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Evaluation of the use of *Pycnoporus sanguineus* fungus for phenolics and genotoxicity decay of a pharmaceutical effluent treatment

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ABSTRACT

If not properly and efficiently treated, wastes produced by the chemical industry can contaminate the environment. Using fungi able to degrade organic compounds (e.g. phenol) seems to be a prominent method to treat pharmaceutical wastewaters, in particular, the white-rot fungus. The aim of this work was to treat pharmaceutical effluent by the *Pycnoporus sanguineus* fungus. Three effluent samples were collected in a pharmaceutical industry. The production of enzymes such as laccase and manganese peroxidase was determined. Their production increased throughout the treatment with the *P. sanguineus* fungus, reaching maximum concentration of 4.48 $U.mL^{-1}$ (Effluent 1), 8.16 $U.mL^{-1}$ (Effluent 2), 2.8 $U.mL^{-1}$ (Effluent 3) and 0.03 $Abs.min^{-1}$ (Effluent 2), respectively, during 96 hours of biological treatment. Genotoxic effects of the raw and treated effluents were also investigated using the *in vivo* mouse bone marrow micronucleus (MN) assay. Results showed the biological treatment reduced the frequency of MN, in a dose-dependent manner, when compared to untreated sample. The decreasing of around 20% and 45% of phenolics concentration was observed throughout the treatment, confirming that laccase production can be related to the degradation of toxic compounds present in the effluent. Therefore, the biodegradation by the *P. sanguineus* fungus seems a promising method for the mineralization of recalcitrant compounds present in pharmaceutical effluents.

Keywords: pharmaceutical effluent, biodegradation, *Pycnoporus sanguineus*, laccase, Manganese peroxidase.

Avaliação do uso do fungo *Pycnoporus sanguineus* no decaimento de compostos fenólicos e genotoxicidade no tratamento de um efluente farmacêutico

RESUMO

Se não for tratado de forma adequada e eficientemente, os resíduos produzidos pela indústria química podem contaminar o meio-ambiente. Utilizar fungos capazes de degradar compostos orgânicos (por exemplo, fenol), pode ser um método para tratar efluentes

proeminentes de insústrias farmacêuticas, em particular, os fungos da podridão branca. O objetivo deste trabalho foi tratar um efluente farmacêutico pelo fungo *Pycnoporus sanguineus*. Três amostras de efluentes foram coletadas em uma indústria farmacêutica. A produção de enzimas como a lacase e manganês peroxidase foi determinado. A sua produção durante o tratamento com o fungo *P. sanguineus* atingiu concentrações máximas de 4,48 $U.mL^{-1}$ (Efluente 1), 8,16 $U.mL^{-1}$ (Efluente 2), 2,8 $U.mL^{-1}$ (Efluente 3) e 0,03 $Abs.min^{-1}$ (efluente 2), respectivamente, durante 96 horas de tratamento biológico. Efeitos genotóxicos dos efluentes brutos e tratados também foram investigados usando o ensaio em que foi avaliada a formação de micronúcleos (MN) na medula óssea de camundongos. Os resultados mostraram que o tratamento biológico reduziu a frequência de MN, de um modo dependente da dose, em comparação com amostras não tratadas. A diminuição de cerca de 20% e 45% da concentração de compostos fenólicos foi observada em todo o tratamento, o que confirma que a produção de lacase pode ser relacionada com a degradação de compostos tóxicos presentes no efluente. Por conseguinte, a biodegradação do fungo *P. sanguineus* parece um método promissor para a mineralização de compostos recalcitrantes presentes em efluentes farmacêuticos.

Palavras-chave: efluente farmacêutico, biodegradação, *Pycnoporus sanguineus*, lacase, manganês peroxidase.

1. INTRODUCTION

Pharmaceutical wastewaters comprise a group that, with regard to the environmental issues, has been given little attention for many years (Hirsch et al., 1999; Roberts and Thomas, 2006).

The impact of discharging pharmaceutical effluent without treatment into rivers (Chen, Wang and Ding, 2008), lakes (Boyd et al., 2004), aquifers (Godfrey, Woessner and Benotti, 2007) is quite strong, affecting both aquatic and human lives.

