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Research Paper

Halotolerance, ligninase production and herbicide degradation ability of basidiomycetes strains

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Abstract

Fungi have been recently recognized as organisms able to grow in presence of high salt concentration with halophilic and halotolerance properties and their ligninolytic enzyme complex have an unspecific action enabling their use to degradation of a number of xenobiotic compounds. In this work, both the effect of salt and polyols on growth of the basidiomycetes strains, on their ability to produce ligninolytic enzyme and diuron degradation were evaluated. Results showed that the presence of NaCl in the culture medium affected fungal specimens in different ways. Seven out of ten tested strains had growth inhibited by salt while *Dacryopinax elegans* SXS323, *Polyporus sp* MCA128 and *Datronia stereoides* MCA167 fungi exhibited higher biomass production in medium containing 0.5 and 0.6 mol.L^{-1} of NaCl, suggesting to be halotolerant. Polyols such as glycerol and mannitol added into the culture media improved the biomass and ligninases production by *D. elegans* but the fungus did not reveal consumption of these polyols from media. This fungus degraded diuron in medium control, in presence of NaCl as well as polyols, produced MnP, LiP and laccase.

Key words: basidiomycetes, halotolerance, ligninases, diuron degradation.

Introduction

The ability to growth and the mechanisms involved in adaptation to low water activity (a_w) has been well understood for prokaryote groups. However, only in the last decade fungi were isolated from saline and hypersaline environments (Moubasher *et al.*, 1990; Cantrell *et al.*, 2006) but their adjustment mechanisms to halophile conditions is still not clear. Two proteins, in the membrane and in the cytoplasm (HwHhk7 and HwSho1) have been reported to act as osmosensors in halophile fungus which is a signal for cascade of the MAPKs, leading to phosphorylation of Hog1 that is translocated into the nucleus, regulating the expression of a number of genes involved in salt tolerance (Gostinc *et al.*, 2011). Lenassi *et al.* (2011) described differences among adaptive, obligate halophiles and salt-sensitive fungi at the molecular level involving enzyme as glycerol-3-phosphate dehydrogenase Gpd1.

Sometimes, high salinity of the medium containing xenobiotics can difficult the biodegradation process, affecting the growth and activities of microorganisms (Petrovic *et al.*, 2002; Abou-Elela *et al.*, 2010). In this way, the utilization of salt-tolerant organisms in biological treatment could be an interesting approach. A successful degradation of xenobiotics by halotolerant *Penicillium* spp, *Trametes* and *Flavodon flavus* (Leitão *et al.*, 2005; Cantrell *et al.*, 2010) has been described.

The increasing in the use of agricultural chemicals results in the accumulation of these compounds and their derivatives in soil and water. Diuron is a phenylurea herbicide (N-(3,4-dichlorophenyl)-N,N-dimethylurea; CAS 330-54-1) and it is one of the most often employed agrochemicals for controlling weeds in sugarcane, citrus and coffee crops. It has high mobility in soils, low susceptibility to natural attenuation and strong toxicity. Besides, more toxic metabolites with genotoxic and teratogenic actions such as 3,4-dichloroanilines, N-3,4-dichlorophenylurea and N-(3,4-dichlorophenyl)-N-methylurea can be generated from diuron by biotic and abiotic reactions (Tixier *et al.*, 2000; Dellamatrice and Monteiro, 2004; Giacomazzi and Cochet,

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2004). The dissipation of phenylurea herbicides in the environment was reported as a predominantly biological process, suggesting that the restoration of polluted sites through bioremediation is a promising tool (Cameron *et al.*, 2000; Sørensen *et al.*, 2003).

Fungi from basidiomycetes group are referred by reason of their noticeable versatility to oxidize a wide range of xenobiotic, phenolic or non-phenolic compounds (Cerniglia, 1997), dyes (Trovaslet *et al.*, 2007), pesticides (Gianfreda and Rao, 2004), chlorophenols and organophosphorus (Keum and Li, 2004). The mechanisms consist of carbon bond breakage with aromatic ring fission, aryl-alkyl breakage and lateral chains removal.

