Extracellular ABTS-oxidizing activity of autochthonous fungal strains from Argentina in solid medium

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Summary

The screening for extracellular oxidases and peroxidases from autochthonous filamentous fungi isolated from different substrates is an important step towards the detection of extracellular fungal oxidative systems. Thirty-one autochthonous fungal strains from Argentina, belonging to different ecophysiological and taxonomic groups, were plate-screened for their ability to produce extracellular oxidoreductases. Modified Kirk solid medium containing the chromogen 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was used to determine the presence of this extracellular activity. The fungi tested were grouped according to the colour intensity of the modified Kirk medium in: a) species without extracellular ABTS-oxidizing activity; b) species with low extracellular ABTS-oxidizing activity; c) species with moderate extracellular ABTS-oxidizing activity; d) species with high extracellular ABTS-oxidizing activity. The assay revealed extracellular ABTS-oxidizing activity in 90% of the strains tested. All species of *Basidiomycetes* used exhibited ABTS-oxidizing activity, except *Laeticorticium roseum*. *Aspergillus terreus* and *Epicoccum purpurascens* (*Deuteromycetes*) did not show extracellular oxidative activity on ABTS. *Agrocybe aegerita*, *Amauroderma boleticeum*, *Cladosporium cladosporioides*, *Coriolopsis rigida*, *Grammothele subargentea*, *Graphium putredinis*, *Hexagona hydnoides*, *Hexagona papyraceae*, *Loweporus lividus*, *Peniophora albobadia*, *Phellinus everhartii*, *Phellinus gilvus*; *Phellinus linteus*; *Pleurotus laciniatocrenatus*, *Pycnoporus sanguineus*, *Rigidoporus ulmarius*, *Steccherinum rawakense*, *Talaromyces helicus*, *Trametes elegans*, *Trametes pavonia*, *Trametes villosa* and *Trichaptum sector* are reported here for the first time as species capable of producing ABTS-oxidizing extracellular oxidorreductases.

Key words Fungal enzymes, Extracellular ABTS-oxidizing activity, Argentina

Actividad extracelular oxidante del ABTS de cepas fúngicas autóctonas de Argentina en medio sólido

Resumen

La búsqueda de cepas fúngicas autóctonas, aisladas a partir de diferentes sustratos, con actividad oxidasa y peroxidasa es la primera aproximación en la detección de los sistemas oxidativos extracelulares fúngicos. Se probaron 31 cepas fúngicas autóctonas de Argentina, pertenecientes a diferentes grupos taxonómicos y ecofisiológicos, en su habilidad para producir oxidorreductasas extracelulares en medio sólido. El medio agarizado Kirk modificado conteniendo ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfónico) (ABTS) se utilizó para determinar la presencia de la mencionada actividad extracelular. Los hongos utilizados se agruparon de acuerdo a la intensidad de coloración del medio Kirk modificado en: a) especies sin actividad extracelular oxidante del ABTS; b) especies con baja actividad extracelular oxidante del ABTS; c) especies con moderada actividad extracelular oxidante del ABTS; d) especies con alta actividad extracelular oxidante del ABTS. El ensayo reveló actividad oxidante del ABTS en el 90% de las cepas utilizadas. Todas las especies de *Basidiomycetes* utilizadas exhibieron actividad extracelular oxidante del ABTS, excepto *Laeticorticium*

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roseum. *Aspergillus terreus* y *Epicoccum purpurascens* (*Deuteromycetes*) no mostraron actividad oxidorreductasa extracelular sobre el ABTS. *Agrocybe aegerita*, *Amauroderma boleticeum*, *Cladosporium cladosporioides*, *Coriolopsis rigida*, *Grammothele subargentea*, *Graphium putredinis*, *Hexagona hydnoides*, *Hexagona papyraceae*, *Loweporus lividus*, *Peniophora albobadia*, *Phellinus everhartii*, *Phellinus gilvus*; *Phellinus linteus*; *Pleurotus laciniatocrenatus*, *Pycnoporus sanguineus*, *Rigidoporus ulmarius*, *Steccherinum rawakense*, *Talaromyces helicus*, *Trametes elegans*, *Trametes pavonia*, *Trametes villosa* y *Trichaptum sector* son citadas por primera vez como especies productoras de enzimas extracelulares oxidantes del ABTS.

