CHAPTER 3

BIODEGRADATION OF DIURON AND PYRUTHIOBAC-SODIUM BY WHITE-ROT AND SOIL FUNGI

Rose M.A. Gondim-Tomaz[1], Telma T. Franco[2] and Lucia R. Durrant[3]
Instituto Argronômico (ICA), Campinas, Brazil[1]; Departamento de Processes Químicos, Faculdade de Engenharia Química, Universidade Estadual de Campinas, Campinas, Brazil [2]; Departamento de Ciências de Alimentos, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas (SP), Brazil[3]

Abstract: Thirteen strains, namely, *Pleurotus* sp BCCB 507, P. sp CCB 068, *Pleurotus* sp. 016, *Agaricus campestris, Phanerochaete chrysosporium* ATCC 24725 and the soil isolates DP24e, DP24o, DRP02n, SP16a, SRP 17c, SRP17g and SRP20e were selected following their cultivation in solid media containing either the pesticide Diuron or Pyrithibac -sodium (Staple 280CS). These fungi were grown in liquid medium for three days when 25 μg/mL of Diuron or 10 μg/mL of Staple were added and cultivation was carried out for up to a 14 -day period. Ligninolytic activities and also the degradation of the pesticides were determined. When Diuron was used the highest degradation was obtained with *Pleurotus* sp BCCB 507 (60.70% - 7th day), *Pleurotus* sp CCB 068 (80.75% - 10th day), *P.* sp. 016 (58.60% - 7th day), and the soil isolates SRP17g (65.46% - 10th day), SRP17c (67.60% – 14th day) and SRP20e (62.20% - 10th day). When Staple 280 CS was used *Pleurotus* sp BCCB 507 (14.25% - 7th day), *A. campestris* (32.90%-14th day) and *P.* sp. CCB 068 (53.20% - 7th day) showed the highest degradation. MnP was the predominant ligninolytic enzyme produced by all the strains, regardless of the pesticide used.

Key words: biodegradation; ligninolytic enzymes; diuron; pyrithiobac-sodium; fungi.

1. INTRODUCTION

Pesticides are widely used in current agricultural practices. Owing to their toxic effects on non-target organisms, most pesticides may produce serious detrimental effects on ecosystems (Bretaud et al, 2000). Pesticide residues persist in the environment and may be incorporated and accumulated via the food chain, affecting human health (Muñoz de la Peña et al, 2003).

The fate of pesticides in edaphic ecosystems is dependent not only on various abiotic mechanisms such as photochemical degradation, adsorption to soil elements, absorption by plants or leakage, but is also governed to a large extent by the degradation activities of microorganisms (Aislabie et al, 1995). Hence, the knowledge of catabolic pathways of pesticide-degrading microorganisms may help to solve some problems of agricultural pollution (Widehem et al., 2002).

The herbicide Diuron, (3-(3,4-dichlorophenyl)-1,1-dimethylurea is one of the most extensively used in Brazilian agriculture (Tien & Kirk, 1988). It is widely used to selectively control annual weeds species in a variety of crops (cotton, sugar cane, vineyards, etc.), or is also used for total weed control on non-cultivated areas (maintenance of roads, railways, parks, etc.). Diuron is very persistent at the soil surface partly due to its low solubility. However, its degradation has been observed in soil and was attributed to biological activity (Dalton et al.,, 1966). It can be degraded or transformed by fungi *(Fusarium oxysporum)* (Kaufman & Blake, 1973); *Cunninghamela echinulata* (Tilmanns et al., 1978), and by mixed bacterial cultures (Shelton et al., 1996 and Cullington & Walker, 1999). The herbicide Pyrithiobac (sodium 2-chloro-6-(4,6-dimethoxypyrimidin-2-ylthio)benzoate), the active ingredient of Staple (DuPont, 1993), is a new herbicide developed for postemergence control of broad-leaf weeds in cotton. Pyrithiobac degrades relatively slowly in soil, primarily by microbially mediated degradation, with an estimated half-life of 60 days based on laboratory studies (DuPont, 1993).