Phenol is one of the main toxic compounds found in pharmaceutical wastewaters and other compounds like that are called phenolics. It belongs to an aromatic organic substances family, by biological or synthetic origin. They can found as a colorless liquid or white solid at ambient temperature, and it has strong and specific odours (Kumaran and Paruchuri, 1997).

Melo et al. (2006) suggested it was necessary to evaluate the efficiency of the decontamination process, and comparing the toxicity of treated and untreated effluents. Bioassays, such as the in vivo micronucleus (MN) assay, have traditionally been used to assess the effluents toxicity, as well as monitoring the wastewater treatment (Wirzinger et al., 2007).

Using fungi able to degrade organic compounds can be a prominent method of pharmaceutical effluent treatment. In particular, the white-rot fungus has got an enzymatic system able to supporting high concentrations of toxic contaminants (Barr and Aust, 1994). These fungus have the ability of degrading recalcitrant organic pollutants such as polyaromatic hydrocarbons, chlorophenols, and polychlorinated biphenyls (Barr and Aust, 1994). This is probably due to the low specificity of their ligninolytic enzymes, such as laccase, lignin peroxidase, and manganese peroxidase (McMullan et al., 2001).

Laccase is a polyphenyloxidase able to oxidise several aromatic compounds (e.g. phenol) coupled with electron reduction of oxygen in water (Durán and Esposito, 1997).

Induced and constitutive laccases are found in many fungal species, but few works have investigated laccases produced by members of the *Pycnoporus* group (Pointing et al., 2000). The *Pycnoporus sanguineus*, a white-rot fungus and comprising the Basidiomycota, was

recognised by biodegrading some textile dyes (Pointing and Vrijmoed, 2000, Trovaslet et al., 2007) and lignosulphonates (Eugenio et al., 2008). This fungus could mineralize both Lignin (recalcitrant compound present in the lignocellulosic wood matrix) and many resistant pollutants (Durán and Esposito, 2000).

Lignin and Manganese Peroxidases are produced by the majority of white-rot fungi such as *Phanerochate chrysosporium*, *Trametes versicolor* and *P. sanguineus*. Lignin peroxidase catalyzes the one-electron oxidation of various aromatic compounds, with subsequent formation of aryl cation radicals, which are decomposed spontaneously by various pathways. Manganese Peroxidase catalyzes the oxidation of Mn(II) to Mn(III), which in turn can oxidize several phenolic substrates (Sayad and Ellouz, 1995). These enzymes were used to decrease phenolic and non-phenolic polluants (Rodríguez et al., 2004).

In Brazil, the country's third largest pharmaceutical and chemical pole is located in the State of Goiás. Within this context, part of the environmental contamination in Goiás is probably attributed to the pharmaceutical industry activities. This work was developed to evaluate the biological treatment of a pharmaceutical effluent by *P. sanguineus* fungi. The genotoxic effects of the raw and treated effluent were also investigated, using the in vivo mouse bone marrow MN assay. Genetic toxicology tests are assays designed to detect direct or indirect genetic damage induced by compounds.

2. MATERIALS AND METHODS

2.1. Effluent

Three samples of 20L each were collected from a pharmaceutical industry in Goiás, Brazil and kept at 4° C. The sampling technique used was the composite sampling.

2.2. Fungus and Growth Media

The fungus used in this work was the *Pycnoporus sanguineus* CCT-4518 which was provided by the Andre Tosello Foundation, São Paulo, Brazil. The fungus was incubated in potato dextrose agar (PDA) media. The Petri dishes were incubated in 37° C for 6 days.

2.3. Treatment Process

For the treatment process, it was used 100 ml of the each effluent sample (samples 1, 2 and 3), and the growth media with the fungus. All the samples were incubated in C24KC Refrigerated Incubator Shaker (Edison NJ. USA) for 24, 48, 72, and 96 hours at 150 rpm and 28° C. The control sample was also treated in the same conditions (as a control, the growth media and the fungus was inoculated to the samples and after that, they were decontaminated at 121°C for 15 minutes). After 24, 48, 72 and 96 hours of treatment, all samples were filtered (filter papers) and they were stored in amber glass flasks at 4° C (adapted from Santiago, 1999).

