Ambiente & Água - An Interdisciplinary Journal of Applied Science
ISSN 1980-993X – doi:10.4136/1980-993X
www.ambi-agua.net
E-mail: ambi.agua@gmail.com

Biodegradation of nitroaromatic compounds in Red Water by white rot fungi *Pleurotus ostreatus* and *floridae*

ARTICLES doi:10.4136/ambi-agua.2594

Received: 10 Jun. 2020; Accepted: 15 Sep. 2020

Cristiane Patrícia Kist[1]; Claudio Eduardo Scherer[2]; Marlene Soares[3]; Marcio Barreto Rodrigues[4*]

[1]Programa de Pós-Graduação em Ciência e Tecnologia Ambiental. Universidade Tecnológica Federal do Paraná (UTFPR), Rua Deputado Heitor Alencar Furtado, n° 5000, CEP: 81280-340, Curitiba, PR, Brazil. E-mail: criskist_ck@hotmail.com
[2]Programa de Pós-Graduação em Tecnologia de Processos Químicos e Bioquímicos. Universidade Tecnológica Federal do Paraná (UTFPR), Via do conhecimento, Km 1, s/n, CEP: 85503-390, Pato Branco, PR, Brazil. E-mail: claudiorx.scherer@gmail.com
[3]Departamento Acadêmico de Química e Biologia. Programa de Pós-Graduação em Ciência e Tecnologia Ambiental. Universidade Tecnológica Federal do Paraná (UTFPR), Rua Deputado Heitor Alencar Furtado, n° 5000, CEP: 81280-340, Curitiba, PR, Brazil. E-mail: marlenesoares@utfpr.edu.br
[4]Departamento Acadêmico de Química. Programa de Pós-Graduação em Tecnologia de Processos Químicos e Bioquímicos. Universidade Tecnológica Federal do Paraná (UTFPR), Via do conhecimento, Km 1, s/n, CEP: 85503-390, Pato Branco, PR, Brazil.
[*]Corresponding author. E-mail: marciorodrigues@utfpr.edu.br

ABSTRACT

Pleurotus fungi are basidiomycetes that stand out in the degradation of recalcitrant organic compounds such as lignin derivatives and phenolic compounds. The aim of this study was to make a comparative evaluation of the capacity of the *Pleurotus ostreatus* POS 560 and *Pleurotus floridae* PSP1 fungi in the degradation of 2,4 and 2,6-dinitrotoluene (DNTs) in effluent from an explosive factory. The characterization of the effluent indicated 318 mg L^{-1} of DNTs, 246 mg L^{-1} of COD and toxicity factor for *Daphnia magna* corresponding to 8. The conduct of a multivariate study estimated the influence of the variables pH (5.0 and 6.0), co-substrate concentration (10 and 20 g L^{-1} of glucose) and species of the fungus *Pleurotus* (*ostreatus* and *floridae*) on the degradation of DNTs, indicating that the variables Fungus and [Glucose] were significant ($p < 0.05$) presenting effects in the order of + 4.45 ± 0.26 and -1.14 ± 0.26, respectively. The reproduction of the best efficiency conditions (*P. floridae*; pH 6.0 and 10 g L^{-1} of glucose) in agitated flasks (100 rpm, 26ºC) was able to carry out, within 14 days of treatment, the removal of organic matter and toxicity factor in levels on the order of 55 and 50%, respectively, in addition to the complete degradation of DNTs which occurred in the first 120 hours of treatment. In this period, the maximum activity of the peroxidase and Mn-peroxidase enzymes was also characterized, suggesting high potential of the bioprocess under study for remediation of effluents contaminated with nitroaromatic compounds.

Keywords: DNT biodegradation, explosives factory effluent, white rot fungi.

