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Growth and extracellular laccase production in liquid cultures of *Minimidochium parvum* LPSC # 548 Strain*

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Summary: *Minimidochium parvum* LPSC # 548, a fungus isolated from litter floating on waters of Río Santiago (Provincia de Buenos Aires, Argentina) polluted with industrial effluents and crude-oil, was grown as a shaking culture on a C-limited medium to evaluate its ability to produce extracellular laccase. The effect of anthracene, $CuSO_4 \cdot 5H_2O$, ethanol, guaiacol, humic acids, Kraft lignin, $MnSO_4 \cdot H_2O$, Tween 20 and veratryl alcohol on its growth and extracellular laccase activity levels was also analyzed. The cultures grown on basal medium produced maximum biomass (over 420 mg/100 ml) and maximum extracellular laccase activity (351.7 ±53.3 pkat/ml) after 5 days of incubation. Among the different factors tested, only the humic acids at 0.1 % (w/v) were found to stimulate the growth of *M. parvum*. However, Tween 20 (0.1 %, v/v) was the only one that produced an increase of laccase activity levels up to 2.5-fold compared to the control.

Key words: growth, laccase, *Minimidochium parvum*.

Resumen: Crecimiento y producción de lacasa extracelular en cultivos líquidos de *Minimidochium parvum* cepa LPSC # 548. *Minimidochium parvum* LPSC # 548, un hongo aislado de materia orgánica colectada en aguas de Río Santiago (Provincia de Buenos Aires, Argentina) contaminadas con efluentes industriales y crudo de petróleo, se cultivó en un medio líquido limitante en carbono bajo agitación para evaluar su habilidad para producir lacasa extracelular. Se analizó también el efecto de ácidos húmicos, alcohol veratrílico, antraceno, $CuSO_4 \cdot 5H_2O$, etanol, guaiacol, lignina Kraft, $MnSO_4 \cdot H_2O$ y Tween 20 sobre el crecimiento fúngico y los niveles de actividad lacasa extracelular. Los cultivos sobre medio basal produjeron máximos niveles de biomasa (superior a 420 mg/100 ml) y actividad lacasa extracelular (351,7 ±53,3 pkat/ml) después de 5 días de incubación. Entre los diferentes agentes químicos testeados, sólo los ácidos húmicos al 0,1 % (p/v) estimularon el crecimiento de *M. parvum*. No obstante, sólo el Tween 20 (0,1 %, v/v) produjo un incremento de los niveles de actividad lacasa (2,5 veces comparado a cultivos control).

Palabras clave: crecimiento, lacasa, *Minimidochium parvum*.

Introduction

Saparrat *et al.* (2002a,b) found that several Fungi Imperfecti isolated from litter polluted with crude-oil, produced extracellular laccase (Lac) activity.

Several fungi and their extracellular enzymes, including phenol-oxidases, are currently being studied since they are useful tools for designing environmentally-sound methods to detoxify and/or degrade aromatic pollutants, which constitute a serious threat to human health (Heinzkill & Messner, 1997; Perestelo *et al.*, 1999; Wong & Yu, 1999; Johannes & Majcherczyk, 2000; Saparrat *et al.*, 2002a; Cing *et al.*, 2003; Eichlerová *et al.*, 2003; Kahraman & Yesilada, 2003; Tomsovsky & Homolka, 2003; Saparrat & Hammer, 2006).

Minimidochium parvum Cabello, Arambarri and Cazau (Hyphomycetes, Fungi Imperfecti) is a fungal species that was isolated from Rio Santiago (Buenos Aires Province, Argentina) (Cabello *et al.*, 1998). This river receives a considerable amount of pollutants such as industrial effluents and crude-oil that produce high levels of contamination (Romero *et al.*, 2002).

Laccases (p-diphenol: oxygen oxidoreductase; EC 1.10.3.2) are a group of phenol-oxidases whose activity may result in fungal detoxification of several

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hazardous pollutants, as well as in the resistance to pollution by crude-oil and xenobiotics (Perestelo *et al.*, 1999; Wong & Yu, 1999; Johannes & Majcherczyk, 2000; Saparrat *et al.*, 2002a; Saparrat & Balatti, 2005). These enzymes catalyse the oxidation of aromatic amines, a wide number of phenolic compounds including chlorophenols, anthraquinone dyes and, to a certain extent, some polycyclic aromatic hydrocarbons (PAHs), such as anthracene (Heinzkill & Messner, 1997; Johannes & Majcherczyk, 2000). However, fungal laccases may also play a role in physiological processes, such as sporulation, pigment production, plant pathogenesis, delignification, humification and morphogenesis (Saparrat & Balatti, 2005). In accordance with this, several patterns and mechanisms for laccase induction that control its regulation and production have been described (Crowe & Olsson, 2001).

