	CONC-F0458	2	Unidentified log
L. betulina	27	CONC-F0461	2	Unidentified log
L. betulina	34	CONC-F0465	2	Unidentified log
L. betulina	67	CONC-F0472	2	log of *Acacia melanoxylon*
L. betulina	111	CONC-F0496	5	Unidentified log
L. betulina	131	CONC-F0503	8	Branch of *Persea lingue*
Paxillus panuoides (Fr.) Fr.	83	CONC-F0479	3	Burnt log of *Pinus radiate*
Phellinus sp.	17	CONC-F0454	1	Unidentified stump
Phellinus sp.	42	CONC-F0468	4	Stem of *Peumus boldus*
Pleurotus ostreatus (Jacq. ex Fr.) Kumm	91	CONC-F0485	7	Stem of *Prunus cerasifera*
P. ostreatus	92	CONC-F0485	7	Stem of *Prunus cerasifera*
Schizophyllum commune Fr.	25	CONC-F0460	2	Wood of *Acacia melanoxylon*
Serpula lacrymans (Wulfen) Karst.	141	CONC-F0511	4	Wood of *Pinus radiata*
Stereum hirsutum (Willd.) Gray	9	CONC-F0448	2	Unidentified branch
S. hirsutum	10	CONC-F0449	2	Stem of *Eucalyptus nitens*
S. hirsutum	15	CONC-F0452	2	Log of *Acacia melanoxylon*
S. hirsutum	19	CONC-F0456	2	Unidentified log
S. hirsutum	69	CONC-F0473	2	Bark of *Eucalyptus nitens*
S. hirsutum	72	CONC-F0475	2	Branch of *Eucalyptus nitens*
S. hirsutum	89	CONC-F0483	2	Unidentified log
S. hirsutum	99	CONC-F0491	7	Log of *Acacia melanoxylon*
S. hirsutum	108	CONC-F0494	5	Unidentified branch
S. hirsutum	113	CONC-F0497	5	Unidentified branch
S. hirsutum	125	CONC-F0501	7	Branch of *Quercus palustris*
Stereum rameale (Pers.: Fr.) Burt*	71	CONC-F0474	2	Branch of *Aextoxicon punctatum*
Trametes versicolor (L.) Pilát	1	CONC-F0445	1	Unidentified stump
T. versicolor	7	CONC-F0446	1	Unidentified stump
T. versicolor	12	CONC-F0451	2	Unidentified log
T. versicolor	24	CONC-F0459	2	Log of *Eucalyptus nitens*
T. versicolor	38	CONC-F0466	2	Unidentified log
T. versicolor	73	CONC-F0476	4	Stem of *Aristotelia chilensis*
T. versicolor	81	CONC-F0477	2	Unidentified log
T. versicolor	88	CONC-F0482	2	Unidentified log
T. versicolor	110	CONC-F0495	5	Unidentified log

1: Concepción, UDEC university campus; 2: La Cantera and el Guindo Forest (native forest and plantation); 3: San Ignacio Estate, Quilacoya, Hualqui (plantation); 4: Concepción center; :5 Tumbes Park, Talcahuano (native forest and plantation); 6: Jorge Alessandri Park, route to Coronel (native forest and plantation); 7: Los Ángeles, UDEC university campus; 8: San Lorenzo Estate, Santa Bárbara (native forest); 9: Saltos del Laja Park, Laja (native forest); 10: Los Patos Sector, Nacimiento (native forest); 11: Collanmahuida Park, Florida (native forest).

bioremediation of contaminated substrates.

A total of 144 collections of basidiomes were taken from a variety of woody substrates at different sites in Southern-Central Chile. Of these, 71 strains of pure cultures were obtained and 59 species of 18 different genera could be identified at the species (55) or genus (4) level (Table I). Most taxa (78.9%) had aphyllophoroid basidiomes; the rest (19.7%) had agaricoid morphology. *Stereum hirsutum, Trametes versicolor*, *Ganoderma australe* and *Lenzites betulina* were the most frequently encountered species and provided several strains (Table I). Other species which could be isolated more than once were *Bjerkandera adusta*, *Flammulina velutipes, Gymnopilus spectabilis* and *Pleurotus ostreatus*. The remaining taxa were isolated only once (Table I).