In spite of their ability to degrade several compounds, a few strains of basidiomycetes were evaluated in relation to their salt-tolerance. Therefore, the aim of this work was to investigate the effect of NaCl concentration on the ability of basidiomycetes species to grow, produce ligninases and degrade herbicide diuron.

Materials and Methods

Fungal cultures

Ten basidiomycetes strains, previously isolated from decaying wood in the Atlantic Rainforest fragments, at "Estação Ecológica do Noroeste Paulista", northeast of São Paulo State, Brazil (Santos *et al.*, 2004; Abrahão *et al.*, 2008) were used in this work. The fungi were being preserved using Castellani's method, under mineral oil and liquid Nitrogen.

Culture medium, inoculum and cultivation condition

Mycelium was scraped from agar plates, under sterile conditions, and transferred to 120 mL Erlenmeyer flasks loosely capped with cotton plugs, containing 50 mL of the basal medium composed of ($g.L^{-1}$): 5.0 glucose, 2.0 yeast extract, 1.0 potassium sodium tartrate, 1.0 ammonium phosphate monobasic, 0.5 magnesium sulfate, 0.2 potassium chloride and 1.0 mL of micronutrient solution. The pH was adjusted to 5.5. After growing for 6-7 days, at 28 °C, the medium was removed and the mycelium washed twice with Knapp buffer (Tixier *et al.*, 2000). After that, fungal biomass was mixed with the buffer in a sterile mixer, and cell suspension was diluted to reach the optical density of 0.5 at the wavelength of 550 nm. This suspension was used as inoculum for experiments using the same medium. The incubation was carried out for 10 days at 28 °C.

Culture conditions for halotolerance, effect of mannitol and glycerol on growth and diuron degradation

In order to evaluate the tolerance of the fungi to salt concentrations, the culture medium was supplemented with 0.5, 0.6 and 0.8 $mol.L^{-1}$ of NaCl. The effect of addition of mannitol and glycerol at 0.5 M to the culture medium was also studied. For diuron degradation experiments, a commercial formulation of diuron Karmex® (dupont) was employed in culture medium. The pesticide was diluted in methanol (12.5 $mg.mL^{-1}$), homogenized by two sonication pulses of 15 min and centrifuged at 10,000 g for 10 min at 10 °C. Then, the solution was sterilized by filtration through a 0.22 μm Millipore membrane. Aliquots were dispensed into sterile 125 mL flasks in a laminar flow. After solvent evaporation, each one received 25 mL of sterile liquid culture medium previously described, resulting in final concentration of 5 and 10 $mg.L^{-1}$ of diuron. Diuron dissipation and ligninolytic enzyme production were evaluated for until 10 days in slight agitation at 28 °C, in the dark. At the end of the cultivation period, cultures were centrifuged at 10,000 g for 10 min and the mycelium was dried at 70 °C to quantification of both biomass and the filtered used for determination of diuron degradation.

HPLC analysis

All media samples were centrifuged and afterwards filtered through a 0.22 μm membrane. Diuron degradation rates were estimated using an isocratic Jasco HPLC, employing a UV-975 detector adjusted to 240 nm and a starchromatography 4.0 Varian software. Analyses were developed with a C18 (Perkin Elmer) column, in an oven stated at 40 °C. The pesticides were eluted by a degassed mobile phase composed of acetonitrile and water (60/40 $v.v^{-1}$), with a flow rate of 1 $mL.min^{-1}$. As standard for calibration curve diuron Ultrachem was employed (99% purity). All samples were injected three times and the results represent the means taken.