Based on the fact that *M. parvum* was isolated from polluted litter and that information on the physiology of laccase production of this fungal species is not available, the aim of this work was to evaluate the ability of *M. parvum* to produce extracellular laccase and to determine the effect of 9 chemical compounds, which are related to different roles of the laccases in fungal processes, on its growth and on the extracellular Lac activity levels.

Materials and Methods

Microorganism, growth and analytical methods

M. parvum LPSC (Culture collection of the La Plata Spegazzini Institute) 548 strain was isolated from floating litter collected from heavily polluted water from Rio Santiago (Buenos Aires Province, Argentina). The fungus was grown on a basal medium (Saparrat *et al.,* 2002b). Several inducers such as 0.1 mM anthracene in 2.5 % (v/v) ethanol, 0.4 mM $CuSO_4 \cdot 5H_2O$, 2.5 % (v/v) ethanol, 1 mM guaiacol, 0.1 % (w/v) humic acids, 0.1 % (w/v) Kraft lignin, 0.4 mM $MnSO_4 \cdot H_2O$, 0.1 % (v/v) Tween 20 and 1 mM veratryl alcohol were added separately to the basal medium before inoculating the fungus, in order to study their effect on the growth and on the oxidative enzyme production. Anthracene was obtained from *E. Merck* (Darmstadt, Germany). Humic acids, Kraft lignin, $MnSO_4 \cdot H_2O$, Tween 20 and veratryl alcohol were purchased from *Sigma* (St Louis, MO, USA). The remaining inducers used were of analytical grade. One hundred ml Erlenmeyer flasks filled with 20 ml medium were inoculated with 2.5 % (v/v) mycelial suspension and incubated in a rotary shaker (2.5 Hz) at 28 ±2.0 °C. Pellets were removed by centrifugation and the supernatant was collected to measure enzyme activity [expressed as pkat/ml liquid medium], pH and reducing sugars. Reducing sugars were assayed by the Somogyi and Nelson method (Somogyi, 1945). Pellets were stained by means of cotton blue and were observed with a microscope to detect the presence of mycelium and asexual spores (chlamydospores and conidia). The lignin associated to the mycelium was dissolved by adding 10 ml 1 M NaOH to the pellets of each culture flask. Fungal growth was estimated by measuring biomass dry mass (mg/100 ml), after drying the pellets overnight at 90 ºC. The effect of the chemical inducers on fungal growth was estimated by determining the level of fungal biomass after culturing the fungus for 5 days in the presence of inducer; the following formula was used to calculate this: [(biomass levels from cultures supplemented with a certain chemical compound – biomass levels from control cultures on basal medium)/ biomass levels from control cultures] x 100. For statistical analysis of data, the Least Significant Difference (LSD) test was used.

Enzyme assays: Lac activity was measured spectrophotometrically with 10 mM 2,6-dimethoxyphenol (DMP) (*Fluka*, USA), in 100 mM tartrate buffer, pH 3.0 (Saparrat & Guillén, 2005). Peroxidase activity was assayed as Lac activity in the presence of 0.1 mM H_2O_2. The enzyme activity is expressed in picokatals (pkat; 1 pkat is the enzyme activity releasing 1 pmol of oxidized product per second). The level of extracellular enzyme activity of cultures supplemented with the chemical inducers was estimated at two sampling times: one related to trophophase, which corresponded to the day when the maximum biomass level on basal medium (5^{th} day) was observed; and another, which was considered in relation to idiophase, after 11 days of culture.

Results and Discussion

The levels of biomass produced by *M. parvum* in cultures grown on basal medium, its extracellular Lac activity and the reducing sugars from the culture medium were monitored for 14 days (Fig. 1). These cultures developed yellowish mycelial pellets, with no spores. The trophophase was restricted to the first 5 days of culture, achieving a biomass level of 423.5 ±14.1 mg/100 ml of basal medium. The cultures subsequently entered into the idiophase under