Among Chilean lignicolous mycobiota, two large groups can be distinguished: endemic fungi that colonize similar endemic tree species and adventives, usually widely distributed species that have been established with introduced trees. It has been suggested that a balance between both fungal groups may develop over time (Butin and Peredo, 1986). The present findings extend the list of known Chilean wood-rot fungi by four newly recorded species: *Antrodia xantha*, *Gloeophyllum abietinum*, *Gloeophyllum protractum* and *Stereum rameale*, all species of wide geographic distribution (Krieglsteiner and Kaiser, 2000). Among these, *A. xantha*, which has also been reported in Argentina (Wright and Albertó, 2006), is a brown-rot species of reference in assays about the effectiveness of wood preservatives, due to its tolerance to Cu (AWPA, 2004).

The enzymatic potential of 59 strains of identified genera and species was determined qualitatively in the MEA medium using different indicators (Table II). All strains hydrolyzed carboxymethylcellulose (CMC) to a greater or lesser extent, proving their ability to produce enzymes from cellulolytic complex. The majority of the native strains (71%) showed a strong positive reaction and the remainder performed only a partial CMC hydrolysis. Among the strains of *B. adusta, G. australe, L. betulina, T. versicolor*, and particularly *S. hirsutum*, differentiated responses in the hydrolysis of CMC were observed (Table II). With birchwood xylan as the indicator substrate for hemicellulolytic enzymes, all strains presented a positive reaction (Table II), with no differences in the color intensity of the reaction zone, but varying in the size of the hydrolysis halos. The reference strains showed similar positive cellulolytic and hemicellulolytic reactions, except *W. cocos*, which reacted only moderately with CMC. The hydrolysis of CMC can be attributed not only to the production of endoglucanase-type cellulases, but also to ß-glycosidases, whereas hydrolysis of xylan is usually performed by endoxylanases and ß-xylosidases (Pointing, 1999), providing good evidence that all the fungal strains analyzed can produce various types of cellulolytic and hemicellulolytic enzymes. The biotechnological applications of these strains or their fungal extracts with hydrolytic activity are of great interest in the textile, detergent and food industries, as well as for bleaching processes in the paper industry (Kuhad *et al*., 1997; Bhat, 2000).

To detect ligninolytic enzymes different enzymatic substrates were used, related to the production of polyphenoloxidases, laccases, peroxidases, lignin-peroxidases (LiP) or Mn-peroxidases (MnP). Most strains demonstrated ability to oxidize at least one of the substrates assayed and only eight strains did not oxidize any of them (Table II). Of these, six are species belonging to the brown-rot fungi group, showing coherence with the results obtained with the reference brown-rot fungi *W. cocos* and *G. trabeum*. Remarkably, two of these strains are of species belonging to the white-rot fungi group (*F. velutipes* 98, *S. rameale* 71). In addition, the strain of *P. panuoides*, a brown-rot fungus, reacted positively with the RBB-R dye, an indicator of ligninolytic activity. A high degree of strain-specific variability in enzymatic activity was observed in *G. australe* and *S. hirsutum*: only strains 64 and 142 of *G. australe* and strain 19 of *S. hirsutum* oxidized all ligninolytic substrates; the rest presented varying reactions to the different indicator substrates. Nevertheless, among other species like *B. adusta*, *L. betulina* and *T. versicolor*, a much more homogenous behavior was observed. All four strains of *B. adusta* behaved similarly, discoloring the polymeric dyes RBB-R and Poly-R 478; only one was able to oxidize tannic acid and none of them oxidized guaiacol. Most of the strains of *L. betulina* reacted positively to all the substrates, and only three of them did not oxidize guaiacol. Most of the strains of *T. versicolor* reacted positively with all the ligninolytic substrates, except strain 7, which did not oxidize guaiacol or tannic acid (Table II). Among native strains, the majority discolored RBB-R (78%) and oxidized tannic acid (73%), and only a smaller proportion reacted with Poly R-478 (58%) and guaiacol (44%).