Biodegradação de compostos nitroaromáticos em Água Vermelha pelos fungos de degradação branca *Pleurotus ostreatus* e *floridae*

RESUMO

Os fungos pleurotus são basidiomicetos que se destacam na degradação de compostos orgânicos de natureza recalcitrante, como derivados de lignina e compostos fenólicos. O objetivo deste estudo foi avaliar comparativamente a capacidade dos fungos *Pleurotus ostreatus* POS 560 e *Pleurotus floridae* PSP1 na degradação de 2,4 e 2,6-dinitrotolueno (DNTs) em efluente da indústria de explosivos. A caracterização indicou 318 mg L^{-1} de DNTs, 246 mg L^{-1} de DQO e fator de toxicidade para *Daphnia magna* correspondente a 8. A condução de um estudo multivariado estimou a influência das variáveis pH (5.0 e 6.0), concentração de co-substrato (10 e 20 g L^{-1} de glicose) e espécies do fungo *Pleurotus* (*ostreatus* e *floridae*) sobre a degradação de DNTs, tendo indicado que as variáveis Fungo e [Glicose] se mostraram significativas ($p<0.05$) apresentando efeitos da ordem de +4.45±0.26 e -1.14±0.26, respectivamente. A reprodução das melhores condições de eficiência (*P. floridae*; pH 6,0 e 10 g L^{-1} de glicose) em frascos agitados (100 rpm, 26ºC) foi capaz de promover em 14 dias de tratamento remoções de matéria orgânica e fator de toxicidade em níveis da ordem de 55 e 50%, respectivamente, além da completa degradação de DNTs a qual ocorreu nas primeiras 120 horas de tratamento, período durante o qual também foi caracterizada atividade máxima das enzimas peroxidase e Mn-peroxidase, sugerindo alto potencial do bioprocesso em estudo para remediação de efluentes contaminados com compostos nitroaromáticos.

Palavras-chave: biodegradação de DNT, efluente de indústria de explosivos, fungos de degradação branca.

1. INTRODUCTION

The nitroaromatic explosives industry produces in its industrial process, especially in the purification stages, wastewater with significant polluting potential, a characteristic especially attributed to the presence of significant amounts of nitroaromatic compounds such as 2,4,6-trinitrotoluene 2,4-dinitrotoluene, 2,6-dinitrotoluene mono and polysubstituted amino and nitrophenols, among others (Barreto-Rodrigues *et al.*, 2009). These effluents have a high degree of toxicity and, when in contact with the intestinal microflora of mammalian organisms, are quickly reduced to a more reactive derivative with characteristic carcinogenic and mutagenic potential (Spain *et al.*, 1994). Due to the low biodegradability that these compounds end up transferring to the effluent, their treatment by conventional remediation technologies is limited. For this reason, different alternatives have been proposed, including filtering catalytic ceramics, modified membranes, different types of advanced oxidative processes and process integration (Bhosale *et al.*, 2019; Ali *et al.*, 2019; Bhanot *et al.*, 2020). As an additional alternative, treatment using fungi has already demonstrated the potential to promote extensive degradation of chemical species of environmental relevance (Bennett, 1994).

Fungi are capable of decomposing wood components, being divided into three groups, according to their morphology: white, brown and mild degradation fungi (Bennett, 1994). White degradation fungi decompose the three components of wood - lignin, cellulose, hemicellulose - at similar speeds. This ability is because they produce several extracellular lignocellulolytic enzymes, mainly laccases, manganese peroxidase and versatile peroxidases, being able to act on several xenophobic compounds with lignolytic characteristics (Šrédlová *et al.*, 2016). In comparison to the enzymatic system of many microorganisms, only the attack of white degrading fungi by specific enzymatic oxidative is able to cause mineralization by the cometabolic process of the aromatic nucleus of the C−TNT ring, which generates CO_2 and carbohydrates, used as a substrate and source of energy and growth (Spain *et al*., 1994, p. 214).

The fungi of the *Pleurotus* genus are known as oyster mushrooms and have about 40 species, occupying the third position (25%) in annual world production. Examples of this species include *Pleurotus pulmonarius*, *Pleurotus sajor-caju*, *Pleurotus floridae* and *Pleurotus ostreatus*. These last two are known as “shimeji” and contain high amounts of proteins, carbohydrates, minerals, vitamins and low fat content, being very important commercially (Maity *et al*., 2011; Wang *et al*., 2015). Microorganisms of the *Pleurotus* genus are generally found in tropical and subtropical forests around the world, and can be grown artificially, due to their ability to degrade a wide variety of substrates that contain cellulose, hemicellulose and lignin, as they have different enzymatic complex, such as cellulases, hemicellulases, ligninases, peroxidases, laccases and proteases (Bennett, 1994).