Enzyme assays

Enzymatic activities in the crude extract of fungal cultures were determined spectrophotometrically, by reactions carried out at 40 °C. Laccase (EC 1.10.3.2) was measured based on ABTS (Sigma) oxidation ($\varepsilon = 3.6.10^4\ M^{-1}\ cm^{-1}$), at 420 nm Buswell *et al.* (1984). Lignin peroxidase (EC 1.11.1.14) (LiP) activity was estimated by means of veratryl alcohol (Fluka) oxidation to veratraldehyde ($\varepsilon = 9.3.10^3\ M^{-1}\ cm^{-1}$) at 310 nm Li *et al.* (2003). Manganese-dependent peroxidase (EC 1.11.1.13) (MnP) had its activity determined by generation of lactate-Mn^{3+} complexes ($\varepsilon = 8.1.10^3\ M^{-1}\ cm^{-1}$), absorbing at 240 nm (Glenn *et al.*, 1986; Aitken and Irvine, 1990). For all cases, 1 unit of enzymatic activity was expressed as the amount of enzyme capable of oxidizing 1 μmol of substrate per minute, under assay conditions, and expressed as U per gram of dry biomass.

Analytical procedures

Reducing sugars were determined by DNS method (Miller, 1959), using D-glucose as standard. The separation and identification of carbohydrate was carried out by

HPAEC-PAD (ICS 3000, Dionex Corporation, EUA), with AS40 automated sampler and carbopac PA-1 anionic column. The standard quadruple E_1 = 0.10 V (t_1 = 0.40 s); E_2 = -2.00 V (t_2 = 0.02 s); E_3 = 0.60 V (t_3 = 0,01 s); E_4 = 0.10 V (t_4 = 0.06 s) was used. The flux was 1 mL.min^{-1}. Glucose, glycerol and mannitol were the standard.

Results and Discussion

The evaluation of effect of NaCl at 0.5 mol.L^{-1} on growth of fungi showed that the majority were sensitive to salt with strong inhibition of the growth, except for *Dacrypionax elegans* SXS323, *Datronia stereoides* MCA167 and *Polyporus* sp MA128 in which the presence of salt improved the biomass production in 90, 39 and 18%, respectively (Table 1). Data from Figure 1 corroborate these results. Concentrations of 0.5 and 0.6 mol.L^{-1} of NaCl improved the growth of *D. elegans*, *D. stereoides* and *Polyporus* sp. in 100, 33 and 25%, respectively. Furthermore, the three fungi were tolerant to 0.8 mol.L^{-1} of the salt.

There is little information about the halophilic and halotolerant fungal specimens. According to Kirk *et al.* (2001), the tolerance to low a_w was demonstrated for fungi from 10 orders among of 106 orders existing. In the Basidiomycota, three orders such as Wallemiales, Trichnosporales and Sporidiales have halophilic or halotolerant groups. While halotolerance is spread in several genus of these orders, a few obligate halophile genus are described. Yeast and yeast-like *Rhodotorula, Debaryomyces, Aureobasidium*, *Trichosporum* as well as filamentous fungi *Cladosporium, Scopulariopsis, Alternaria, Aspergillus* spp*., Penicillium* spp. are described as halotolerant. Halophilic spices are found in the genus *Wallenia, Hortea, Phaetotheca, Trimmatostroma* (Cantrell *et al.*, 2006; Gunde-Cimerman *et al.*, 2009). It was reported that basidiomycetes *Microsporus xanthopu*s AN 24, *Pycnoporus sanguineus* Gc23 and GC67 and *Schizophyllum commune* Gc41, tolerates 35, 60 and 70 g.L^{-1} of NaCl Castillo and Demoulin (1997). *Phlebia* sp tolerated 30 to 50% (26) and *Formitopis*, 47 g.L^{-1} (Miyazaki *et al.*, 2007; Li *et al.*, 2002).

