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Production of ligninolytic enzymes by *Fusarium solani* strains isolated from different substrata

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Summary

A comparative study on the extracellular ligninolytic enzymatic activity of five strains of *Fusarium solani* in a carbon-limited medium under shaking, revealed a differential production of these enzymes. Aryl alcohol oxidase (AAO) activity was observed only in the supernatant of strain CLPS no. 568 with levels higher than 57 mU ml^{-1}. Free extracellular laccase activity was detected in strains CLPS nos. 493, 568 and 570, strain no. 568 being the one which showed the highest activity (over 8.6 mU ml^{-1}). Free extracellular lignin peroxidase (LiP) activity was not detected in any isolate tested, whereas low levels of manganese-dependent peroxidase (MnP) and manganese-independent peroxidase (MIP) activities were detected in certain isolates used. The AAO activity of *F. solani* on primary α-alcohols such as veratryl alcohol, is reported for the first time; this enzyme activity is hydrogen-peroxide independent. This is also the first report for extracellular MnP and MIP activities of *F. solani*.

Introduction

The genus *Fusarium* produces wilt diseases in a wide variety of plants and the ability of the pathogen to cause wilt symptoms is correlated to its enzyme production (Cooper 1984). Usually these fungi preferentially degrade carbohydrates and pectin in wood; different enzymes involved in cellulose, hemicellulose and pectin degradation have been characterized previously (Alconada & Martínez 1994). However, these fungi are also able to mineralize lignin (Norris 1980; Regalado *et al.* 1997). Ligninolytic systems have been mainly investigated in white-rot fungi (Szklarz *et al.* 1989; Peláez *et al.* 1995; Heinzkill & Messner 1997). These enzymatic systems include ligninolytic peroxidases, lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and manganese-independent peroxidase (MIP), laccases and the enzymes responsible for the production of H_2O_2, such as glyoxal oxidase or aryl-alcohol oxidase (AAO) (Guillén *et al.* 1992; Heinzkill & Messner 1997). These enzymes present a wide substrate specificity (Hofrichter & Fritsche 1996) and they are also involved in different aspects of fungal physiology (breakdown of plant polymers, detoxification of phenolic compounds, conidial pigmentation, morphogenesis and pathogenesis) (Binz & Canevascini 1996; Heinzkill & Messner 1997). Due to the lack of specificity of the system involved in the depolymerization and mineralization of lignin, fungi are important organisms to be taken into account in biorremediation processes (Field *et al.* 1993).

Fusarium solani is able to mineralize ^{14}C-labelled milled wood lignin from wheat straw, degrading about 25% of carbohydrates and lignin (Rodríguez *et al.* 1996). There have also been reports on the capacity of *F. solani* to oxidize α,β-unsaturated alcohols, such as coniferyl alcohol, to the corresponding aldehyde, as well as the non-etherified side chain of dehydroconiferyl alcohol (Iwahara *et al.* 1980). In other species of *Fusarium* a laccase-type intracellular enzyme was isolated capable of oxidizing guaiacol (Janshekar & Fiechter 1983); molecular evidence of a lignin peroxidase has been reported in *F. oxysporum* (Mönkemann *et al.* 1996).

The aim of the present work was to get a better insight into the ability of five strains of *F. solani*, isolated from

different substrata and growning in C-limited medium, to produce free extracellular ligninolytic enzymes: AAO, laccase, LiP, MnP and MIP.

Materials and Methods

Fungal strains

The strains of *F. solani* used for this work were isolated from different substrata and habitats; they belong to the culture collection of the Spegazzini Institute (CLPS). Stock cultures were maintained on agar slants (Table 1) at 4 °C until used.

Medium and culture conditions

Fungal strains were cultivated in 250 ml Erlenmeyer flasks containing 50 ml of the modified Czapek Dox medium (Peláez *et al.* 1995). The flasks were incubated at 25 °C in a rotary shaker at 150 rev min^{-1}. The cultures were performed in quadruplicate.