Although several previous studies report the use of *Pleurotus* species in processes of bioremediation and degradation of different chemical species of environmental relevance, the applications are restricted mainly to *P. ostreatus*, *P. sajor-caju*, *P. eryngii* and *P pulmonarius*, with few studies with some species like *Pleurotus floridae*. For example, using *Pleurotus ostreatus* HAUCC 162, Zhuo *et al.* (2019) characterized the role of laccase enzyme in the degradation process of textile dyes, Malachite green (MG), Remazol Brilliant Blue R (RBBR), Bromophenol blue (BB) and Methyl orange (MO) and in 24h the decolorization rates were of 91.5%, 84.9%, 79.1%, and 73.1% for MG (100 mg L^{-1}), RBBR (100 mg L^{-1}), BB (100 mg L^{-1}) and MO (100 mg L^{-1}), respectively. Kamida *et al*. (2005) studied the bioremediation of synthetic effluent (textile dye - indigo) with *Pleurotus sajor-caju*, and obtained in 14 days of treatment the total removal of the color from the midst. Hadibarata and Kristanti (2013) studied the bioremediation of synthetic fluorene effluent using *Pleurotus eryngii*, and during 23 days of treatment they obtained complete removal of the contaminant. Zhang *et al*. (2015) studied the remediation of bisphenol A synthetic effluent (10 mg/L) by *Pleurotus ostreatus*, observing total biodegradation after 7 days. Finally, Šrédlová *et al*. (2016) studied the remediation of polychlorinated biphenyl, obtaining 72 to 80% removal of the total polychlorinated biphenyl content, after a week of treatment with the *Pleurotus ostreatus* fungus.

The present work evaluated the potential of *Pleurotus floridae* and *Pleurotus ostreatus* for the degradation of nitroaromatic compounds in wastewater from the explosives industry. The scientific contribution and novelty presented is related to the comparison between species of the same fungus applied to the treatment of industrial effluent, since, as mentioned above, most research involving fungi of the genus *Pleurotus* uses compounds or synthetic effluents as models, and applications with *floridae* species are relatively scarce.

2. MATERIAL AND METHODS

2.1. Chemicals and supplies

All chemicals used were of analytical grade and were obtained from Merck, Reagen or Sigma. The wastewater samples were collected from IMBEL Explosives Materials Industry, located in São Paulo State, Brazil. The wastewater resulted from the trinitrotoluene manufacturing process purification stage. After being collected at room temperature, it was stored under refrigeration (4ºC) for later analysis. All experiments used diluted samples (1:200).

2.2. Analytical control

The following parameters were used to determine the pollution potential of the effluent and ensure analytic control of the system:

UV-visible spectroscopy (UV–Vis): Spectroscopic analyses were performed on a spectrophotometer Thermal Scientific Evolution 60S model-UV-visible, using quartz cuvettes with an optical path of 1 cm. Absorbance measurements at ʎmax = 275nm were performed to quantify the rate of degradation of nitroaromatic compounds in the effluent.

Chemical oxygen demand (COD): A sample was refluxed in a strongly acidic solution with a known excess of $K_2Cr_2O_7$. The consumed oxygen was measured against the standard at 600nm with a spectrophotometer (APHA *et al.*, 2005).

Gas chromatograph/mass spectrometer analysis: One hundred millimeters (100 mL) of effluent were dried under reduced pressure and the residue was solubilized with methanol chromatographic grade. After treatment with anhydrous sodium sulfate for moisture extraction and filtering on 0.45µm cellulose membrane, it was transferred to a sample vial. The proportional sample amount (0.4 µL) was injected into a gas chromatograph/mass spectrometer (Varian 431-GC/210MS) equipped with a capillary column (DB5, 30 m×0.25mm, film thickness 0.25µm), operated from 313 to 573K at a programming rate of 20 $K.min^{-1}$. The obtained mass spectra were used to identify 2,4 and 2,6-dinitrotoluene involved in the wastewater when compared to authentic standard compounds.

Red water acute toxicity with Daphnia magna: The procedures followed the NBR 12.713 (ABNT, 2009) and evaluated the acute toxicity of samples from wastewater, surface or subterranean waters and from continental soluble chemicals or from chemicals dispersed in the water. The *D. magna* factor toxicity (FTD) was calculated according to the lowest sample dilution, which presented immobility in not more than 10% of the organisms.