White-rot fungi are defined by their physiological capacity to degrade lignin, a very complex and heterogeneous polymer (Hataaka, 1994). The white-rot fungi's enzyme system is non-specific, enabling the degradation of compounds which resemble lignin structure to occur, having been implicated in the degradation of a wide variety of aromatic xenobiotics, including polyaromatic hydrocarbons (PAHs), polychlorinated bifenyls, pentachlorophenol and various groups of pesticides (Higson, 1991 and Prado & Airoldi, 2002). The present study was designed to investigate the ability of 13 fungal strains to degrade two herbicides, diuron and pyrithiobac-

sodium (Staple 280 CS) and also to determine the production of the ligninolityc enzymes during their growth in these herbicides.

2. MATERIAL AND METHODS

2.1 Chemicals

Diuron and Pyrithiobac-sodium were a gift from Millenia Agro Ciência S.A., Londrina-PR, and Iharabras S.A., Sorocaba-SP, Brazil. The purity of each compound was checked by HPLC prior to use and was higher then 98%.

2.2 Fungal strains and inocula preparation

The 13 fungal strains: 5 ligninolytic white-rot fungi belonging to the culture collection of the Laboratório de Sistemática e Fisiologia Microbiana, UNICAMP, Campinas-SP, Brazil) and 8 strains isolated from soil treated with either Diuron or Pyrithiobac-sodium, were maintained on PDA (potato dextrose agar - DIFCO) slants. Petri dishes containing PDA were inoculated with a mycelium portion of each strain and incubated at 30° C for 8-10 days, after which the agar was cut and either 1 cm^2 or 5 x 1 cm^2 mycelial pieces were removed with a thin spatula and used as the inocula for the experiments described below.

2.3 Culture conditions

Fungal strains were inoculated (5 x 1 cm^2), in liquid medium (50 mL/250 mL Erlenmeyer flasks) containing per liter: 0.5 g $(NH_4)_2$ HPO_4; 0.8 g KH_2PO_4; 0.3 g K_2 HPO_4; 0.3g $MgSO_4$ $7H_2O$; 0.055 g $CaCl_2$ H_2O; 4.0 mg Zn SO_4 $6H_2O$; 0.2 g yeast extract and 1.0 mL of thiamine (2,0 mg/mL). The final pH of the culture media was 5.0 Following inoculation all cultures were incubated under stationary conditions at 30°C. Diuron (25μg/mL) or pyrithiobac-sodium (10μg/mL) was added on the 3rd day of cultivation and incubated for a further 3, 7, 10 and 14 days. To correct for possible abiotic losses controls, consisting of uninoculated flasks, were also run.

2.4 Enzyme assays

The assays were carried out as described previously (Clemente et al., 2001). Lignin-peroxidase (LiP), manganese-peroxidase (MnP), Laccase

(Lac.) and peroxidase activities were assayed spectrophotometrically in the extracellular fluid of the culture supernatants. LiP was determined by measuring veratryl alcohol oxidation (Tien, & Kirk, 1984). MnP determinations were based on the oxidation of phenol red, according to Kuwahara *et al.* (1984). Laccase was determined by the oxidation of syringaldazine to its quinone form and peroxidase was determined by the oxidation of syringaldazine to its quinone form in the presence of hydrogen peroxide (Szklarz, 1989). The supernatants were obtained following centrifugation of the content of whole flasks at 17,000 rpm for 15 min at 4°C. Enzyme activities were expressed in units per liter, where one unit is equal to 1 μmol of substrate oxidized per minute.

2.5 High performance liquid chromatography (HPLC)

All HPLC analyses were performed using a C_{18} reverse-phase column (SUPELCO). Separation was achieved by isocratic elution in methanol: water (70:30) as mobile phase (Esposito, 1998), with a flow rate of 0,7 mL/min and UV absorbance detector set at 254 nm for diuron. Pyrithiobac-sodium was eluted using 43% ACN/ 57% pH 3, 30 mM potassium phosphate buffer as the mobile phase (Sumpter, & Peterson, 1994), with a flow rate of 0,8 mL/min, 40°C, and UV absorbance detector set at 254 nm.