2.4. Total Phenolics Determination

Phenolis concentrations were determined by the colorimetric method using Folin-Ciocalteu reagent (APHA, 1992). The reaction mixture was: 1000 μL of effluent, 250 μL of sodium carbonate solution (12g.L^{-1}) and 25 μL of Folin-Ciocalteau 2 mol.L^{-1}. All samples were kept at 20°C for 30 min. The phenolics concentrations were determinated at 700 nm, in a spetrophotometer. The results were expressed in mg.L^{-1} of phenolics, using the calibration curve in the range of 0 to 14 mg.L^{-1} which was obtained using the same procedure, using phenol as a standard.

2.5. Determination of Laccase (Benzenediol: oxygen oxidoreductase, EC 1.10.3.2) Enzymatic Activity

The enzymatic activities were determined in treated and untreated effluent. The control samples were used to detect likely interferences in the enzymatic methodology. The laccase enzymatic activity was determined in duplicates at 30° C for 3 min and 5 min using 1 mol.L^{-1} Syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehydeazine) as substrate. The reaction mixture consisted of a buffered solution of 300 µL of sodium acetate 0.05 mol.L^{-1} (pH = 5.0), 600 µL of filtrate (previously centrifuged at 12,000 rpm during 15 min at 4°C) and 100 µL of Syringaldazine 1 mol.L^{-1}. The enzymatic activities were determined in 525 nm (absorbance). The reaction started by the addition Syringaldazine (ε_{525} = 6.5×10^4 mol.L^{-1}.cm^{-1}) and the enzymatic activity was measured after 3 and 5 min later. From each value found, the control value was subtracted in order to remove possible disturbances of other compounds. The arithmetic average of the absorbance values of each treatment period was calculated. One unit laccase activity was defined as the amount of enzyme causing one unit increase in absorbance per min per mL of the assay (adapted from Szklarz et al., 1989).

2.6. Determination of Manganese Peroxidase (MnP, EC 1.11.1.7) Enzymatic Activity

The activity of peroxidase dependent on Mn (II) was determined by the oxidation of red phenol. The reaction mixture (1.0 mL) consisted of 500 µL filtrate juice, 100 µL of Sodium Lactate 0.25 mol.L^{-1}, 200 µL of bovine albumine 0.5%, 50 µL of Manganese Sulphate 2.0 mmol.L^{-1}, 50 µL of Hydrogen Peroxide solution 2.0 mol.L^{-1} prepared in Sodium Succinate buffer 0.2 mol.L^{-1} (pH = 4.5) and 100 µL of red phenol 0.1%. The mixture was incubated at 30°C for 5 min and the reaction was stopped by adding 40 µL sodium hydroxide 2.0 mol.L^{-1}. The absorbance was set at 610nm and the enzymatic activity was expressed in Abs.min^{-1} (Kuwahara et al., 1984).

2.7. Determination of Lignina Peroxidase (LiP, EC 1.11.1.7) Enzymatic Activity

Lignina peroxidase activity was determined by the oxidation of veratryl alcohol (ε_{310} = 9,300 mol.L^{-1}.cm^{-1}). The reaction mixture (1 mL) consisted of 600 µL of filtrate, 200 µL of hydrogen peroxide, 2.0 mmol.L^{-1} and 200 µL of veratryl alcohol solution 2.0 mmol.L^{-1} in sodium tartarate buffer 0.4 mol.L^{-1} (pH = 3.0). The reaction was started by adding the hydrogen peroxide and the appearance of veratryl alcohol oxidized was determined by absorbance at 310 nm (Tien and Kirk, 1984).

2.8. Toxicity Tests

The *in vivo* genotoxicity of the raw and treated pharmaceutical effluent (0.1; 0.2 or 0.3 mL.day^{-1} per mouse) was investigated in mice (n=5) orally exposed, for 10 days, using the bone marrow cells micronuclei assay. The experiments were carried out on adult male Swiss mice (8-12 week) obtained from the Chemical Industry of Goiás (IQUEGO). All mice weighed between 28 g and 30 g and were kept under constant environmental conditions with 12:12 light-dark cycle. All were fed standard granulated chow and given drinking water *ad libitum*. Animal experiments were done in accordance with Institutional Protocols of Animal Care. The micronuclei frequency was performed 24 hours after the exposure.