2.3. Effluent biotreatment in shake flasks

Microorganism and inoculum preparation: The ligninolytic *P.ostreatus* POS 560 and *P. floridae* PSP 1 fungus (Fungibrás/SP) were initially grown on potato dextrose agar (PDA) in Petri dishes at 37°C for 7 days and then transferred to the liquid culture (Malt Broth, MB) by punching out 5mm of the agar plate with a sterilized self-designed cutter. Twenty disks were used in each flask as standard inoculum.

Red water effluent treatment: After 72 h, the pre-inoculum was filtered through sterile gauze and the fungal biomass (20 g) was transferred to 250 ml of effluent diluted with distilled water (1:200) and subjected to treatment according to conditions described by factorial planning for a period of 14 days. Samples were collected daily, filtered and analyzed according to parameters described in 2.2.

Experimental Design: A complete 2^3 factorial design with three genuine repetitions was conducted in shaker flasks (100 rpm, 26 ºC, 7 days) to evaluate the effects X_1: fungus species (*P.ostreatus* and *P. Floridae*), X_2: pH (5.0 and 6.0) and X_3: concentration of co-substrate in the form of glucose (10 and 20 g L^{-1}) on the removal of nitroaromatic compounds as measured by the percentage reduction in absorbance at λ_{max} = 275nm. For the purpose of statistical calculations, the values of the independent variables were coded in two levels (− and +), as shown in Table 1.

Table 1. Factorial planning matrix and Effects for nitroaromatic removal in Red Water.

Test	Codified levels			Actual values			Nitroaromatic Removal (%)	
	X_1	X_2	X_3	Fungus	pH	[glucose] (g L^{-1})		
1	-1	-1	-1	*P. ostreatus*	5.0	10	14.3	± 0.53
2	+1	-1	-1	*P. floridae*	5.0	10	17.5	± 0.58
3	-1	+1	-1	*P. ostreatus*	6.0	10	18.4	± 0.42
4	**+1**	**+1**	**-1**	***P. floridae***	**6.0**	**10**	**21.0**	**± 0.74**
5	-1	-1	+1	*P. ostreatus*	5.0	20	15.6	± 0.65
6	+1	-1	+1	*P. floridae*	5.0	20	20.9	± 0.74
7	**-1**	**+1**	**+1**	***P. ostreatus***	**6.0**	**20**	**11.7**	**± 0.75**
8	+1	+1	+1	*P. floridae*	6.0	20	18.4	± 0.44

X_1: Fungus; X_2: pH and X_3: *Pleurotus* fungus specie.

For better interpretation, a statistical analysis carried out with Statgraphics plus 5.1 software estimated the effects of the variables of interest on the removal rate of nitroaromatic compounds. The results are shown in Table 2, in which it is possible to observe estimated effect values, regression coefficients, interactions with significant and non-significant parameters, in addition to associated errors and level of significance attributed to each parameter. Factors in bold and marked with an asterisk were considered significant for the 95% confidence interval (X_1: Fungus, X_3: glucose; interactions $X_1.X_3$ and $X_2.X_3$).

Table 2. Effects, regression coefficients and interaction to Red Water biotreatment.

Factors	Effect	Effect error	tcalc	p Value	Coefficient	Coefficient error
Average	17.22	± 0.134	128.5		17.225	± 0.067
X_1:fungus*	**4.45**	**± 0.268**	**16.60**	**0.0000***	**2.225**	**± 0.134**
X_2: pH	0.325	± 0.268	1.212	0.2115	0.1625	± 0.134
X_3:[glucose]*	**-1.137**	**± 0.268**	**-4.244**	**0.0002***	**-0.5687**	**± 0.134**
X_1. X_2	0.225	± 0.268	0.8395	0.3829	0.1125	± 0.134
X_1. X_3*	**1.53**	**± 0.268**	**5.376**	**0.0001***	**0.7687**	**± 0.134**
X_2. X_3*	**-3.51**	**± 0.268**	**-13.10**	**0.0001***	**-1.756**	**± 0.134**

*Statistically significant factors ($p<0.05$). t_{tab} 0.05; 24 = 2.060.