Analytical methods

The fungal cultures were harvested at days 7, 14 and 21 of incubation. Each sample was centrifuged ($10{,}000 \times g$ for 30 min) at 4 °C and filtered through tared Whatman no. 1 filter paper. The mycelial pellet was used to estimate fungal biomass, and the dry weight was determined at 60 °C. The supernatant of the liquid culture was collected for the determination of reducing sugars, proteins, pH and for enzymatic assays. The reducing sugars were determined by the Somogyi and Nelson method (Somogyi 1945). The extracellular proteins were determined by the Lowry method. The pH was determined using a Beckman 50 pH-meter.

Table 1. Fungal isolates used in this study.

CLPS strain no.	CLPS culture medium of maintenance	Year of isolate	Substrate of isolate
256[a]	m	1993	Organic matter floating in freshwater[c]
273[a]	m	1993	Organic matter floating in freshwater[c]
493[a]	m	1995	Crude-oil polluted soil[d]
568[a]	h	1995	Non hydrocarbon polluted agricultural soil[d]
570[b]	m	1997	*Eustoma grandiflora* (host), with symptom of base rot[e]

[a] Saprotrophic (saprophytic) strain.
[b] Phytopathogen strain.
Collection source
[c] Santiago river (Province of Buenos Aires), Argentina.
[d] Ensenada (Province of Buenos Aires), Argentina.
[e] La Plata city (Province of Buenos Aires), Argentina.
Culture media strain type: m: 2% (w v^{-1}) malt-agar; h: Czapek with 1% (v v^{-1}) crude oil.

Enzyme assays

AAO activity was estimated by following veratraldehyde formation spectrophotometrically (ε_{301}: 9,300 M^{-1} cm^{-1}) from 5 mM veratryl alcohol (Fluka) in 100 mM phosphate buffer at pH 6.0 (Guillén *et al.* 1992). Laccase activity was measured spectrophotometrically with 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma) in 100 mM acetate buffer at pH 5.5 (ε_{436} of the ABTS cation radical: 29,300 M^{-1} cm^{-1}) (Peláez *et al.* 1995). The LiP activity was determined by H_2O_2-dependent veratraldehyde formation from 2 mM veratryl alcohol (Fluka) in 100 mM sodium tartrate buffer at pH 3.0, with H_2O_2 0.4 mM (Tien & Kirk 1988), and Azure B oxidation was determined spectrophotometrically by utilizing 32 μM Azure B, in 100 mM Na tartrate (pH 4.5) and H_2O_2 0.4 mM (ε_{651} of Azure B: 48,800 M^{-1} cm^{-1}) (Archibald 1992). Total phenol-red (PR)-determined peroxidase activity was estimated under conditions described by Paszcynsky *et al.* (1988) using 0.01% of this phenolic substrate (Sigma) in presence of 0.1 mM H_2O_2 and 1 mM $MnSO_4$ in 100 mM Na tartrate (pH 5.0). MIP activity was similarly assayed as described above except the final assay contents contained 1 mM EDTA and $MnSO_4$ was excluded. MnP activity was calculated by subtracting the MIP activity from the total PR peroxidase activity.

Oxidative enzyme activities were determined on aliquots of the supernatant. The enzyme reactions were carried out in duplicate, and control assays were performed without the addition of the enzyme or hydrogen peroxide for oxidase and peroxidase assays, respectively. Based on preliminary studies, optimal assay times were known to fall in the linear range of enzyme kinetics. All oxidation rates were determined at 25 °C using a Beckman DU 640 u.v.-visible spectrophotometer. 1 U of enzymatic activity was defined as the amount of enzyme that transforms 1 μmol substrate min^{-1}.

Results

Table 2 shows fungal biomass from five strains of *F. solani* grown in modified Czapek Dox medium, at days 7, 14 and 21 of incubation. The strain CLPS no. 273 reached the highest biomass value (over 400 mg 100 ml^{-1} of culture medium) at the seventh day of incubation, decreasing at days 14 and 21. A similar pattern was observed in the rest of the strains tested.

The reducing sugars were already undetectable in the supernatant at the seventh day of incubation. Under these conditions, and referring to the values of fungal biomass, the cultures were found in idiophase or stationary phase of growth under carbon-limited conditions, restricting the trophophase to the first days.