Palabras clave Enzimas fúngicas, Actividad extracelular oxidante del ABTS, Argentina

Several extracellular fungal oxidative enzymes, oxidases and peroxidases, have been involved in the degradative process of different recalcitrant compounds of natural origen (lignin, humic acids) and xenobiotics (chlorinated phenols, polycyclic aromatic hydrocarbons) [1-4]. These enzymes present a non-specific activity [3,5] and are able to form reactive radicals [6], properties that determine their capacity to oxidize and degrade different compounds of high-molecular-mass structure. This group of enzymes exhibits differential characteristics depending on the species, strains and culture conditions [3]. In spite of these enzymes have been mainly investigated in white-rot species (Order *Aphyllophorales*, *Basidiomycetes*) due to their high ligninolytic power [7-8], representatives of other taxonomic and ecophysiological groups also produce them [3,9-12].

Different qualitative and quantitative methodologies to detect extracellular oxidases and peroxidases have already been used by several investigators [4,7,11]. Exist previous works [13-14] on enzymatic characterization of different white-rot *Basidiomycetes* using several aromatic compounds in spot-tests. However, there is no information available as regards the production of extracellular oxidorreductases by autochthonous fungal strains belonging to different ecophysiological and taxonomic groups. Thus, the aim of this work is to detect the extracellular ABTS-oxidizing activity of different autochthonous fungal strains of Argentina in an agar medium containing the chromogen 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS).

MATERIALS AND METHODS

Fungal strains. Fungal strains representative of different ecophysiological and taxonomic groups (Table 1.) were selected for this study. The strains belong to the culture collection of the Instituto Spegazzini (LPSC). Stock cultures of the *Basidiomycetes* strains tested were kept at 4°C on 2% (w/v) malt-agar slants supplemented with yeast extract and a wood chip. Strains of *Deuteromycetes* and *Ascomycetes* were maintained on different solid culture media according to fungal type in slants at 4°C until used.

*Medium and culture conditions.*The ability of the fungal strains to produce extracellular oxidorreductases was performed in an agar medium containing the chromogen 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma), a very sensitive substrate that allows a rapid screening of fungal strains producing the extracellular oxidative enzymes by means of a colour reaction [4,15-16].

Screening was performed in Petri dishes (90 mm diameter) with 20 ml of the modified Kirk medium containing: 10 g of glucose, 2 g of KH_2PO_4, 0.5 g of $MgSO_4$ x $7H_2O$, 0.1 g of $CaCl_2$, 2.2 g of 2,2-dimethylsuccinate, 0.5 g of ammonium tartrate, 0.2 g of yeast extract, 0.2 g of ABTS, 22 g of agar-agar, per liter of medium. The pH was adjusted to 5.0 before autoclaving at 1 atm and 120°C for 20 min. Plates were inoculated with agar disks, 5 mm diameter, of active mycelia previously cultured in 2% malt-agar extract. Each strain was processed in triplicate under controlled conditions at 25°C.

Fungal growth estimation and enzymatic activity. Fungal growth was estimated in terms of diameter of fungal colony. Extracellular ABTS-oxidizing activity was measured by the colour intensity (oxidation of ABTS) of the medium used. A scale of colour intensity was used to express the results: 0= negative, no colouration (without ABTS-oxidizing activity); 1= low colouration; 2= moderate colouration ; and 3= high colouration. Plates were observed once a week for three consecutive weeks. After three weeks no variation in growth or increment in the colouration of the medium with ABTS was observed.

RESULTS AND DISCUSSION

This work represents the first report on the production of extracellular oxidorreductases by different autochthonous fungal strains of Argentina using a test based in an agar medium with the chromogen ABTS. The assay revealed extracellular ABTS-oxidizing activity in 90% of the strains tested. Only *Laeticorticium roseum* (*Basidiomycetes*), *Aspergillus terreus* and *Epicoccum purpurascens* (*Deuteromycetes*) failed to produce detectable ABTS-oxidizing activity under test conditions. Mycelial growth on modified Kirk medium of all fungi tested did not show differences in comparison to those on media used to maintain strains in the culture collection.