3. RESULTS AND DISCUSSION

3.1. Multivariate study of red water biotreatment with *Pleurotus*

Table 1 shows the tests, coded and real levels, in addition to the results obtained in each experiment carried out with the same experimental errors. The data (responses) obtained based upon the statistical design were assessed by analysis of variance (ANOVA). The homogeneity of variance was checked by Levene's test, and the normal distribution of results was checked using the Shapiro-Wilk test with a 5% significance level. In general, degradation rates of nitroaromatic compounds ranged from 11.7 to 21%, obtained in tests 4 and 7, which used *P. ostreatus* (pH 6, 20 g L^{-1} of glucose) and *P. floridae* (pH 6, 10 g L^{-1} of glucose), respectively.

Equation 1 was generated considering only the significant coefficients listed in Table 2 and it explains mathematically how each variable affects the nitroaromatic compounds removal during the biological treatment of red water effluent.

$$\text{DNT removal (\%)} = 17.22 + 2.22X_1 - 0.568X_3 + 0.768X_1.X_3 - 1.756X_2.X_3 \qquad (1)$$

As it would be of interest to use Equation 1 as a model for predictive and interpretative purposes, the adjustment of Equation 1 was assessed with the aid of analysis of variance (ANOVA) (Barros Neto *et al*., 2002) and the results are shown in Table 3. In this context, it can be verified with 95% confidence that the model is satisfactory. Approximately 95% of variation around the mean can be verified, in addition, the value for the F_{calc} ratio was higher than the F_{tab} value, suggesting that a regression involving the study variables can be considered significant and adequate to be used for predictive purposes (Box *et al.,* 1978). Thus, Equation 1 was used for the construction of response surfaces "Fungus *versus* Glucose" ($X_1.X_3$) and "pH *versus* Glucose" ($X_2.X_3$) illustrated in Figure 1 and useful for the interpretation of the interaction of significant effects on the rate of removal of dinitrotoluenes from the red water effluent.

Table 3. Variance analysis to Red Water Discoloration.

Variance source	GL	SQ	QM	F_{calc}
Model	6	287.633	47.938	93.5
Lack of adjust	1	2.10125	2.10125	4.09
Pure error	24	12.305	0.512708	
Total	31	302.0392		

R^2 = 95.23; F_{tab} 0.05; 6; 25 = 2.490

Both surfaces illustrated in Figure 1 show a variable [glucose] slope of the response of nitroaromatics removal (%) from the lower level to the upper level, indicating that lower concentrations of co-substrate provide significant efficiency gains. Regarding pH, although its main effect was not significant, its interaction with the glucose illustrated by surface 1 (a) reveals better efficiency when the tests were conducted at the lowest glucose concentration and the highest pH level (6.0). These results show that even in treatments of different contaminants, there is a relationship in the efficiency presented by the fungi when influenced by pH and glucose as the study of Kunjadia *et al*. (2016) shows, where the efficiency of biodegradation of three types of azo dyes was evaluated by three varieties of white degrading fungi of the genus *Pleurotus*, namely *P. ostreatus*, *P. sapidus*, and *P. floridae*, which respectively showed 88, 92 and 98% efficiency in decolorization in 10 days of treatment where the influence by the pH of the medium in the process of biosorption carried out by the fungi had the maximum removal, being achieved with pH varying between 6−6.5 and, outside of that, it was verified that the the removal rates reduce quickly. The influence of sugar, especially glucose as a co-substrate in cultures showed use of up to 0.4 mg/mL for the *P. ostreatus* compared to the use of 0.30 and 0.35 mg mL^{-1} for *P.* sapidus, and *P. floridae*, in that order. For the fungus variable, the high slope from the level (-1) to the level (+1) of Figure 1 (b) towards the dependent variable associated with the calculated effect (Table 2) shows a gain of 4.4 percentage points when the *P. ostreatus* is replaced by *P. floridae*.