2.8.1 Micronucleus test

The mutagenicity of the effluents was evaluated using the micronucleus test by scoring 1.000 bone marrow cells per each of 05 animals per treatment group, as described by MacGregor et al. (1987). The cells were stained with Leishman, the slides were coded, and the cells blindly scored by light microscope at 1000 magnification. The frequency of MN in

individual mice was used as the experimental unit, with variability (standard deviation) based on differences among animals within the same group.

3. RESULTS AND DISCUSSION

The higher laccase production was determined in 96 hours of treatment (4.48 U.mL-1, 8.16 U.mL-1 and 2.8 U.mL-1) for the effluent samples 1, 2 and 3 respectively. It was not determined laccase production in control samples, showing the relation between fungus growth and enzymatic production, as studied by Garcia (2007). Laccase production during the treatment process is shown in Figure 1.

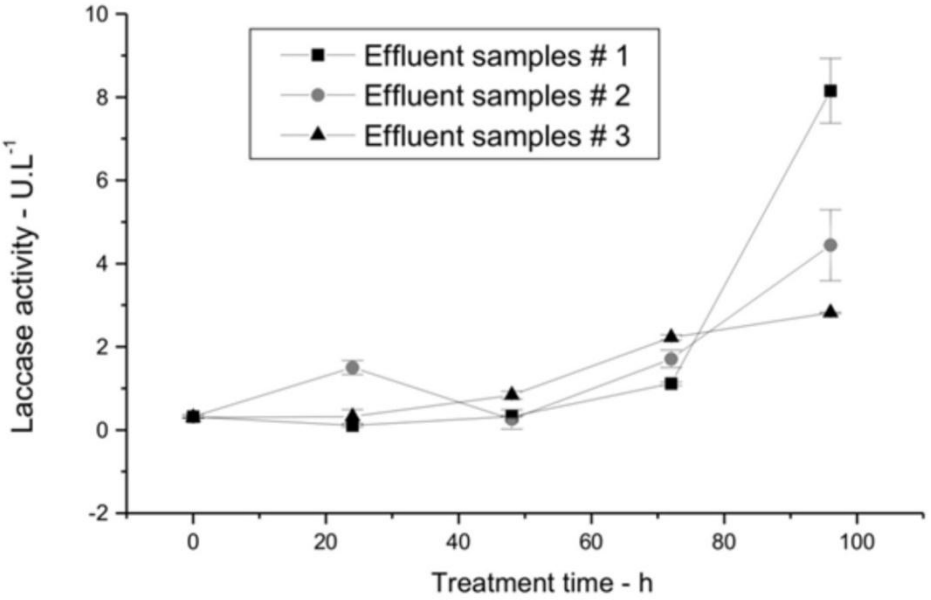

Figure 1. Laccase production for the pharmaceutical effluents 1, 2 and 3 treated by *Pycnoporus sanguineus.*

Manganese Peroxidase enzyme production, also responsible for toxic compound degradation, by the P. sanguineus fungus was determinated in the treated effluent 2 (Figure 2), was also higher in 96 hours of treatment, equal to 0.03 ABS.min-1. The Manganese peroxidase production of was not detected in raw effluent 2 and in control samples. Studies showed the Manganese Peroxidase production is regulated by the nitrogen and carbon substrate concentration and they are inversely proportional to (Linko, 1992). Therefore, the increasing of MNp production towards the biological treatment indicates the decreasing of organic compounds in the effluent (not shown in Figure 2).

In the samples 1 and 3 and in their controls, the manganese peroxidase production was null. Similar results were found for the production of lignin peroxidase in the effluent samples 1, 2 and 3 treated by the P. sanguineus fungus and their control samples (not treated).

The pH of the samples 1 and 2 (treated effluent - Figure 3) presents a drop (i.e. acidification) between 24 and 48 hours of treatment and it increased up to its initial value (pH = 5.5 - 6.0). For the effluent 3, the pH increased with time during whole treatment time (Figure 3). For the control, the pH was approximately constant and equal to 4.7, 6.4 and 4.6 respectively for the samples 1, 2 and 3.