The establishment of limit between halotolerant and halophilic fungi is not straightforward. In prokaryote this limit has been clearly defined, that is to say, there are obligate halophilic microorganisms which require salt for optimal growth and, according to the concentration required, they can be categorized as extremely halophilic or moderately halophilic. Halotolerant microorganisms can survive and grow in high concentration of salt, although the best growth occurs in the absence of salt (Kivisto and Karp, 2011). It was reported that halophilic fungi do not require salt in order to grow, nor for the adjustment of cell activity and stability (Tangavelu *et al.*, 2006; Plemennita *et al.*, 2008). However, our results indicate that the basidiomycetes strains studied required salt for optimal growth and consequently could be considered as moderately halophilic.

Effect of NaCl, mannitol and glycerol on the growth and diuron degradation by *D. elegans*

The ability of fungi to grow in medium with high salt concentration is an important tool for bioremediation, since industrial effluent frequently contains solute concentration that could inhibit enzymes and microorganisms sensitive to low a_w. On the other hand, crop soil, where herbicides are frequently used, can have its salinity increased by irrigation or by use of chemical fertilizer.

Considering that *D. elegans* required salt for growth and that polyol such as glycerol and mannitol have been described as osmolytes produced and/or accumulate by fungi as strategy for survival in environment with high salt concentration (Adler *et al.*, 1985) this fungus was cultivated in medium containing sodium chloride, mannitol and glycerol and it growth and ability to degrade herbicide diuron was evaluated (Table 2).

Data from Figure 2a indicate that the growth of *D. elegans* was not affected by diuron, although the presence

Table 1 - Biomass production by basidiomycetes strains in media containing none or 0.5 Mol.L^{-1} of NaCl after 48 h of cultivation.

	Control	NaCl (0.5 Mol L^{-1})	Improvement (+) or inhibition (-) of growth (%)
Datronia Caperata MCA5	1.2 ± 0.8	0.1 ± 0.08	-92
Gloeophyllum striatum MCA7	2.5 ± 0.3	0.2 ± 0.1	-92
Hexagonia hirta MCA131	1.5 ± 0.6	0.2 ± 0.04	-87
Gloeophyllum striatum MCA2	1.8 ± 0.5	0.25 ± 0.09	-86
Pycnoporus sanguineus MCA16	2.3 ± 0.8	0.6 ± 0.05	-74
Polyporus tennuiculus MCA9	0.5 ± 0.06	0.18 ± 0.03	-64
Polyporus tenuiculus MCA11	1.0 ± 0.2	0.5 ± 0.09	-50
Dacrypionax elegans SXS323	2.0 ± 0.04	3.8 ± 0.08	+90
Datronia stereoides MCA167	2.6 ± 0.4	3.5 ± 0.2	+39
Polyporus SP. MCA128	2.7 ± 0.4	3.2 ± 0.2	+18

of both salt and diuron in the culture medium (Figure 2b) resulted in a lower biomass production suggesting therefore the increase in the sensitivity of fungi to herbicide in presence of salt. However when glycerol was added into the culture media, the biomass reached 7 g.L^{-1} (Figure 2e), twice the maximum production obtained in medium where these compounds were absent.

Considering the high biomass production by fungus in medium in presence of glycerol, the remaining concentrations of this polyol in the culture medium in different phases of growth was determined. Figures 2b shows that in