3. RESULTS AND DISCUSSION

All the fungal strains used in this work exhibited some ligninolytic activity and degradation after growth in the herbicides, which varied with the time of growth and also with the carbon source. Table 1 shows the greatest values of the enzymes' activities produced following growth of the fungi in diuron.

Table 1. Maximal Ligninolytic activities and degradation produced following growth o f the fungal strains in Diuron

Strains	LiP	MnP	Lac.	Perox.	Degradation (%)
BCCB 507	0.19	19.46	ND	0.05	60.74
A.camp.	0.16	7.85	ND	0.49	11.76
P.crysosp.	0.13	13.36	0.06	0.30	3.88
CCB068	0.68	63.94	4.79	3.38	80.75
Pleur.016	0.39	5.96	ND	0.08	58.63
DP24e	0.22	9.33	0.10	0.23	33.80
DP24o	0.13	5.92	ND	0.03	37.58
DRP02n	0.17	19.01	0.07	0.20	5.30
DRP02e	0.21	12.33	0.05	0.20	44.06
SP16a	0.17	10.58	ND	0.10	21.03
SRP17g	ND	14.89	0.02	0.09	65.46
SRP17c	0.16	11.61	ND	0.07	67.03
SRP20e	0.22	12.02	ND	0.03	62.60

ND: Not Detected.

Extracellular LiP, MnP, laccase and peroxidase were determined in the supernatant of the cultures grown in diuron and higher levels of MnP were produced by most of the fungal strains, when compared with the other ligninolytic enzymes. MnP catalyzed the H_2O_2-dependent oxidation of aromatic compounds via a single-electron oxidation mechanism, followed by a series of non-enzymatic reactions yielding various types of degradation products (Yoshidaet al., 1996). It has been suggested that manganese-peroxidase could be the only enzyme responsible for the oxidation of the aromatic structure of diuron (Esposito et al., 1998).

Pleurotus sp (BCCB 507), *Pleurotus* sp (CCB 068) and the non-identified soil fungi SRP17c showed the highest degradation of diuron (table 1 and fig. 2) among the 13 strains, following 7, 10 and 14 days of growth. *Pleurotus* sp CCB 507 exhibited maximal LiP, MnP and Peroxidase activities on the 3^{rd}, 10^{th} and 7^{th} day, whereas Laccase was not detected. *Pleurotus* sp CCB 068 presented the highest LiP, MnP, Laccase and Peroxidase activities on the 7^{th}, 10^{th}, 10^{th} and 7^{th} day, respectively. The soil fungus SRP17c exhibited maximal LiP, MnP and Peroxidase activities on the 10^{th}, 3^{rd} and 7^{th} day, whereas Laccase was not detected. These results indicate that these three stains expressed their ligninolytic system differently when growing in diuron.

Table 2 shows the highest values for the enzyme activities produced following growth of the 13 strains in Pyrithiobac-sodium. *Agaricus campestris* and *Pleurotus* sp CCB 068 showed highest degradation of this herbicide following 14 and 7 days of growth (table 2 and fig. 4),

respectively. Maximal values for MnP were produced on the 10th day by *Pleurotus* sp CCB 068, whereas a low level of MnP was produced by *Ag.campestris* on the 3rd day (table 2). LiP was produced by these two strains on both the 3rd and 7th days of growth. Laccase was not detected in the culture supernatants of *Ag.campestris* and *Pleurotus* sp CCB 068 showed a peak for laccase activity on the 10th day. Maximum values for peroxidase activities by these two strains were on the 7th day.

Table 2. Maximal ligninolytic activities and degradation produced following growth of the fungal strains in Pyrithiobac-sodium.

Strains	LiP	MnP	Lac.	Perox.	Degradation (%)
BCCB 507	0.21	6.10	1.09	0.34	14.25
A.camp.	0.17	0.90	ND	0.36	32.94
P.crysosp.	0.33	34.12	0.20	0.46	3.55
CCB068	0.41	96.68	2.38	4.39	53.23
*Pleur.*016	0.13	4.53	0.01	0.44	ND
DP24e	0.32	5.43	0.33	0.41	ND
DP24o	0.68	7.58	0.19	0.54	ND
DRP02n	0.09	19.64	0.27	0.56	ND
DRP02e	0.07	9.06	0.24	0.59	ND
SP16a	1.94	10.90	0.23	0.24	ND
SRP17g	0.23	11.03	0.32	0.52	ND
SRP17c	0.04	8.78	0.14	0.42	ND
SRP20e	ND	7.49	0.11	0.28	ND

ND: Not Detected.