The extracellular ABTS-oxidizing activity and the growth of the colonies of the fungal strains into the modified Kirk medium are showed in the Table 2. The fungi tested are grouped according to the scale cited above in:

a) Species lacking extracellular ABTS-oxidizing activity: *Aspergillus terreus*, *Epicoccum purpurascens* and *Laeticorticium roseum* did not reveal extracellular ABTS-oxidizing activity under our test conditions.

Table 1. Fungal strains studied, habitats, and ecophysiological groups to which they belong.

Fungal species and taxonomic groups.	Strain #	Substrate/ habitat	Ecophysiological groups
Basidiomycetes			
Agrocybe aegerita (Brig.) Singer	344	trunk of living tree *Acer negundo*[LP]	LF [24]; WRF[ru]
Amauroderma boleticeum (Pat. & Gaill.) Torr	157	decaying wood of subtropical rain forests[M]	WRF[wr] [14]
Coriolopsis rigida (Berk. Et Mont.) Murrill	133	decaying wood of subtropical rain forests[M]	WRF[ru]
C. rigida (Berk. Et Mont.) Murrill	232	rotten wood of subtropical rain forests[M]	WRF[ru]
Grammothele subargentea (Speg.) Rajch.	436	trunk of living tree Angiosperm of subtropical rain forests[M]	WRF[ru]
Hexagona hydnoides (Swartz: Fr.) K. Fidalgo	136	decaying wood of subtropical rain forests[M]	WRF[ru]
H. papyraceae Berkeley	380	rotten wood of subtropical rain forests[M]	WRF[ru]
Laeticorticium roseum (Fr.) Donk.	164	rotten wood of subtropical rain forests[M]	WRF[ru]
Loweporus lividus (Kalchbrenner) Wright	289	rotten wood of subtropical rain forests[M]	WRF[ru]
Peniophora albobadia (Schw.: Fr.) Boidin	285	rotten wood of subtropical rain forests[M]	WRF[ru]
Phanerochaete sordida (Kartz) Eriksson et Ryv.	179	trunk of living tree *Lauraceae* of subtropical rain forests[M]	WRF[wr] [3]
Phellinus everhartii (Ellis & Gall) Pilát	439	trunk of living tree of subtropical rain forests[M]	WRF[ru]
Phellinus gilvus (Schw.) Pat. var. *licnoides* (Mont.)	156	rotten wood of subtropical rain forests[M]	WRF[wr] [13-14]
Phellinus linteus (Berk. Et Curt.) Teng.	338	trunk of living tree of subtropical rain forests[M]	WRF[wr] [14]
Pleurotus laciniatocrenatus (Speg.) Speg.	39	trunk of living tree *Taxodium* sp. of urban forest area[P]	WRF[ru]
Pleurotus laciniatocrenatus (Speg.) Speg.	332	fallen trunk *Populus* sp. of urban forest area[LP]	WRF[ru]
Pycnoporus sanguineus (L.: Fr.) Murr.	163	trunk of living tree *Leguminosae* of subtropical rain forests[M]	WRF[wr] [13]
Rigidoporus ulmarius (Sow.: Fr.) Imazeki	27	trunk of living tree *Lauraceae* of subtropical rain forests[M]	WRF[wr] [25]
Steccherinum rawakense (Pers. Apud Gaud) Banker.	28	rotten trunk of subtropical rain forests[M]	WRF[ru]
Stropharia rugoso-annulata Farlow	105	upper soil layer[LP]	LF [4]
Trametes elegans (Spreng.: Fr.) Fr.	234	decaying trunk of subtropical rain forests[M]	WRF[ru]
Trametes hirsuta (Wulf.: Fr.) Pl.	343	trunk of living tree *Lauraceae* of subtropical rain forests[M]	WRF[wr] [3]
Trametes pavonia (Hook.) Ryv.	437	rotten trunk of subtropical rain forests[M]	WRF[ru]
Trametes villosa (Fr.) Kreisel	233	rotten trunk of subtropical rain forests[M]	WRF[wr] [3]
Trichaptum sector (Ehrenb.: Fr.) Kreisel	287	rotten wood of subtropical rain forests[M]	WRF[wr] [13]
Deuteromycetes			
Aspergillus terreus Thom	558[m]	Organic matter floating in freshwater[S]	SF [26]
Cladosporium cladosporioides (Fresen.) de Vries	556[m]	Hydrocarbon-polluted soil[E]	SF [26]
Epicoccum purpurascens Ehrenb. ex Schlecht	554[m]	Hydrocarbon-polluted soil[E]	SF [26]
Fusarium solani (Martius) Saccardo	493[h]	hydrocarbon-polluted soil[E]	WRF[sr] [27]; SF [26, 28]
Graphium putredinis (Corda) Hughes	423[h]	hydrocarbon-polluted soil[E]	SF [26]
Ascomycetes			
Talaromyces helicus (Raper & Fennell) C. R. Benjamin	553[m]	Hydrocarbon-polluted soil[E]	SF [26, 28]