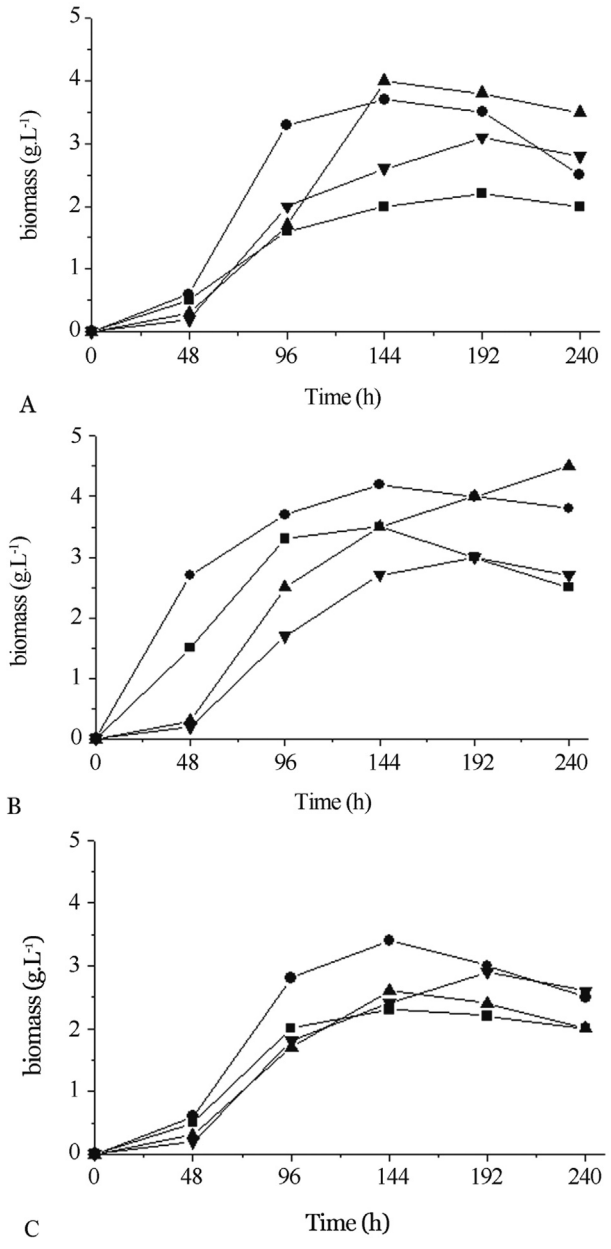

Figure 1 - Biomass production by *Dacripionax elegans* SXS323 (a), *Datronia stereoides* MCA167 (b) and *Polyporus* sp MA128 (c) in medium containing none (■), 0.5 mol L^{-1} (●), 0.6 mol L^{-1} (▲) and 0.8 mol L^{-1} (◆).

Table 2 - Biomass production (g.L^{-1}) by *Dacryopinax elegans* SXS323 in medium with 5.0 g.L^{-1} of glucose added diuron, mannitol and/or glycerol.

Culture time (h)	Biomass (g L^{-1})							
	Control		Sodium Chloride 0.5 M		Mannitol 0.5 M		Glycerol 0.5 M	
	None diuron	Diuron (10 mg.L^{-1})	None diuron	Diuron (10 mg.L^{-1})	None diuron	Diuron (10 mg.L^{-1})	None diuron	Diuron (10 mg.L^{-1})
0	0	0	0	0	0	0	0	0
48	0.54 ± 0.1	0.9 ± 0.3	1.4 ± 0.08	0.3 ± 0.2	1.8 ± 0.1	1.2 ± 0.3	1.3 ± 0.1	1.1 ± 0.09
96	1.2 ± 0.8	1.8 ± 1.7	2 8 ± 0.8	0.6 ± 0.07	3.6 ± 0.5	3.9 ± 0.3	3.4 ± 0.7	3.6 ± 1.1
144	1.8 ± 0.7	1.5 ± 0.7	3.0 ± 0.4	0.7 ± 0.05	4.8 ± 1.2	4.3 ± 0.7	3.7 ± 0.2	4.8 ± 1.6
192	1.9 ± 0.9	1.4 ± 0.7	2.6 ± 0.2	0.6 ± 0.2	7.0 ± 1.1	5.8 ± 0.5	5.4 ± 0.2	4.4 ± 0.2
240	1.3 ± 0.7	1.3 ± 0.8	2.2 ± 0.5	0.4 ± 0.1	6.4 ± 1.2	7.2 ± 0.9	6.2 ± 1.7	4.2 ± 0.2