According to Garcia et al. (2007), laccase tends to react differently to different substrates depending on the pH. The purified laccase, using syringaldazine as substrate, presented optimum pH in acid interval (around 4.2) and the same was observed to other laccases from

other fungi. pH range in acid interval is within the interval of optimum pH for laccase, MnP and LiP activities (Wesenberg et al., 2003). Figure 3 shows the treatment time that all samples pH values were below 7.0 (acid). However, the pH values of all samples were between 5.0 and 6.0 at the end of treatment (at 96 hours).

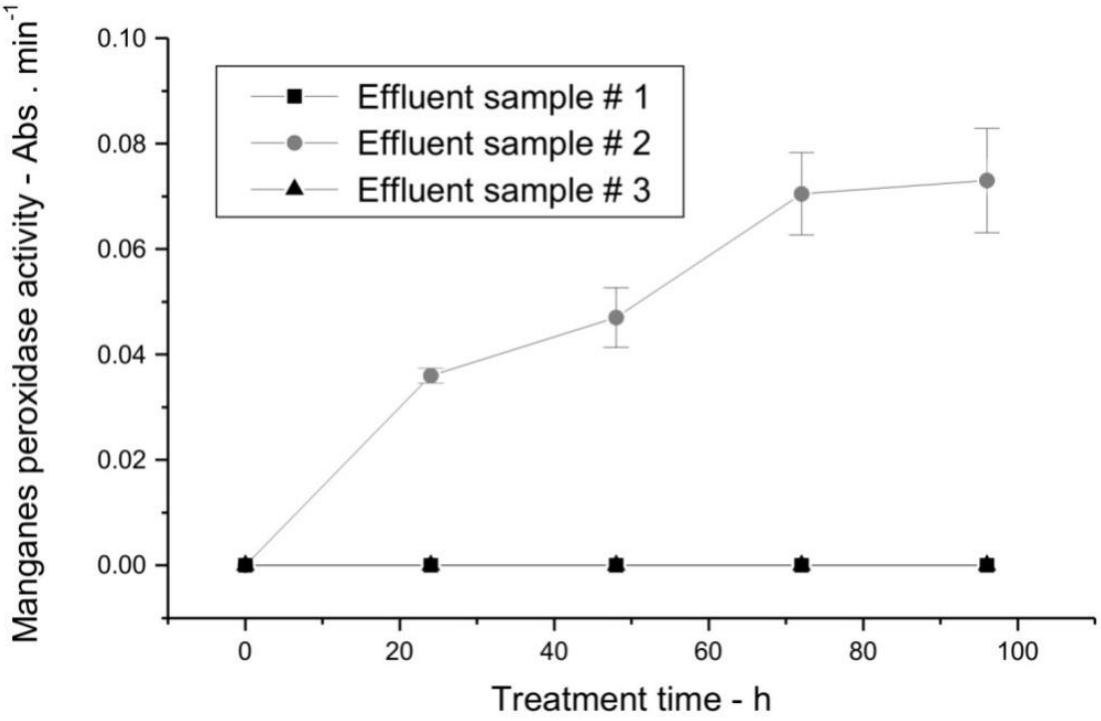

Figure 2. Manganese peroxidase production in the effluent 2 treated by the *Pycnoporus sanguineus*, during 96 hours.