Strain #: is the LPSC culture number. Culture media type *Deuteromycetes* and *Ascomycetes*: [m]: 2% (w/v) malt-agar; [h]: Czapek with 1% crude oil. Ecophysiological groups: LF: litter-decaying fungi; WRF: wood-rotting fungi ([wr]: white-rot; [sr]: soft-rot; [ru]: type of rot unknown); SF: soil fungi. [M]: Province of Misiones, Argentina; [P]: Parque Pereyra woodland (Province of Buenos Aires), Argentina; [S]: Santiago river (Province of Buenos Aires), Argentina; [E]: Ensenada (Province of Buenos Aires), Argentina; [LP]: City of La Plata (Province of Buenos Aires), Argentina.

Milstein *et al*. [17] reported that *Aspergillus terreus* did not shown neither phenol-oxidase nor hydroxyquinol-dioxygenase activity. Nevertheless, *A. terreus* is able to decarboxylate, to demethoxylate and to ring-cleave aromatic compounds [17]. On the other hand, two laccases were characterized from *Aspergillus nidulans*, which indicate the presence of phenoloxidases in the genus [18]. In spite of *Epicoccum purpurascens* not showing extracellular oxidorreductase activity on ABTS, a phenoloxidase has already been detected for the benzidine reaction [19]. Cerniglia [20] referred to this specie as a suitable fungus to oxidize naphtalene and other hydrocarbons, suggesting non-specific oxidative enzyme activity. The absence of extracellular ABTS-oxidizing activity does not necessarily imply the lack of capacity to produce these oxidative enzymes but could reflect a possible inhibition of their expression; the oxidative enzyme system is not homogeneous; its production and properties depend on the conditions and culture media [3,21-22].

b) Species with low ABTS-oxidizing activity.This group includes the *Basidiomycetes*: *Phanerochaete sordida* and *Trametes pavonia*, and the *Deuteromycete*: *Fusarium solani*, which is able to use lignin as the sole source of carbon and energy [23]. *P. sordida* have been quoted as a producer of manganese-dependent peroxidases (Mn-P) [3]. *T. pavonia* is reported for the first time as producer of extracellular oxidorreductases under test conditions. *Fusarium solani* has already been reported as a producer of an aromatic alcohol oxidase [9] and an enzyme of the laccase type [10].

c) Species with moderate ABTS-oxidizing activity. This group is represented by the wood-rotting *Basidiomycetes*: *Coriolopsis rigida*; *Peniophora albobadia*; *Phellinus everhartii*; *Steccherinum rawakense*; *Trametes villosa*; and soil fungi: *Cladosporium cladosporioides*; *Graphium putredinis*; and *Talaromyces helicus*, reported for the first time as producers of extracellular enzyme activity on ABTS.

d) Species with high ABTS-oxidizing activity: *Agrocybe aegerita*; *Amauroderma boleticeum*; *Grammothele subargentea*, *Hexagona hydnoides*; *H. papyraceae*; *Loweporus lividus*; *Phellinus gilvus*; *P. linteus*; *Pleurotus laciniatocrenatus*; *Pycnoporus sanguineus*; *Rigidoporus ulmarius*; *Stropharia rugosoannulata*; *Trametes elegans*; *Trametes hirsuta*; *Trichaptum sector*. This is the group with the most active

Table 2. Extracellular ABTS-oxidizing activity and growth estimation of distincts fungal strains into the modified Kirk medium.