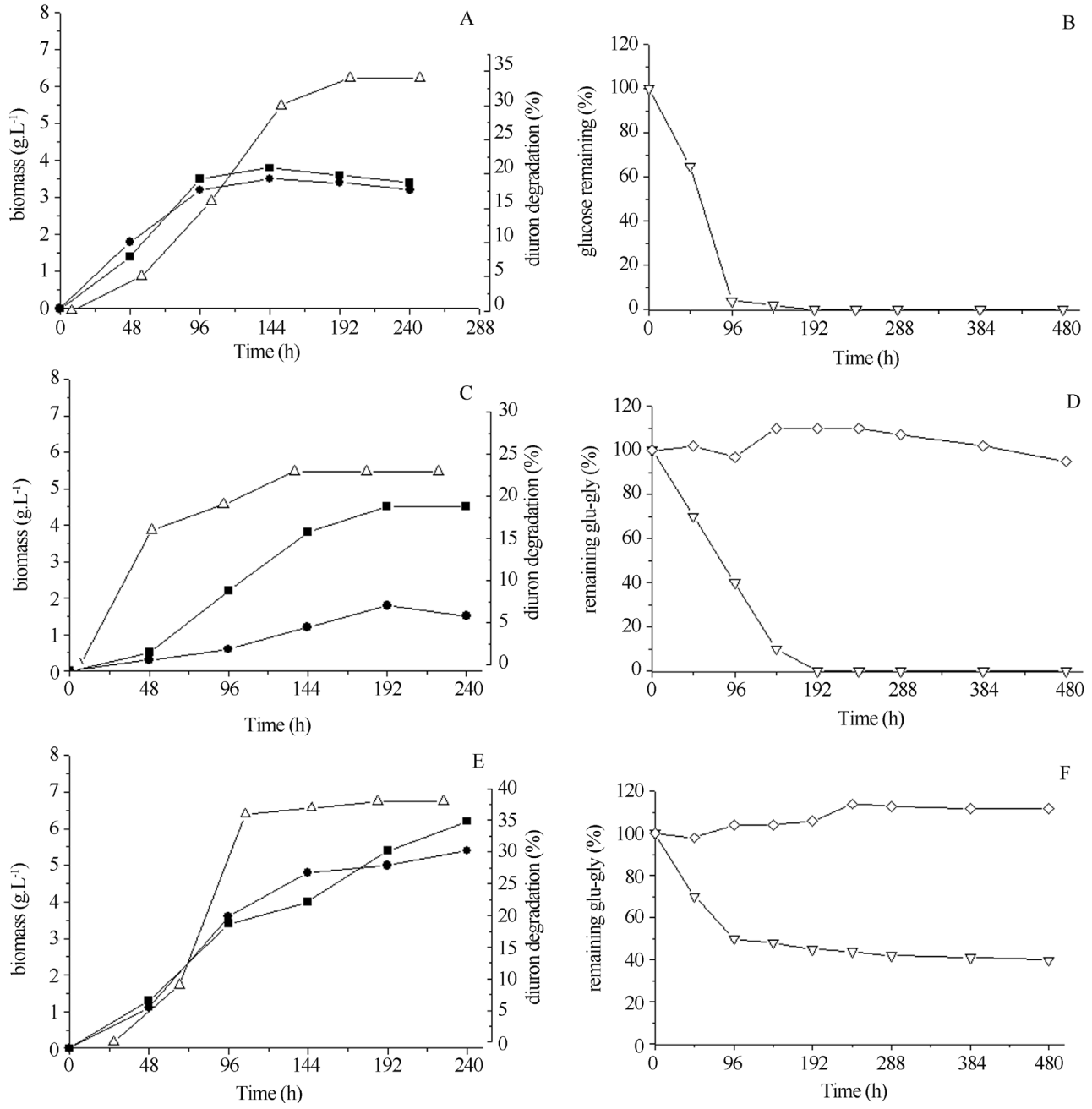

Figure 2 - Production of biomass and diuron degradation by *Dacrypionax elegans* SXS323. a, b = medium containing 0.03 M of glucose and 10 mg.L^{-1} of diuron; c, d = medium containing 0.03 M of glucose, 0.5 M of glycerol and 0.5 M of NaCl; e, f = medium containing 0.5 M of glucose and 0.5 M of glycerol. ■ = biomass without diuron, ● = biomass with diuron, Δ = diuron degradation; ◇ = remaining glycerol;▽ = remaining glucose.

medium with 0.03 M of glucose this sugar was totally exhausted in 96 h. When NaCl at 50 g.L^{-1} was added to the medium, the glucose was completely consumed only after 144 h of cultivation and glycerol level decreased to 96 h but afterward it increased up to 110% (Figure 2d). In medium with a higher glucose concentration (0.5 M) and 0.5 M of glycerol, it was observed that the sugar was not totally consumed and 0.2 M were still left after 480 h of cultivation. Also, glycerol concentration in the medium had an increase of 14% in the stationary phase of culture (240 h) (Figure 2f). The results indicate that the fungus did not take up glycerol in quantity for assimilative metabolism and biomass production not even in absence of glucose. However, variations in glycerol concentrations suggest accumulation and secretion in response to growth phase, osmotic stress and/or possible presence of diuron. It was demonstrated that fungi produce and/or accumulate polyols such as glycerol, mannitol, ribitol from environment, as a compatible solute to maintain the internal Na^+ below the toxic level in high saline concentration, independently of the ability of