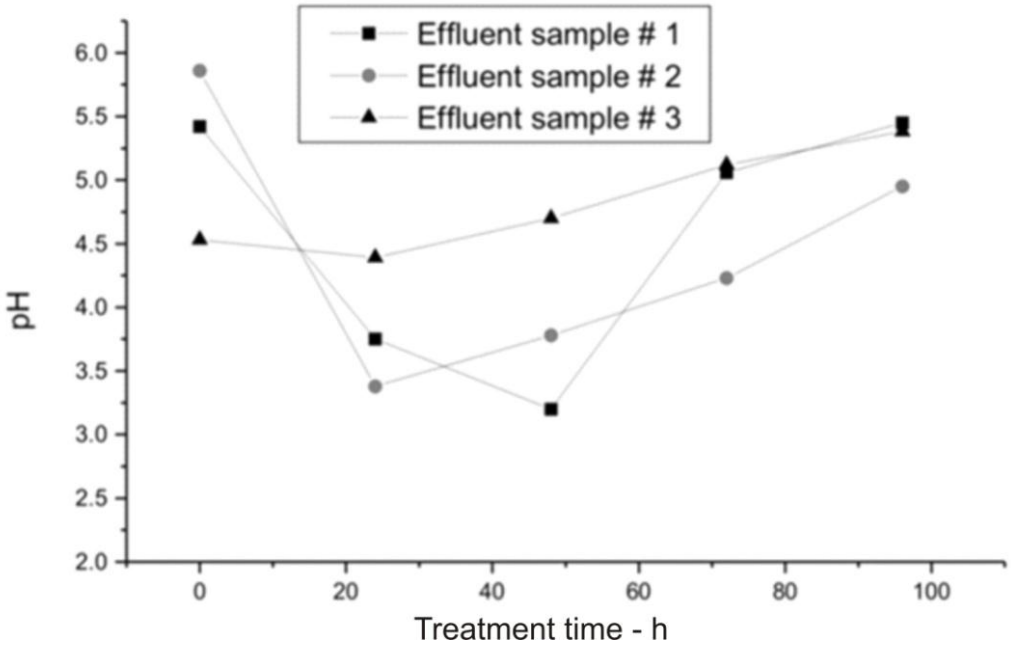

Figure 3. Variation of pH values during the biological treatment.

The phenolics concentration during the biological treatment can be seen in Figure 4, that shows the decreasing around 20% and 45% of all samples, comparing to untreated samples.

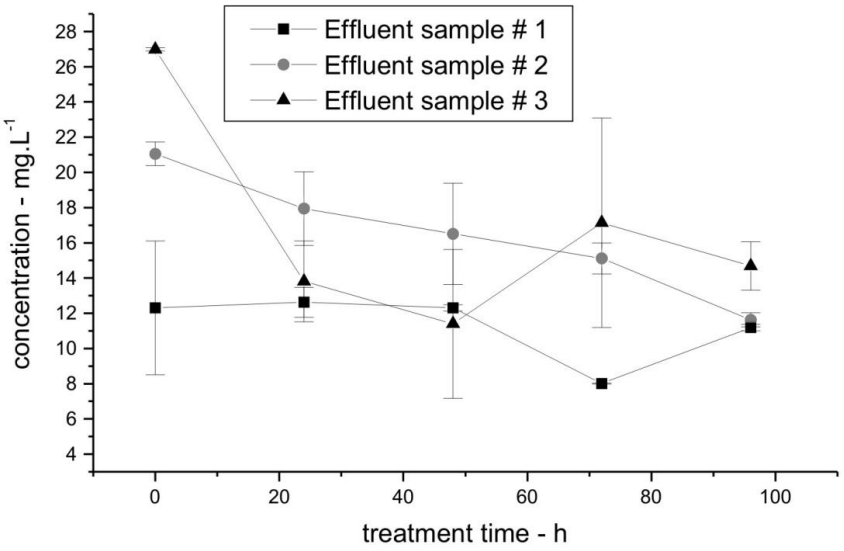

Figure 4. Phenolics concentration for the pharmaceutical effluents 1, 2 and 3 treated by the *P. sanguineus* fungus.

Comparing the enzyme production and the phenolics degradation, it was observed the Laccase and Managese Peroxidase productions increasing, as a result of decreasing of the phenolics, during the biological treatment. This result confirms Laccase production can be related to the degradation of toxic compounds into effluents. In the same way, when the in vivo genotoxicity was investigated it was observed the reduction of frequencies of micronucleated erytrocytes from mice, in a dose-dependent manner, when it was compared to untreated sample (Figure 5).