The strains responsible for the greatest degradations of the herbicides, *Pleurotus* sp CCB 507, *Pleurotus* sp (CCB 068) and SRP17c, in diuron, and *Ag.campestris* and *Pleurotus* sp CCB 068 in Pyrithiobac-sodium, were selected and used for more detailed studies on the growth and degradation of these herbicides, as described under culture conditions.

Figure 1 shows the activities of the enzymes produced following the growth of *Pleurotus* sp CCB 507, *Pleurotus* sp CCB 068 and the soil fungi SRP17c in diuron.

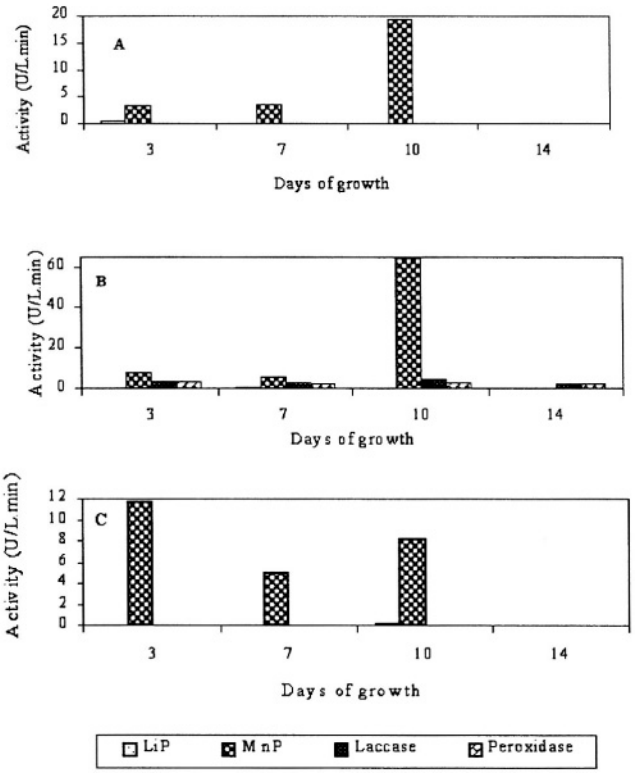

Figure 1. Ligninolytic activities produced following growth of the fungal strains in diuron . A) Pleurotus sp. CCB 507; B) Pleurotus sp. CCB 068; C) Soil fungus SRP17c