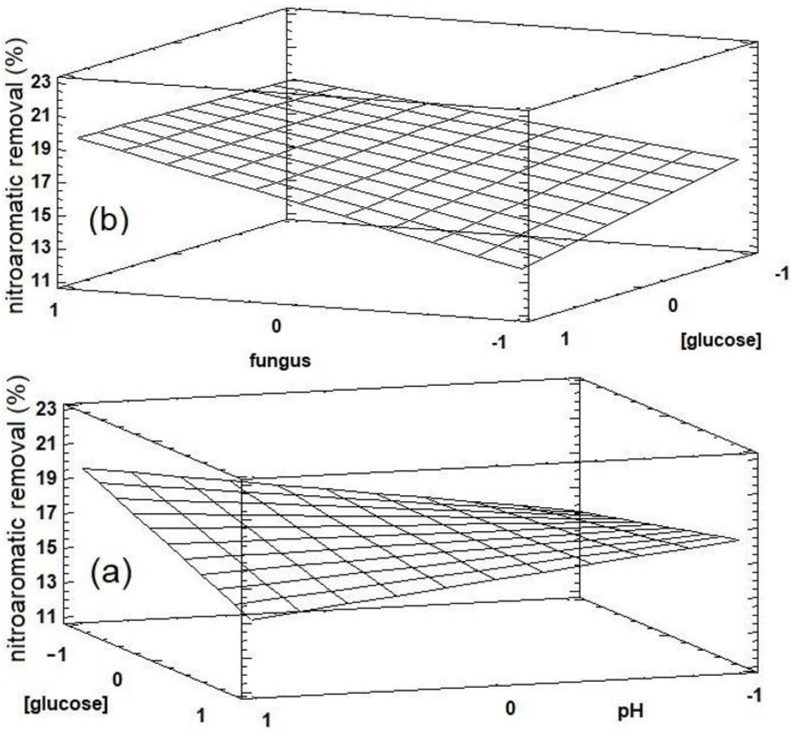

Figure 1. Response surface for the effects on DNT removal: (a) glucose x pH; (b) fungus x glucose.

3.2. Biological treatment of Red Water effluent in shake flasks

The reproduction of the conditions of better efficiency identified through the factorial design (species of fungus *P. floridae*; pH 6.0 and 10 g L^{-1} of glucose) in shaker flasks generated the results illustrated in Figure 2 and Table 4.

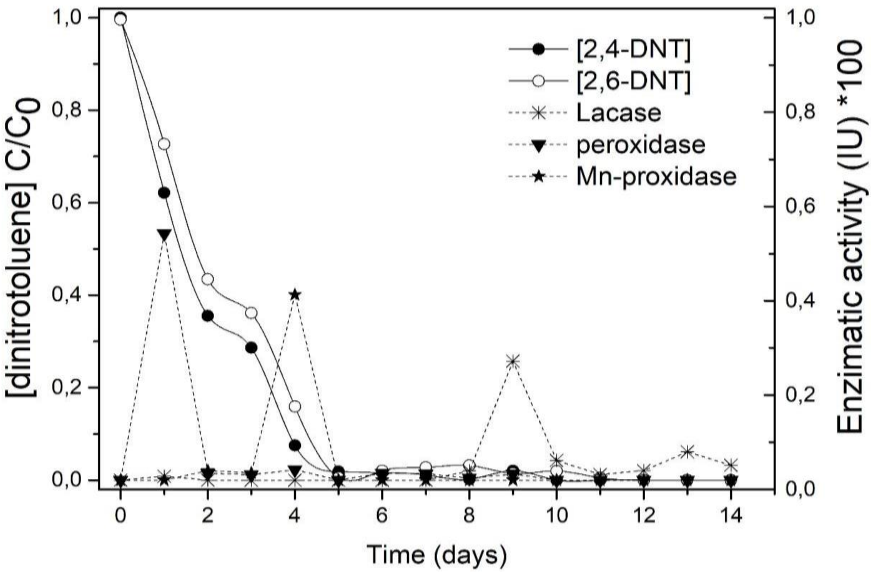

Figure 2. Reduction of dinitrotoluenes and Enzymatic activity of Red Water effluent under treatment in shake flasks with *Pleurotus floridae* PSP. Experimental conditions: initial pH of 6.0; 10 g L^{-1} glucose and 14 days fermentation time.

Table 4. Physical-chemical parameters and ecotoxicity of red water before and after fungal treatment (dilution 1: 200; treatment time: 14 days).

Parameters	Untreated effluent	Treated effluent	Reduction (%)	Limits of environmental legislation*
COD, mg L^{-1}	246	135	55	225
Total dinitroaromatic, mgL^{-1}	318	n.d	100	---
Toxicity Factor	8	4	50	8

* Brazilian resolution CEMA 81/2010, CONAMA 430/2011 (CEMA, 2010; CONAMA, 2011); n.d: not detected.