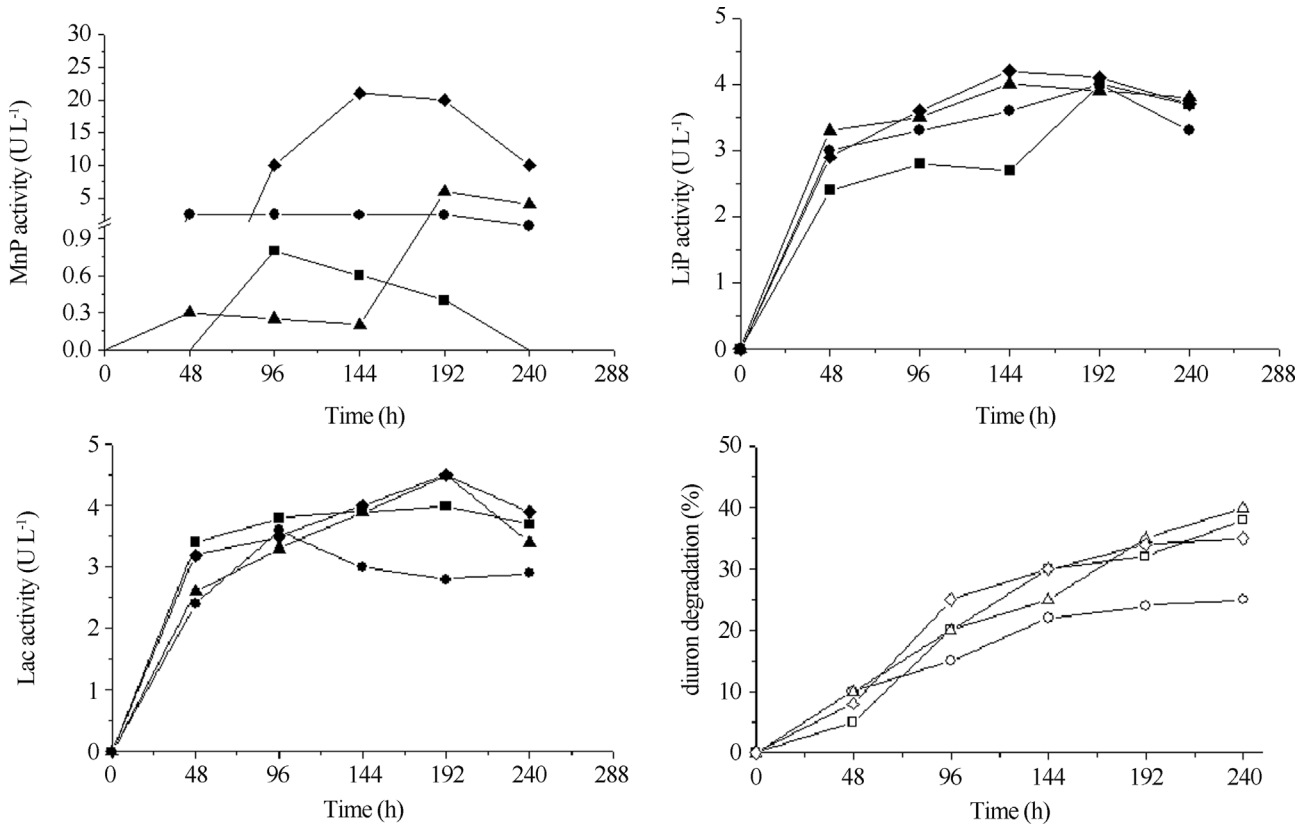

Figure 3 - Manganese peroxidase (a), lignin peroxidase (b), laccase production (c) and diuron degradation (d) by *D. elegans* in medium control (■), containing 0.5 M of NaCl (●), 0.5 M of manitol (▲) or 0.5 M of glycerol (◆).

microorganisms to metabolize them (Adler *et al.*, 1985; Gunde-Cimerman *et al.*, 2009).

D. elegans was able to degrade the diuron in all the culture medium used (Figure 2). In spite of inhibition of the growth by presence of 0.5 M of NaCl plus diuron (Figure 2b), the diuron degradation was considerable (25%) compared to control medium (33%) (Figure 2a). The highest degradation of the herbicide was observed in medium with glycerol (38%) (Figure 2e). Considering that glycerol improved the fungal growth and the diuron degradation, in spite of it was not had been used as carbon source, we could infer that glycerol roll in the cell is further of osmotic regulation.

Effect of NaCl, mannitol and glycerol on growth and ligninases production by *D. elegans*

According to Figure 3a, the presence of glycerol and salt in the culture medium increased the manganese peroxidase production by *D. elegans*, but did not influence the lignin peroxidase and laccase production. The action of NaCl on laccase activity has been described in different ways depending on the microorganism. The presence of low concentrations of