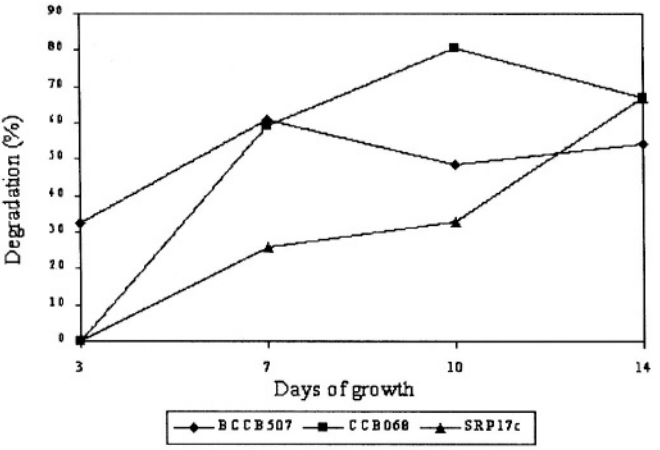

Figure 2. Degradation produced following growth of the fungal strains in diuron

When diuron was used as the carbon source for growth of *Pleurotus* sp CCB 507, *Pleurotus* sp CCB 068 and SRP17c, MnP was the enzyme presenting the highest activities. For each day on which activity was measured, *Pleurotus* sp CCB 507 (Fig.1A) produced MnP, very low levels of LiP and no peroxidase and laccase activities were detected. *Pleurotus* sp CCB 068 (Fig. 1B) exhibited low levels of LiP only on the 3rd and 7th days; laccase and peroxidase activities were always detected when assayed. MnP activity was detected on the 3rd, 7th and 10th days. The soil isolate SRP17c (Fig. C) exhibited MnP on the 3rd, 7th and 10th days, but not on the 14th day. LiP was detected only on the 10th day. Low levels of peroxidase and no laccase activities were detected. These three strains were able to degrade diuron, and maximum levels of degradation 80.75%, 67.03% and 60.74% were produced by the strains CCB 068, SRP17c and BCCB 507, respectively (Fig. 2).

Figure 3 shows the activities of the enzymes produced following growth of *Pleurotus* sp CCB 068 and *Ag.campestris,* in pyrithiobac-sodium. MnP was the enzyme exhibiting the highest activities. *Pleurotus* sp (CCB 068) (Fig. 3A) produced LiP activity on the 3rd, 7th and 10th days. MnP was produced always when assayed. Laccase and peroxidase activities were not detected on the 3rd day. *Ag.campestris* (Fig. 3B) produced LiP and peroxidase on the 3rd and 7th days; MnP on the 3rd and 10th days but no laccase activity was detected. The best degradation rates were obtained on the 7th and 14th days by *Pleurotus* sp CCB 068 (53.23%) and *Ag.campestris* (32.94%), respectively (Fig. 4).

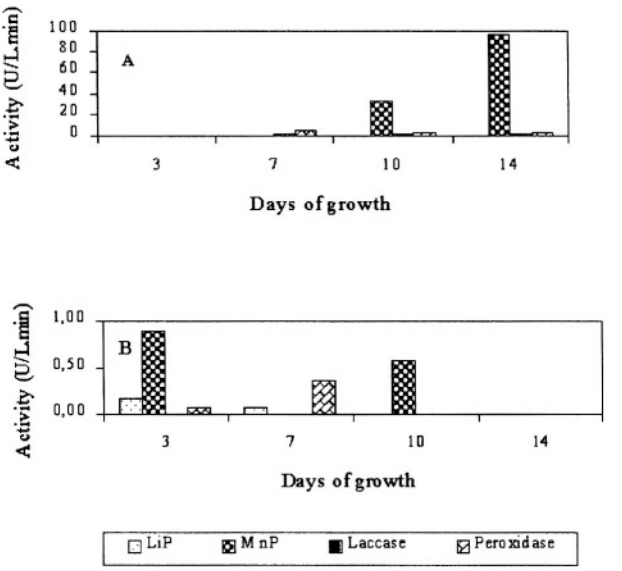

Figure 3. Ligninolytic activites produced following growth of the fungal strains in Pyrithiobac-sodium. A) Pleurotus sp CCB 068; B) Agaricu campestris

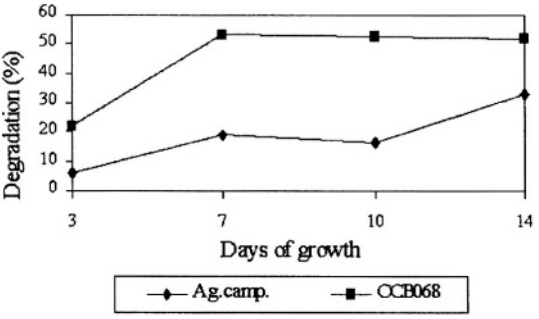

Figure 4. Degradation produced following growth of the fungal strains in Pyithiobac -sodium.