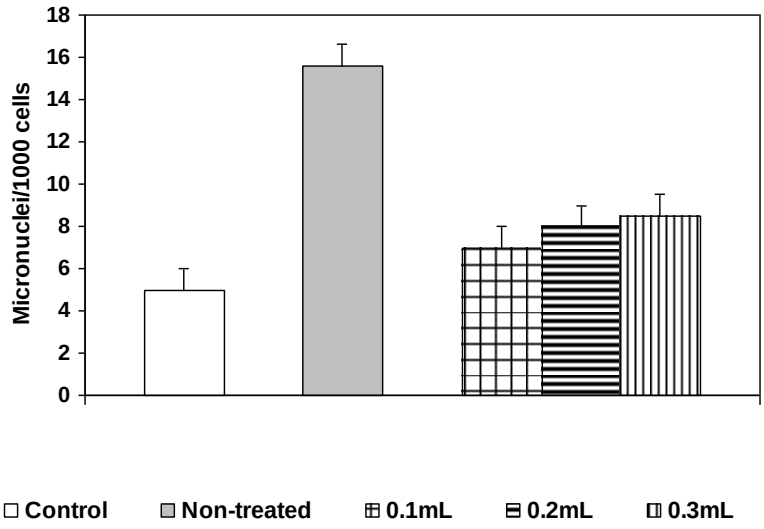

Figure 5. Effects of the raw (non-treated) and treated pharmaceutical effluent (0.1; 0.2 and 0.3 mL.day^{-1}.mouse^{1}), per oral, for 10 days on the frequencies of micronucleated erytrocytes (MN) from mice (n=5). The control group received saline solution only.

Wirzinger et al. (2007) demonstrated that genotoxic assays including the MN test, can be applied routinely for the evaluation of the genotoxic potential of waters.

4. CONCLUSIONS

Based on the results, *P. sanguineus* fungus was able to produce high amount of laccase and small amount of manganese peroxidase enzymes for the treatment of the pharmaceutical effluent investigated in this work. The decreasing of phenolics concentration during the biological treatment showed *P. sanguineus* has a high potential of reducing the genotoxic for this kind of effluent, with a high laccase production during the biological treatment.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

AMERICAN PUBLIC HEALT ASSOCIATION (APHA). **Standard Methods for the Examination of Water and Wastewater.** 17. ed. Washington DC, USA, 1992.

BARR, D. P.; AUST, S. D. Mechanisms white rot fungi use to degrade pollutants. **Environmental Science & Technology**, 28 (2), 1994, 79 – 87.

BOYD, G. R.; PALMERI J. M., ZHANG S.; GRIMM D. A. Pharmaceuticals and personal care products (PPCPs) and endocrine disrupting chemicals (EDCs) in storm water canals and Bayou St. John in New Orleans, Louisiana, USA. **Science of the Total Environment**, Amsterdan, 333 (1-3), 137-148, 2004.

CHEN, H.; WANG P.; DING W. Using liquid chromatography–ion trap mass spectrometry to determine pharmaceutical residues in Taiwanese rivers and wastewaters. **Chemosphere**, 6, 72, 863-869, 2008.

DURÁN, N.; ESPOSITO, E. 1997. Lignin biodegradation and effluent treatment by ligninolytic fungi. In: I. S. De Melo, J. L. Acevedo (Eds.). **Microbiologia Ambiental,** CNPMA/EMBRAPA Publishers. São Paulo, Brazil, Chapter 12, pp. 269.

DURÁN, N.; ESPOSITO, E. Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. **Applied Catalysis B: Environmental**, 28, 2000, 83 – 89.

EUGENIO, M. E.; CARBAJO, J. M.; TERRÓN, M. C.; GONZÁLEZ, A. E.; VILLAR, J. C. Bioremediation of lignosulphonates by lignin-degrading basidiomycetous fungi. **Bioresources Technology**, 99 (11), 2008, 4929-4934.

GARCIA, T. A.; SANTIAGO, M. F.; ULHOA, C. J. Studies on the *Pycnoporus sanguineus* CCT-4518 laccase purified by hydrophobic interaction chromatografy. **Applied Microbiology and Biotechology**, 75 (2), 2007, 311 – 318.

GODFREY, E.; WOESSNER, W. W.; BENOTTI, M. J. Pharmaceuticals in On-Site Sewage Effluent and Ground Water, Western Montana. **Groundwater**, 45 (3), 2007, 263-271.

HIRSCH, R.; TERNES, T.; HABERER K.; KRATZ, K. L. Occurence of antibiotics in the aquatic environment. **Science of the Total Environment**, Amsterdan, 225, 1999, 109-118.