A considerable intra- and interspecific variety of responses was obtained among the 59 native fungal strains and only eight strains were unable to react with any of the substrates used. These results were expected since seven of the strains belong to species classified as brown-rot fungi. Interestingly, *S. rameale* 71, a white-rot fungus, was also among the strains that showed no reaction. This may be related to the selected substrate indicators, because when α-naphthol, p-cresol and pyrogallol were used as substrates, *S. rameale* reacted positively, oxidizing these compounds (Stalpers, 1978). On the other hand, some white-rot species (*B. adusta*, *F. velutipes* 95 and 98, *G. australe* 28 and 86, *S. rameale* 71 and *T. versicolor* 7) showed inconsistent results with the Bavendamm test as they reacted negatively. These results confirm the importance of using more than one enzymatic substrate in studies regarding the selection of ligninolytic enzyme-producing fungi. Confirming the type of wood rot (white or brown) through enzymatic assays in solid medium is considered of great importance for the taxonomy of xylophagous fungi (Stalpers, 1978). In the reviewed literature there is no prior information with respect to the wood-rot type or lignocellulolytic enzymes produced by *B. guaitecasensis*. The assay-based classification of this species as a white-rot fungus (Table II) is consistent with the same status described for *B. montana* (Quél.) Singer, from the Northern Hemisphere (Breitenbach and Kränzlin, 1986; Krieglsteiner and Kaiser, 2000).

It is worthy of note that *P. panuoides* 83, of the brown-rot fungi group, decolorizes the RBB-R dye, suggesting that in addition to the expected hydrolytic enzyme production, this strain is also able to produce oxidative enzymes. Although few studies exist on the detection of ligninolytic enzymatic activity in brown-rot fungi, some describe the production of laccases by this type of

TABLE II
DETECTION OF LIGNOCELLULOLYTIC ENZYME ACTIVITY AND TOLERANCE TO METAL IONS IN MEA MEDIUM OF SPECIES OF WOOD-ROT FUNGI COLLECTED IN SOUTHERN-CENTRAL CHILE