Fungal Species	Incubation Time					
	1ª week		2ª week		3ª week	
	R.	G.	R.	G.	R.	G.
Agrocybe aegerita (344)	3	2	3	4	2	6
Amauroderma boleticeum (157)	1	6	3	9	1	9
Aspergillus terreus (558)	0	1	0	2	0	2
Cladosporium cladosporioides (556)	2	2	2	4	2	7
Coriolopsis rigida (133)	2	6	0	9	0	9
Coriolopsis rigida (232)	2	4	2	6	1	7
Epicoccum purpurascens (554)	0	2	0	3	0	5
Fusarium solani (493)	1	7	0	9	0	9
Grammothele subargentea (436)	3	8	0	9	0	9
Graphium putredinis (423)	2	2	2	2	1	2
Hexagona hydnoides (136)	3	4	1	5	1	5
Hexagona papiraceae (380)	3	1	3	3	2	4
Laeticorticeum roseum (164)	0	8	0	9	0	9
Loweporus lividus (289)	3	5	3	7	0	8
Peniophora albobadia (285)	2	8	0	9	0	9
Phanerochaete sordida (179)	1	7	1	9	1	9
Phellinus everhartii (439)	2	1	2	3	2	4
Phellinus gilvus (156)	3	2	3	4	3	6
Phellinus linteus (338)	2	0	2	2	1	2
Pleurotus laciniatocrenatus (39)	3	3	3	4	1	5
Pleurotus laciniatocrenatus (332)	3	4	3	9	0	9
Pycnoporus sanguineus (163)	3	3	3	6	0	8
Rigidoporus ulmarius (27)	3	2	2	5	1	5
Stecherinum rawakense (28)	2	1	2	1	2	2
Stropharia rugoso-annulata (105)	3	2	3	4	3	6
Talaromyces helicus (553)	1	5	2	6	1	9
Trametes elegans (234)	3	4	2	8	1	9
Trametes hirsuta (343)	3	7	0	9	0	9
Trametes pavonia (437)	1	3	1	6	1	7
Trametes villosa (233)	2	8	0	9	0	9
Tricaptum sector (287)	3	1	3	2	3	4

Data are mean of 3 replicates. In parentheses, numbers of LPSC culture. R: intensity of the oxidation enzymatic reaction of ABTS (see scale of intensities in Materials & Methods) G: fungal colony growth (diameter in cm).

extracellular oxidorreductases producers. It was restricted to the *Basidiomycetes*, especially the *Aphyllophorales*, which are wood-rotting fungi, with the exception of the litter-decaying fungi: *Agrocybe aegerita* and *Stropharia rugoso-annulata* (*Agaricales*). Interestingly, Peláez *et al.* [7] reported that *A. aegerita* did not produce any extracellular ligninolytic oxidative enzyme in shaken liquid cultures. Neverthelss, *A. aegerita*, under controlled conditions, mineralized the 1.5% and 38.5% of ^{14}C pyrene present in sterile and non-sterile, artificially contaminated soil [24]. Except for *Stropharia rugoso-annulata* and *T. hirsuta*, all the species present in this group are reported here for the first time as having extracellular ABTS oxidative enzymes.

This work allowed us to identify the extracellular oxidorreductase-producing autochthonous fungi by using a suitable, easy and inexpensive method. Neverthelss, more detailed research on the production and induction of that kind of enzymes by fungal strains and species belonging to the different groups considered here will help to further understand the properties of these catalysts as well as the optimal conditions for the expression of ABTS-oxidizing activity in the different fungi tested.

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