KUMARAN, P.; PARUCHURI, Y. L. Kinetics of phenol biotransformation. **Water Resources Research**, 31(1), 1997, 11-22.

KUWAHARA, M.; GLEN, 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*. **FBS Letters**, 169, 1984, 247-250.

LINKO, S. Production of Phanerochaete chrysosporium Lignin Peroxidase. **Biotechnology Advances**, 10 (2), 1992, 191 -236.

MACGREGOR, J. T.; HEDDLE, J. A.; HITE, M., MARGOLIN, B. H.; RAMEL, C. SALAMONE, M. F., TICE, R. R., WILD, D. Guidelines for the conduct of micronucleus assay in mammalian bone marrow erythrocytes. **Mutatation Research**. 189, 1987, 103–112.

MCMULLAN., G.; MEEHAN, C.; CONNEELY, A.; KIRBY, N.; ROBINSON, T.; NIGAM, P.; BANAT I.M., MARCHANT, R.; SMYTH, W. F. Microbial decolourisation and degradation of textile dyes. **Applied Microbiology and Biotechology**, 56, 2001, 81–87.

MELO, P. S.; FABRIN-NETO, J. B.; MORAES, S. G.; ASSALIN, R.; DURÁN, N.; HAUN, M. Comparative toxicity of effluents processed by different treatments in V79 fibroblasts and the Algae *Selenastrum capricornutum*. **Chemosphere**, Oxford, 62, 2006, 1207-1213.

POINTING, S. B.; JONES, E. B. G.; VRIJMOED, L. L. P. Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. **Mycologia**, V 92, 2000, 139-144.

POINTING, S. B.; VRIJMOED L. L. P. Decolorization of azo and triphenylmethane dyes by *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase. **World Journal f. Microbiology and Biotechnology**, 16, 2000, 317-318.

ROBERTS P. H., AND THOMAS K.V. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. **Science of the Total Environment**, Amsterdan, 356 (1-3), 2006, 143-153.

RODRÍGUEZ, E.; NUERO, O.; GUILLEN, F.; MARTINEZ, A.T.; MARTINEZ, M.J.. Degradation of phenolic and non-phenolic aromatic pollutants by four Pleurotus species: The role of laccase and versatile peroxidase. **Soil Biology & Biochemistry**, 36 (6), 2004, 909-916.

SANTIAGO, M. F. **Estudo de substâncias de baixa massa molar que mimetizam as fenoloxidases com aplicações em tratamento de efluentes industriais**. Tese de doutorado, Universidade Estadual de Campinas, São Paulo, Brazil. 1999.

SAYAD, S.; AND ELLOUZ, R. roles of lignin peroxidase and manganese peroxidase from phanerochaete chrysosporium in the decolorization of olive mill wastewaters. **Applied Environmental Microbiology**, 61(3), 1995, 1098–1103.

SZKLARZ, G. D.; ANTIBUS, R. K.; SINSABAUGH, R. L.; LINKINS, A. E. Production of phenoloxidases and peroxidases by wood-rotting fungi. **Mycologia**. 81 (2), 1989, 234-240.

TIEN, M.; KIRK, K. Lignin degrading enzyme from *Phanerochaete chrysosporium*: purification characterization and catalytic properties of a unique H_2O_2 requiring oxygenase. **Proceedings of the National Academy of Sciences of the United States of America**. 81, 1984, 2280-2284.

TROVASLET, M.; ENAUD, E.; GUIAVARC'H, Y.; CORBISIER, A. M.; VANHULLE, S. Potential of a *Pycnoporus sanguineus* laccase in bioremediation of wastewater and kinetic activation in the presence of an anthraquinonic acid dye. **Enzyme and Microbial Technology**, 41 (3), 2007, 368-376.

WESENBERG, D.; KYRIAKIDES, I.; AGATHOS, S. N. White-rot fungi and their enzymes for the treatment of industrial dye effluents. **Biotechnology Advances,** 22, 2003, 161-187.

WIRZINGER, G.; WELTJE, L.; GERCKEN, J.; SORDYL, H. Genotoxic damage in field-collected three-spined sticklebacks (*Gasterosteus aculeatus* L.): a suitable biomonitoring tool? **Mutat. Res.** 30,628(1), 2007, 19-30.