The fungal strains used in this work were able to degrade herbicides and exhibited ligninolytic activities following their growth in diuron or pyrithiobac-sodium (Staple 280CS). MnP was the predominant ligninolytic enzyme detected in the supernatants of all strains. Some actinomycete strains are able to grow in diuron-contaminated soil producing high levels of MnP activity (Esposito, 1998). Similar to the fungi used here, these actinomicete also produced MnP as the predominant ligninolytic enzyme. Laccase, peroxidase and LiP were also produced. It is suggested that MnP could be the unique enzyme responsible for the oxidation of the aromatic structure of diuron. The actinomycete strain CCT 4916 presented a good potential for bioremediation of contaminated soils with diuron. This organism degraded up to 37% of the herbicide in the *in vitro* assays. Here, it was shown that *Pleurotus* sp. CCB 068 degraded up to 80% of diuron and 53% of pyrithiobac-sodium, an indication of its potential for the treatment of diuron and Staple-contaminated soils and sediments.

The ability of nine species of white rot fungus was investigated, from a variety of basidiomicete orders to degrade distinct mono-aromatic pesticides (Bending, et al., 2002). Three members of the Polyporaceae are known to produce a different ligninolitic enzyme systems, i.e., *C.versicolor* produces LiP and MnP, whereas *D.squalens* and *P.ostreatus* produce MnP and Laccase, but not LiP. While all of these are very effective degraders of natural lignin and synthetic polymers, the nature of the ligninolytic enzyme as well as the detoxification systems they produce may determine their ability to degrade xenobiotics.

Phanerochaete crysosporium BKM-F-1767, the most widely studied ligninolytic fungus, when incubated in synthetic and natural media degraded 14 and 10 mg/L diuron, respectively. In this study, diuron could be degraded by *Phanerochaete crysosporium* BKM-F-1767 in synthetic, N-limited liquid medium with a maximum efficiency of 75%. When this fungus was grown on ash wood chips as its sole nutrient source, it produced MnP and degraded diuron with an efficiency of 95%. The ligninolytic system produced by this fungus in both, natural and synthetic media, seems to be involved in diuron degradation since a relationship was observed between the onset of ligninolytic activity and diuron disappearance (Fratila-Apachitel et al., 1999).

As far as we know, there are no published studies on the degradation of pyrithiobac-sodium in soils because it is a new compound and has yet to be classified into a chemical family of herbicides. The technical information on pyritiobac reports that it degrades relatively slowly in soil, primarily by microbial mediated degradation, with an estimated half-life of 60 days based on laboratory studies (DuPont, 1993). The more complex chemical structure

of the pesticide Pyrithibac-sodium (Staple 280 CS), when compared to diuron, may be responsible for the lower levels of degradation and of strains able to attack it. Here, two strains of white-rot fungi were able to grow and degrade this herbicide (~53%) in seven days.

Considering both pesticides, *Pleurotus* sp CCB 068 was the best among all the strains tested. It is important to mention that the highest levels of enzymes activities were produced by this strain, regardless of the pesticide. Our results indicate that this fungus has a great potential for application in the bioremediation of soil and sediments contaminated with herbicides.

ACKNOWLEDGEMENTS

We would like to thank the EMBRAPA for financial support.

REFERENCES

Bretaud,S.; Toutant,J.-P.; Saglio,P. Effects of carbofuran, diuron, and nicosulfuron on acetylcholinesterase activity in goldfish (*Carassiues auratus) Ecotoxicology and Environmental safety* v.47,p. 117-124,2000.

Muñoz de la Peña, A.; Mahedero, Bautista -Sánchez, A. Monitoring of phenylu rea and propanil herbicides in river water by solid -phase-extraction high performance liquid chromatography with photoinduced -fluorimetric detection. *Talanta,* v.00, p.1 -7, 2003.

Aislabie, J.; Lloyd-Jones, G. A review of bacterial degradation of pesticides. *Aust.J.Soil Res.* v.33, p.925-942, 1995.

Widehem,P.; Aït-Aïssa, S.; Tixier, C.; Sancelme,H.V.; Truffaut, N. Isolation, characterization and diuron transformation capacities of a bacterial strain *Arthrobacter* sp. N2. *Chemosphere.* v.46, p.527-534, 2002.

Tien,M. & Kirk, T.K. Lignin Preoxidase of *Phanerochete chrysosporium Methods in Enzymology* v.161, p.238-249, 1988.