The extracellular protein levels varied in the different strains used (Table 3). The highest levels of extracellular

Table 2. Estimation of fungal biomass (mg 100 ml^{-1}) of different strains of *F. solani*[a].

Fungal strain no.	Day 7	Day 14	Day 21
256	382.2 ± 3.3	363.4 ± 6.6	373.9 ± 2.3
273	405.3 ± 22.5	370.1 ± 10.0	360.7 ± 2.9
493	365.6 ± 6.9	333 ± 7.5	355.3 ± 6.3
568	261.9 ± 25.9	190.9 ± 46.6	169.7 ± 35.4
570	388.8 ± 12.2	380.7 ± 5.2	364.1 ± 6.8

[a] All values are reported as mean ± SD for four replicate cultures.

Table 3. Production of extracellular proteins (μg ml^{-1}) of different strains of *F. solani*[a].

Fungal strain no.	Day 7	Day 14	Day 21
256	267.0 ± 22.0	520.6 ± 5.1	363.2 ± 175.1
273	845.9 ± 122.7	335.2 ± 76.7	471.2 ± 111.0
493	287.7 ± 25.5	376.2 ± 22.4	407.2 ± 16.4
568	506.8 ± 53.6	416.6 ± 56.2	381.3 ± 91.2
570	629.4 ± 101.1	599.5 ± 54.5	342.2 ± 74.1

[a] All values are reported as mean ± SD for four replicate cultures.

proteins correspond to the culture of strain no. 273 at 7 days of incubation.

pH measurements revealed an increase of more than two units in the supernatant of the cultures of the different strains after the first 7 days of incubation. Values close to neutrality were also detected on days 14 and 21, which corresponded to the optimal pH for *in vitro* growth (7.8).

The AAO activity was only detected in the supernatant of isolate no. 568 at all the incubation times tested, attaining the highest level at day 21 of incubation (>57 mU ml^{-1}) (Table 4).

Free extracellular laccase activity (Table 4) was detected in the cultures of strains nos. 493, 568 and 570 with the strain no. 568 showing the highest activity (over 8.6 mU ml^{-1}). In the strain no. 493, laccase activity was detected only on day 21 of incubation. However, strains nos. 568 and 570 showed laccase activity at three incubation times, reaching the highest levels on days 21 and 14 of incubation, respectively.

Table 5. Free extracellular MnP (MIP) activity (mU ml^{-1}) in different strains of *F. solani*[a].

Fungal strain no.	Day 7	Day 14	Day 21
256	0.3 ± 0.1	0[b]	0
273	0	0	0
493	0.5 ± 0.1	0	0
568	0.4 ± 0.1	0.7 ± 0.2 (0.7 ± 0.5)	0.4 ± 0.1
570	0	0	0

[a] All values are reported as mean ± SD for four replicate cultures.
[b] No activity.

The LiP activity, assayed by two different methods (the oxidation of veratryl alcohol and the oxidation of Azure B) was not detected in any of the strains tested; whereas low levels of MnP activity (Table 5) were detected in the cultures of strains nos. 256, 493 and 568; this latter strain showed the highest activity (0.7 mU ml^{-1}) of MIP.

Discussion

Lignin is a recalcitrant heteropolymer of phenyl-propanoid units present in woody plant tissues (Higuchi 1990). Lignin biodegradation is a natural process developed mainly by basidiomycetous fungi (Kirk & Farrell 1987). However, other microorganisms such as *Ascomycetes* and *Deuteromycetes* are involved in the lignin decay process (Drews & Kadam 1978; Ferraz *et al.* 1991). *Fusarium solani* and other *Fusarium* sp. are able to colonize, modify and degrade lignin (Norris 1980; Rodríguez *et al.* 1996), as well as model compounds of this polymer (Ohta *et al.* 1979; Nazareth & Mavinkurve 1986). The physiological variability of *F. solani* (Domsch *et al.* 1993), led us to examine the production of different extracellular ligninolytic enzymes by strains isolated from different sources.