Species	Strain[a]	CMC	X	G	TA	RBB	PolyR	WR	GR (mm/day) Control[b]	GR (mm/day) 3mM Cu[c]	GR (mm/day) 1mM Cd[d]
Agrocybe sp.	97	+	+	-	-	-	-	B	3.24 ±0.03	NG	NG
Anthracophyllum discolor	18	+	+	+	+	+	+	W	1.45 ±0.00	0.34 ±0.0 (76)	NG
Antrodia xantha	139	+/-	+	-	-	-	-	B	5.82 ±0.32	3.23 ±0.06 (44)	0.75 ±0.04 (87)
Bjerkandera adusta	30	+	+	-	-	+	+	W	4.90 ±0.46	1.27 ±0.07 (74)	NG
B. adusta	62	+	+	-	-	+	+	W	8.10 ±0.10	1.50 ±0.36 (81)	0.26 ±0.04 (97)
B. adusta	119	+	+	-	-	+	+	W	3.94 ±0.53	1.65 ±0.02 (58)	NG
B. adusta	122	+/-	+	-	+	+	+	W	8.90 ±0.03	1.18 ±0.12 (87)	1.22 ±0.09 (86)
Bondarzewia guaitecasensis	140	+/-	+	+	+	+	+	W	1.08 ±0.16	NG	NG
Clitocybula dusenii	144	+	+	+	+	+	+	W	1.32 ±0.02	0.40 ±0.03 (70)	0.32 ±0.02 (76)
Flammulina velutipes	95	+	+	-	-	+	+	W	5.43 ±0.08	NG	NG
F. velutipes	98	+	+	-	-	-	-	W	3.48 ±0.39	NG	NG
F. velutipes	117	+	+	-	+	-	-	W	3.94 ±0.05	NG	NG
Ganoderma australe	28	+	+	-	-	+	+	W	8.80 ±0.04	3.36 ±0.30 (62)	NG
G. australe	64	+/-	+	+	+	+	+	W	5.28 ±0.04	NG	0.67 ±0.03 (87)
G. australe	86	+	+	-	-	+	+	W	3.40 ±0.03	1.35 ±0.01 (60)	NG
G. australe	87	+	+	+	+	+	-	W	3.90 ±0.14	1.62 ±0.17 (58)	0.29 ±0.03 (82)
G. australe	100	+	+	-	+	+	-	W	2.74 ±0.36	2.10 ±0.29 (23)	NG
G. australe	105	+	+	-	+	+	-	W	2.63 ±0.04	1.49 ±0.07 (43)	0.26 ±0.04 (90)
G. australe	142	+	+	+	+	+	+	W	5.14 ±0.20	1.87 ±0.17 (64)	0.39 ±0.06 (92)
Gloeophyllum abietinum	132	+	+	-	-	-	-	B	9.08 ±0.35	2.30 ±0.27 (75)	NG
G. protractum	31	+	+	-	-	-	-	B	2.52 ±0.27	0.07 ±0.00 (97)	NG
Gloeophyllum sp.	8	+/-	+	-	-	-	-	B	3.90 ±0.16	2.80 ±0.40 (28)	NG
Gymnopilus spectabilis	137	+	+	-	+	+	+	W	3.13 ±0.00	0.93 ±0.09 (70)	NG
G. spectabilis	138	+/-	+	-	+	+	+	W	3.38 ±0.10	1.44 ±0.03 (57)	NG
Lenzites betulina	20	+/-	+	-	+	+	+	W	5.67 ±0.07	NG	NG
L. betulina	22	+	+	-	+	+	+	W	7.42 ±0.26	NG	NG
L. betulina	27	+	+	+	+	+	+	W	6.37 ±0.17	NG	NG
L. betulina	34	+	+	+	+	+	+	W	4.29 ±0.12	NG	NG
L. betulina	67	+/-	+	-	+	+	+	W	6.02 ±0.10	NG	NG
L. betulina	111	+	+	+	+	+	+	W	5.19 ±0.06	NG	NG
L. betulina	131	+	+	+	+	+	+	W	5.62 ±0.62	NG	NG
Paxillus panuoides	83	+/-	+	-	-	+	-	B	4.35 ±0.25	1.63 ±0.08 (62)	1.27 ±0.31 (71)
Phellinus sp.	17	+/-	+	+	+	+	+	W	1.61 ±0.31	0.47 ±0.05 (71)	NG
Phellinus sp.	42	+	+	+	+	+	+	W	1.60 ±0.05	0.56 ±0.01 (65)	NG
Pleurotus ostreatus	91	+	+	+	+	+	-	W	5.52 ±0.02	0.38 ±0.01 (93)	NG
P. ostreatus	92	+	+	+	+	+	-	W	4.58 ±0.35	1.03 ±0.02 (77)	NG
Schyzophyllum commune	25	+	+	-	+	+	-	W	4.08 ±0.10	NG	NG
Serpula lacrymans	141	+/-	+	-	-	-	-	B	0.39 ±0.05	0.60 ±0.01 (0)	0.66 ±0.01 (0)
Stereum hirsutum	9	+/-	+	-	+	+	-	W	6.98 ±0.18	0.50 ±0.04 (93)	0.15 ±0.02 (98)
S. hirsutum	10	+	+	-	+	-	-	W	5.45 ±0.22	0.59 ±0.07 (89)	NG
S. hirsutum	15	+	+	+	+	+	-	W	6.15 ±0.52	0.71 ±0.19 (88)	0.33 ±0.06 (95)
S. hirsutum	19	+	+	+	+	+	+	W	1.58 ±0.02	0.58 ±0.01 (63)	NG
S. hirsutum	69	+	+	-	+	-	-	W	5.50 ±0.01	NG	NG
S. hirsutum	72	+/-	+	-	+	+	+	W	6.20 ±0.30	1.16 ±0.14 (81)	NG
S. hirsutum	89	+	+	-	+	-	+	W	2.99 ±0.12	1.04 ±0.09 (65)	0.40 ±0.02 (87)
S. hirsutum	99	+	+	+	+	-	-	W	6.88 ±0.05	0.42 ±0.06 (94)	NG
S. hirsutum	108	+/-	+	-	+	+	-	W	6.90 ±0.13	0.36 ±0.01 (95)	NG
S. hirsutum	113	+/-	+	-	+	+	-	W	6.40 ±0.70	0.40 ±0.14 (94)	0.11 ±0.01 (98)
S. hirsutum	125	+	+	+	+	+	-	W	6.75 ±0.32	0.40 ±0.10 (78)	NG
Stereum rameale	71	+/-	+	-	-	-	-	W	5.56 ±0.08	1.21 ±0.03 (97)	NG
Trametes versicolor	1	+/-	+	+	+	+	+	W	8.15 ±0.75	0.28 ±0.01 (80)	NG
T. versicolor	7	+	+	-	-	+	+	W	10.6 ±0.40	2.15 ±0.40 (80)	0.88 ±0.11 (92)
T. versicolor	12	+	+	+	+	+	+	W	7.42 ±0.12	NG	1.03 ±0.06 (86)
T. versicolor	24	+	+	+	+	+	+	W	11.10 ±0.57	2.34 ±0.04 (79)	NG
T. versicolor	38	+	+	+	+	+	+	W	7.34 ±0.01	2.57 ±0.84 (65)	NG
T. versicolor	73	+	+	+	+	+	+	W	5.41 ±0.09	2.09 ±0.12 (71)	NG
T. versicolor	81	+	+	+	+	+	+	W	7.02 ±0.02	2.34 ±0.04 (67)	NG
T. versicolor	88	+	+	+	+	+	+	W	3.70 ±0.31	1.60 ±0.04 (57)	NG
T. versicolor	110	+	+	+	+	+	+	W	8.60 ±0.50	1.42 ±0.03 (83)	1.11 ±0.05 (87)
Wolfiporia cocos	ATCC62778	+/-	+	-	-	-	-	B	11.36 ±1.21	5.29 ±0.63 (53)	0.81 ±0.39 (93)
G. trabeum	ATCC11539	+	+	-	-	-	-	B	4.16 ±0.07	0.68 ±0.30 (84)	0.44 ±0.04 (89)
T. versicolor	DEBIO	+	+	+	+	+	+	W	7.15 ±0.20	1.15 ±0.30 (84)	0.82 ±0.31 (89)
G. australe	A464	+	+	+	+	+	+	W	4.04 ±0.72	0.71 ±0.11 (82)	NG