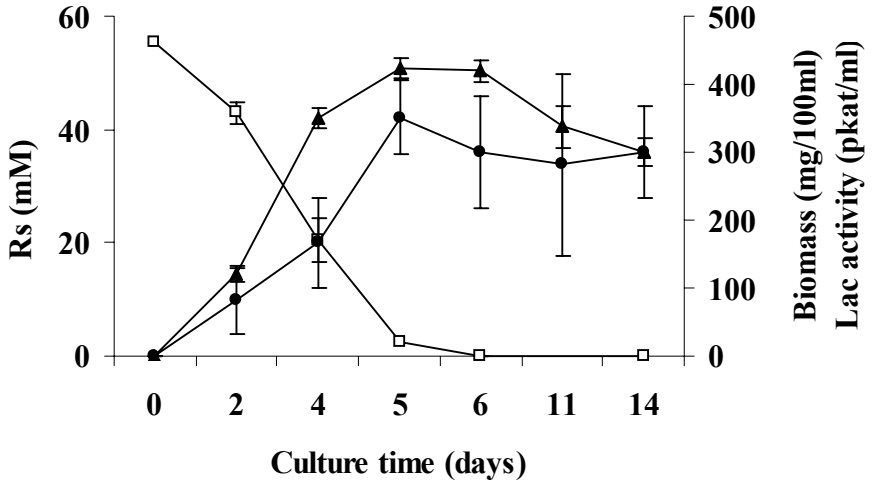

Fig. 1. Time course of biomass (*filled triangles*) of *M. parvum* cultures grown on basal medium as well as its Lac activity (*filled circles*) and reducing sugars (Rs) (*hollow squares*) present in the extracellular fluid. All values are means of three replicates. Error bars correspond to standard deviation.

carbon-limited conditions. Extracellular Lac activity increased during the trophophase, reaching maximum levels after 5 days of culture (351.7 53.3 pkat/ml of liquid medium). Then, the levels of enzyme activity remained constant during 14 days of incubation. The addition of H_2O_2 to reaction mixture to detect Lac activity did not exert any effect on the oxidation of DMP, suggesting that extracellular peroxidase activity was absent from cultures of *M. parvum* on basal medium.

The effect of several chemical inducers on the biomass and extracellular Lac activity levels of *M. parvum* cultures was compared to control cultures grown on basal medium. These putative inducers, which are related to several roles that laccases may play in fungal processes, were chosen in relation to either their known or suspected action as substrates (anthracene, guaiacol, humic acids, Kraft lignin) and cofactors of fungal laccases ($CuSO_4 \cdot 5H_2O$), or their role in controlling the regulation and production of laccase (ethanol, $MnSO_4 \cdot H_2O$, Tween 20, veratryl alcohol; Gomez-Alarcon *et al.*, 1989; Crowe & Olsson, 2001; Lo *et al.*, 2001; Tribak *et al.*, 2002). Most of the chemical inducers added to the culture medium did not affect the morphology and/or other characteristics of the fungus when grown on basal medium. Only two cultures, one supplemented with humic acids and the other one with Kraft lignin, exhibited dark-coloured pellets as a result of the adsorption of the inducers and their metabolites to the mycelium. Furthermore, in cultures supplemented with humic acids, the fungus formed chlamydospores and produced a remarkable loss of brown color to the medium.

The level of biomass of *M. parvum* cultures grown on basal medium (control) and in the presence of different inducers after 5 and 11 days of incubation is presented in Fig. 2. *M. parvum* produced the largest biomass (over 670 mg/100 ml of culture medium at the 5th day of incubation) in cultures supplemented with humic acids at 0.1 %, which were the only compounds found to stimulate growth of *M. parvum* (34.4 %; $P \leq 0.01$). Dehorter & Blondeau (1992) reported for cultures of *Phanerochaete chrysosporium* and *Trametes versicolor* (Basidiomycota) that their growth was also stimulated by humic acids. Although in other inducer-supplemented *M. parvum* cultures biomass production was also abundant, in those supplemented with lignin it reached low levels (less than 300 mg/100 ml after 11 days of incubation). Likewise, anthracene, ethanol and Kraft lignin markedly inhibited growth of *M. parvum* (65 to 75 %; $P \leq 0.01$) after 5 days of incubation. This reduction in *M. parvum* growth might be due to the effect of the oxidative stress and the presence of toxic compounds in the culture medium as a result of fungal transformation of the inducers. Accordingly, Crowe & Olsson (2001) and Lo *et al.* (2001) reported the deleterious effect of several chemicals, including ethanol and several Lac substrates, on fungal growth, via the production of oxidative stress and toxic compounds. Most *M. parvum* cultures exhibited lower amounts of biomass after 11 days of culture