In regards to the ligninolytic enzymatic activities studied in this work, it is necessary to comment that LiP activity has not been detected in the five strains of *F. solani* in the culture conditions used. Evidence of LiP in *F. oxysporum* has been detected by Northern blot

Table 4. Free extracellular AAO and laccase activities in different strains of *F. solani*[a].

Fungal strain no.	Enzymatic activity (mU ml^{-1})					
	AAO			Laccase		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
256	0[b]	0	0	0	0	0
273	0	0	0	0	0	0
493	0	0	0	0	0	0.4 ± 0.1
568	51.6 ± 11.2	51.9 ± 7.6	57.5 ± 17.1	2.2 ± 1.1	5.2 ± 1.2	8.7 ± 3.2
570	0	0	0	1.0 ± 0.4	4.0 ± 1.7	1.4 ± 0.3

[a] All values are reported as mean ± SD for four replicate cultures.
[b] No activity.

analysis (Mönkemann *et al.* 1996). The AAO, laccase, MnP and MIP activities have only been detected in certain isolates. These results are in accordance with those reported on the variability of ligninolytic enzymes in the different fungal species, and their production depends on the composition and conditions of the culture (Rogalski *et al.* 1991; Schoemaker *et al.* 1991). Likewise, and according to Hammer & Schauer (1997), the oxidative enzyme system is a strain-specific characteristic and not a species-dependent feature. The differential production and the inability to secrete extracellular oxidases and peroxidases by certain strains of *F. solani*, could be due to the maintenance of the strains in culture for long periods (Szklarz *et al.* 1989). In this sense, the total absence of enzymatic activity in the supernatant of non-producer cultures does not indicate the lack of capacity to produce them. Enzyme systems can be inhibited by different factors that interfere with their expression (Bollag & Leonowicz 1984; Deshpande *et al.* 1992).

According to Iwahara *et al.* (1980), the AAO of *F. solani* is able to oxidize α,β-unsaturated alcohols such as coniferyl alcohol, the non-etherified side chain of dehydroconiferyl alcohol and different treated lignins, but shows no activity on veratryl alcohol. However, in spite of this latter activity not being detected in the supernatant of the strains nos. 256, 273, 493 and 570 of *F. solani*, it was found in the supernatant of isolate no. 568; the veratryl alcohol was oxidized to veratraldehyde. This reaction was independent of hydrogen peroxide. Therefore, this oxidase activity detected in *F. solani* strain no. 568 was different to those reported for this species by Iwahara *et al.* (1980). The lack of LiP activity in the supernatant of this latter strain, not detected by any of techniques used, confirms the extracellular oxidase activity of *F. solani* on veratryl alcohol. This is the first report of AAO activity of *F. solani* on primary α-alcohols, enlarging the spectrum of substrates for this oxidase.

The levels of MnP and MIP estimated were comparatively lower than AAO and laccase activities, as has been reported for ligninolytic systems of different white-rot fungi (Peláez *et al.* 1995). As far as we know, this is the first report of extracellular MnP and MIP activities in *F. solani*.

The AAO activity detected in strain no. 568 of *F. solani* assayed in this work is higher than that found in strains isolated from different white wood-rot *Basidiomycetes* assayed under the same conditions (Peláez *et al.* 1995). Many ligninolytic Basidiomycetous fungi only secrete oxidative lytic enzymes under some nutrient-limited conditions (Faison & Kirk 1985; Peláez *et al.* 1995). These strains generally require long incubation times, 15–20 days or even more, to complete their growth and reach the idiophase (Pal *et al.* 1980). The high growth rate and plasticity of *F. solani* (Domsch *et al.* 1993), may make this species useful in biotechnology related to lignin biodegradation and biorremediation process.

Variations among strains represent a crucial problem in the study of the oxidative enzymology of this species with such a broad physiological heterogeneity (Domsch *et al.* 1993; Hammer & Schauer 1997). In this respect, it is worth emphasizing the importance of working with accurately identified strains, deposited in a culture collection. Under such *in-vitro* culture conditions, each isolate could be maintained for long periods of time, and the effect of different metabolites studied.

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