[a] Number of collection of the strain, [b] growth rate (mm/day) in MEA medium without metals, and [c] with 3mM copper and [d] with 1mM cadmium. CMC: carboxymethylcellulose, X: xylan, G: guaiacol, TA: tannic acid, RBB: Remazol Brilliant Blue R, Poly-R 478, WR: wood-rot type, W: white, B: Brown, +: positive reaction with formation of coloration or discoloration halo of the substrate indicator, +/-: weak reaction, -: negative reaction, NG: no growth observed. Values of GR ±SD. Values in parenthesis indicate the percentage of growth inhibition with respect to the control (without metal).

fungi and the detection of the genes responsible for coding these enzymes (Peláez *et al*., 1995; D'Souza *et al*., 1996; Lee *et al*., 2004). The discoloration and/or degradation of RBB-R and Poly R-478 have been correlated with the production of peroxidases (LiP and MnP) and laccases, and also with organopollutant degradation. This suggests that the fungi that react positively with these dyes present a wider range of ligninolytic enzymes, making them potential candidates for the treatment of effluents from the textile industry, biopulping, kraft pulp bleaching or the bioremediation of organopollutants, among others (Freitag and Morrell, 1992; Okino *et al*., 2000; Pointing *et al*., 2000; Minussi *et al*., 2001; Hakala *et al*., 2004; Hernández-Luna *et al*., 2008).

Additionally, the ability of the native strains to grow in solid medium containing Cu or Cd ions was evaluated through the radial growth rate of the colonies. The metal tolerance of fungi is of importance for some biotechnological applications, such as the bioremediation of contaminated substrates. Soil contamination with polycyclic aromatic hydrocarbons (PAHs) is frequently accompanied by contamination with metal ions, which makes it essential that the fungi selected for bioremediation programs, in addition to being able to degrade PAHs, also show tolerance to metals (Baldrian *et al*., 2000; Riis *et al*., 2002; Baldrian, 2003). Some metals can also interfere significantly with the activity of extracellular enzymes and colonization capacity of fungi (Baldrian, 2003).

Radial growth rates in MEA medium varied between 0.39 and 11.6mm/day among all species and strains (Table II). Twelve strains, mainly belonging to white-rot fungi, presented the fastest growth rates (>7mm/day), among which two strains of *B. adusta* and most of the *T. versicolor* strains stood out, followed by *G. australe* 28, *L. betulina* 22 and *G. abietinum* 132. The remaining strains showed a moderate growth rate and only *S. lacrymans* 141 grew very slowly under the assay conditions. Generally, growth rates were comparable and in some cases superior to those of the reference strains. The addition of 3mM Cu or 1mM Cd to the MEA medium reduced the growth rate in all strains tested compared to the control (without metal), with the exception of *S. lacrymans* 141, a dry and brown-rot fungus. This strain, despite its slow growth rate, displayed unusual behavior in that it was not inhibited by either of the metal ions, whereas all the strains of white-rot fungi *F. velutipes* and *L. betulina* exhibited a similar sensitivity to both. The majority (43) of the native strains grew in the presence of Cu, showing varying degrees of tolerance to the metal, reflected in rates that fluctuated between 0.07 and 3.36mm/day (Table II). The remaining strains showed a high sensitivity to the metal. Most strains of the white-rot fungi *G. australe*, *T. versicolor* and *S. hirsutum* were tolerant to 3mM Cu and only one strain of each species exhibited sensitivity. Among the native strains, the white-rot fungus *G. australe* 100 and brown-rot fungus *Gloeophyllum* sp. 8 displayed the lowest growth inhibition in the presence of Cu (23 and 28%, respectively), compared with their respective controls. On the other hand, the majority of the native strains were unable to grow in the presence of 1mM Cd and only 17 strains were tolerant to the metal, with growth rates varying between 0.11 and 1.27mm/day (Table II). Growth inhibitions caused by Cd were always elevated and higher than those observed in the assays with Cu. However, some strains such as *G. australe* 64 and *T. versicolor* 12 showed tolerance to Cd but not to Cu. A group of 15 native strains belonging to different white-rot (*B. adusta*, *C. dusenii*, *G. australe*, *S hirsutum* and *T. versicolor*) and brown-rot (*A. xantha*, *P. panuoides* and *S. lacrymans*) species showed tolerance to both metal ions.