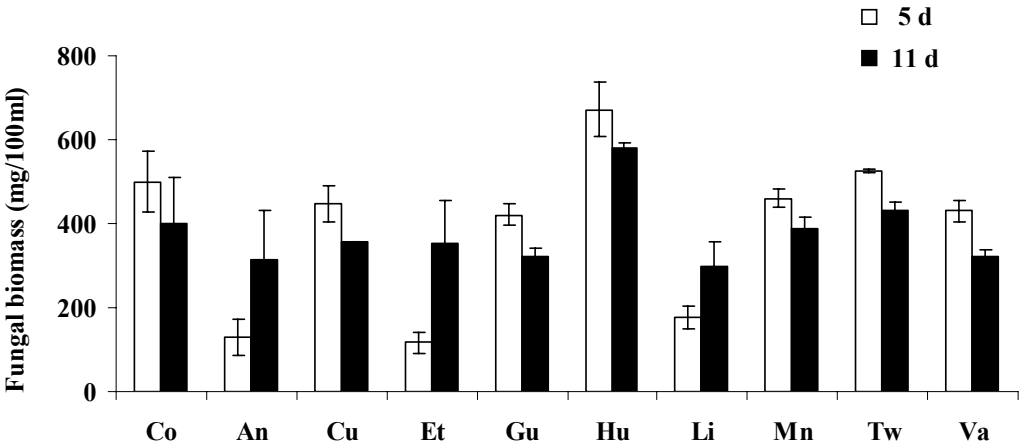

Fig. 2. Biomass production of *M. parvum* cultures grown in absence (**Co**) and in presence of different inductors (**An**, 0.1 mM anthracene in 2.5 % (v/v) ethanol; **Cu**, 0.4 mM $CuSO_4 \cdot 5H_2O$; **Et**, 2.5 % (v/v) ethanol; **Gu**, 1 mM guaiacol; **Hu**, 0.1 % (w/v) humic acids; **Li**, 0.1 % (w/v) Kraft lignin; **Mn**, 0.4 mM $MnSO_4 \cdot H_2O$; **Tw**, 0.1 % (v/v) Tween 20; **Va**, 1 mM veratryl alcohol) at 5th and 11th day (**d**) of culture. All values are means of three replicates and error bars correspond to standard deviation.

compared to the biomass levels observed after 5 days of culture. However, those cultures supplemented with anthracene, ethanol and Kraft lignin showed an inverse profile, suggesting that these inducers might have been slowly detoxified along the fungal culture, thus allowing later growth of *M. parvum* under these conditions. However, *M. parvum* grown in the presence of $CuSO_4$, guaiacol, $MnSO_4$, Tween 20 or veratryl alcohol, produced a similar amount of biomass to that of the control. Although Tween 20 at 0.1 % did not stimulate growth of *M. parvum*, several reports have described its action as a stimulator for fungal growth (Leìstan *et al.,* 1993; Tribak *et al.,* 2002).

While *M. parvum* grown in the presence of veratryl alcohol for 5 days exhibited levels of extracellular Lac activity (400.1 ±76.7 pkat/ml) similar to those of the control, none of the cultures grown in the presence of the remaining inducers showed any Lac activity. However, after 11 days of incubation those cultures supplemented with $MnSO_4 \cdot H_2O$, Tween 20 and veratryl alcohol exhibited Lac activity (Fig. 3). Tween 20 at 0.1 % was, among the inducers tested, the only one that resulted in a 2.5-fold increase of Lac activity in *M. parvum* cultures compared to the control ($P \leq 0.01$). This increment in the levels of Lac activity might have been the result of the surfactant properties of Tween 20 and/or its ability to induce enzyme production. In agreement with this, Gomez-Alarcon *et al.* (1989) and Tribak *et al.* (2002) found that several surfactants also induced the production of fungal extracellular enzymes, including

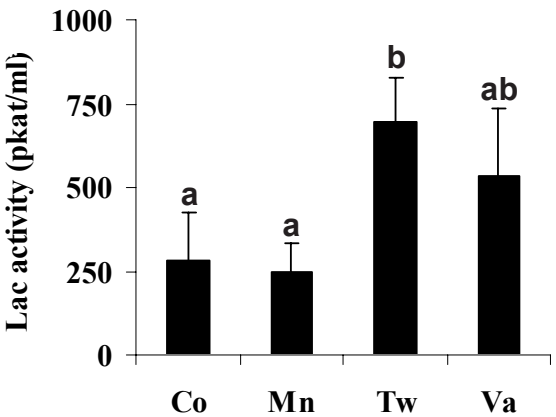

Fig. 3. Extracellular Lac activity of *M. parvum* cultures grown in absence (**Co**) and in presence of $MnSO_4 \cdot H_2O$ (**Mn**), Tween 20 (**Tw**), or veratryl alcohol (**Va**) after 11 days of culture. Values are means of three replicates. Error bars correspond to standard deviation. Bars with the same letter are not significantly different (LSD, $P \leq 0.01$).

laccases. However, no extracellular peroxidase activity was detected in cultures of *M. parvum* supplemented with 9 inducers after 5 and 11 days of incubation, suggesting that the only enzymatic component of the extracellular oxidative system of *M. parvum* is the Lac activity. Since laccases catalyse the oxidation of several toxic and recalcitrant aromatic compounds, this enzyme activity in *M. parvum* might contribute to the resistance mechanisms of the fungus to xenobiotic pollution.

This study reports that *M. parvum* shaken cultures grown on basal medium increased their Lac

activity levels during the trophophase. In these cultures humic acids at 0.1 % and Tween 20 at 0.1 % proved to be the best effectors for supplementing production of biomass and laccase, respectively. These findings have implications in the culture conditions choice and design for further investigations at larger scales and in the potential application of *M. parvum* and its laccase in the biotechnology field, mainly for xenobiotic transformation and detoxification processes.

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