Dalton, R.L.; Evans, A.W.; Rhodes, R.C. Disappearance of diuron from cotton fields soils. *Weeds.* v.14, p. 14-31, 1966.

Kaufman, D.D.; Blake, J. Microbial degradation of several acetamide, acylanilide, carbamate, toluidine and urea pesticides. *Soil Biol.Biochem.* v. 5, p. 297-308, 1973.

Tilmanns, G.M.; Wallnöffer, P.R.; Engelhardt, G.; Olie, K.; Hutzinger, O. Oxidative dealkylation of five phenylurea herbicides by the fungus *Cunninghamella echinulata* Thaxter. *Chemosphere.* v.1, p.59-74, 1978.

Shelton, D.R.; Khader, S.; Karns, J.S.; Pogell, B.M. Metabolism of twelve herbicides by *Streptomyces. Biodegradation,*v.7, p.129-136, 1996.

Cullington, J.E. & Walker, A. Rapid biodegradation of diuron and other phenylurea herbicides by a soil bacterium. *Soil Biol. Biochem.* v. 31, p. 677-686, 1999.

DuPont Agricultural Products. *Staple herbicide, technical information ;* DuPont Agricultural Products: Wilmington, DE, 1993

Hataaka, A. Lignin-modifying from selected white-rot fungi: production and role in lignin degradatin. *FEM Microbiol.Rev.* v.13, p.125-135, 1994.

Higson, F.K. Degradation of xenobiotics by white-rot fungi. *Rev.Environ.Contam.Toxicol.* v.l22,p.111-152, 1991.

Prado, A.G.S. & Airoldi C. The toxic on soil microbial activity by the free immobilized pesticide diuron *Thermochimica Acta* v.394, p.155-162, 2002.

Clemente, A.R.; Anazawa, T.A.; Durrant,L.R. Biodegradation of polycyclic aromatic hydrocarbons by soil fungi. *Brazilian Journal of Microbiology.* v. 32, p.255-261, 2001.

Tien, M. & Kirk, T.K Lignin-degrading enzyme from *Phanerochaete chrysosporium:* purification, characterization and catalytic properties of a unique H_2O_2 requiring oxygenase. *Proceedings of National Academy of Science USA ,* v.81, p.2280-2284, 1884.

Kuwahara, M., Glenn, J.K., Morgan, M.A. & Gold, M.H. Separation and Characterization of two extracellular H_2O_2 – dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium. FEBS Letters,* 169(2):247-250, 1984.

Szklarz, G.D., Antibus, R.K., Sinsabaugh, R.L. & LinkIins, A.E. Production of Phenol oxidases and Peroxidases by Wood -Rotting Fungi. *Mycologia,* 81(2), p.234-240, 1989.

Esposito, E., Paulillo, S.M. & MANFIO, G.P. Biodegradation of the Herbicide Diuron in Soil by Indigenous Actinomicetes *Chemosphere,* Great Britain, **37**(3): 541-548, 1998.

Sumpter, S.R. & Peterson, B.A. *Analytical Method for the Determination of KIH-2031 (DPX-PE350) in Water Using Column-Switching Liquid Chromatography. DuPont Agricultural Products,* Experimental Station Wilmington, DuPont Report N^0AMR 2746-93, p.1-58, 1994.

Yoshida, S.; Yonehara, S.; Ha, H -c, Iwahara, K.; Watanabe, T.; Honda, Y., Kuwahara, M. Production and characterization of ligninol ytic of *Bjerkandera adusta* on wood meal/wheat bran culture and production of these enzymes using a rotary-solid fermenter. *Mycoscience* v.37, p. 417-425, 1996.

Bending, G.D.; Friloux, M.; Walker, A. Degradation of contra sting pesticides by white rot fungi and its relationship with ligninolitc potencial. *FEMS Microbilogy Letters,* v. 212, p.59-63, 2002.

Fratila-Apachitel, L.E.; Hirst, J.A.; Siebel, M.A. & Giyzen, M.J.; Diuron Degradation by *Phanerochaete crysosporium* BKMF-F. 1767 in Synthetic and Natural Media *Biotechnology Letters.* v.21, p.147-154, 1999.