After mercury (Hg), Cd is considered one of the most toxic metals for some species of wood-rot fungi (Baldrian and Gabriel, 1997; Tham *et al*., 1999). This explains why Cu exerted a lower inhibiting effect on the growth of most of the native strains than Cd, even though the later was used at a lower concentration. The growth of colonies in the presence of metals showed a great intra-specific variability between strains of *G. australe*, *S. hirsutum* and *T. versicolor*, all white-rot fungi (Table II). Some strains of *G. australe* were tolerant to Cu, but not to Cd; others were tolerant to both metals and only one was tolerant to Cd, but not to Cu. In the case of *S. hirsutum* and *T. versicolor*, most strains were tolerant to Cu, but not to Cd. Only among the strains of *F. velutipes* and *L. betulina,* also white-rot fungi, the sensitivity to Cu and Cd was similar. The results do not allow to generalize the behavior of the different brown-rot and white-rot fungi species analyzed in this study with respect to Cu and Cd tolerance, since in the case of brown-rot fungi, only one strain of each species was assayed. However, among the brown-rot species, *A. xantha* and *P. panuoides* presented relatively low growth inhibition in the presence of both metals. Furthermore, *G. abietinum* and *Gloeophyllum* sp. showed tolerance to Cu but not to Cd. Among the reference strains, the brown-rot fungus *W. cocos* displayed the greatest tolerance to Cu, but in the presence of Cd its growth was significantly inhibited (Table II).

In order to avoid erroneous generalizations regarding the metal tolerance of brown-rot and white-rot fungi, relevant studies should always include the maximum possible number of strains of a given species (Collet, 1992; Woodward and De Groot, 1999; Clausen *et al*., 2000). According to various studies, brown-rot fungi present a higher Cu tolerance than white-rot fungi, which could be related to an increased production and accumulation of oxalic acid in the brown-rot fungi cultures (Murphy and Levy, 1983; Green and Clausen, 2003; Baldrian, 2003). Organic acids, together with other extracellular metal-chelating agents such as siderophores, have been considered among detoxification mechanisms that allow various organisms to tolerate high concentrations of some metal ions (Gadd, 1993, 2004). Most studies have been conducted, however, comparing only single strains of a range of species.

Conclusions

As the result of the collection of wood-rot fungal basidiomes from different sites in Southern-Central Chile, four species are reported for the first time in Chile: *Antrodia xantha*, *Gloeophyllum abietinum*, *G. protractum* and *Stereum rameale.* In addition, all the fungal strains analyzed through qualitative assays produced cellulases and xylanases, according to the observed hydrolysis of CMC and birchwood xylan, respectively, and only some strains produced ligninolytic activity. With regard to metal sensitivity of the strains the great majority showed tolerance to 3mM Cu in solid medium, but were inhibited by 1mM Cd. According to the lignocellulolytic enzymatic potential and/or the degree of tolerance to metal

ions presented by some native strains identified in this study, these could be selected for future biotechnological applications. *A. xantha*, *G. abietinum*, *Gloeophyllum* sp. and *P. panuoides*, for example, will be used in future studies on tolerance to wood copper preservatives and preserved wood waste biodegradation, whereas some strains of *G. australe*, *S. hirsutum* and *T. versicolor* will be used in studies on soil bioremediation or industrial effluents contaminated with organopollutants and heavy metals.

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