NaCl inhibited the activity of laccase from basidiomycetes *Ganoderma lucidum* and *Pycnosporus sanguineus*, but activated laccase from *Bacillus halodurans* (Ruijssenaars and Hartmans, 2004; Trovaslet *et al.*, 2007; Zilly *et al.*, 2011).

Figure 3d show that profiles of ligninase activities are similar to diuron degradation. Laccases are referred as versatile enzymes that degrade a wide range of aromatics compounds like PAHs, PCBs (Johannes and Majcherczyk, 2000; Bending *et al.*, 2002; Keum and Li, 2004; Rodríguez *et al.*, 2004). Nonetheless, the degradation of diuron could be a process independent of ligninases or other enzymes secreted to the medium, as reactions mediated by cytochrome P-450 monooxigenase and epoxide hydrolase (Bezalei *et al.*, 1997; Rabinovich *et al.*, 2004).

In conclusion, basidiomicetes strains responded in different ways to saline concentration in the medium, varying from very sensitive to moderate halophilic profiles. The halophilic *D. elegans* had both an improvement of its growth in presence of NaCl as well as a positive answer to presence of glycerol in the culture medium with increase in the biomass production and in its ability to degrade diuron, suggesting that glycerol has other rolls in the cell besides to be a compatible solute in mechanisms of response to high salinity.

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