The complete removal of nitroaromatic compounds occurred in 5 days of treatment (120 hours) and was related to the activity of the enzyme peroxidase and Mn-peroxidase, which presented maximum activity on the first and fourth days, respectively, coinciding with the periods of higher removal of 2,4 and 2,6-dinitrotoluene. Additionally, the laccase enzyme was also detected, but only after the ninth day of treatment; therefore, its performance seemed to be more related to the degradation of possible intermediates of 2,4 and 2,6-DNT. Such enzymes are recognized for their ability to break down lignin and its derivatives, and also other types of xenobiotic compounds such as pesticides, hormones, drugs and nitroaromatic compounds (Morsi *et al*., 2020). For example, Levin *et al*. (2016) obtained 98.4% removal of 4-nitrophenol (70 mg L^{-1} aqueous solution) with *Trametes versicolor* in 4 days of treatment, the present study also observed the importance of the process performance of the laccase and Mn-peroxidase and peroxidase enzymes. Bettin *et al*. (2019) also found activities of several phenoloxidases, including laccase in the degradation of phenol in aqueous solution.

As shown in Table 4, in addition to the complete removal of 2,4 and 2,6-dinitroaromatics, the data suggests that the enzymatic activity shown by *Pleurotus floridae* was also sufficient to, in 14 days of treatment, remove organic matter and toxicity factor for *Daphnia magna* at levels in the order of 55 and 50%, respectively. Although the contribution of the adsorption / biosorption processes in the mycelium in relation to biodegradation has not been evaluated, the results reveal that fungal biotreatment promoted a sufficient reduction in the levels of the monitored parameters, making them compatible with the limits provided for in the current environmental legislation. An analysis of the efficiency of Red Water treatment processes available in the scientific literature (Table 5) reveals that physical (adsorption) and chemical (electrochemical) processes present, in comparison to the results obtained in this work, equivalent performances, which can also reach 100% of removal of DNT, however, in shorter intervals of treatment time (between 3 and 30 hours). On the other hand, it is important to highlight that such processes generally have limitations related to the need to dispose of contaminated phases in the case of adsorbents, in addition to the typical complexity of electrochemical processes, which hinder scaling up operations. In comparison to the biological process with immobilized microorganisms, the results of which are also illustrated in Table 5, the present study required less treatment time for relatively greater removal of the DNT (5 days). Finally, it should be noted that the fact that the % of COD removal (55%) was inferior to the other treatments may be related to the residual cosubstrate (glucose) present in the effluent after treatment.

Table 5. Performance of treatment process for Red Water treatment.

Process	**Experimental Conditions**	**Treatment time**	**Removal Efficiency**	**Ref.**
Adsorption in Activated Coque (AC)	160 g L^{-1} AC, pH 6.28, 20°C, red water dilution 1:100	3 h	64.8% COD 84% DNT	Zhang *et al.* (2011)
Electrochemical Treatment (Ti/IrO2)	Anodic Ti/IrO_2 electrode and Ti cathode. Red water dilution 1:100	30 hours	68.5% COD 100% DNT	Jiang *et al.* (2018)
Immobilized ana/aerobic microbial filters	upflow, Bionetix B925 microorganisms, red water dil. 1:200	25 days → 100 days →	88% DNT 77% COD	Zhang *et al.* (2015)
Fungal biotreatment	10 g L^{-1} glucose, pH 6, *P. floridae* fungal, red water dilution 1:200	5 days → 14 days →	100% DNT 55% COD 50% toxicity	**This work**

4. CONCLUSIONS

The statistical analysis of the factorial design indicated that the variables of greatest influence on the degradation of 2,4 and 2,6-dinitrotoluene were the concentration of co-substrate [Glucose] and species of fungus. Treatability studies conducted in an optimized condition (*P. floridae*, initial pH of 6.0 and 10 g L^{-1} glucose) revealed significant performance for the reduction of chemical oxygen demand, toxicity factor for *Daphnia magna* and also for the total removal of dinitroaromatic compounds constituents of the Red Water effluent. Additionally, it was possible to infer that the observed mechanism of degradation of DNTs is directly related to the activity of identified phenoloxidase enzymes, especially peroxidase and Mn-peroxidase. These results suggest the potential of the bioprocess studied for applications in the treatment of effluents from the nitroaromatic explosives industry.

5. ACKNOWLEDGMENTS

The authors would like to thank UTFPR, Câmpus Pato Branco, CAPES and the Analysis Center availability for the use of the laboratories and for the analysis carried out.

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