Vol. 17(52), pp. 1445-1454, 26 December, 2018
DOI: 10.5897/AJB2018.16426
Article Number: 317FE1F59637
ISSN: 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Degradation kinetics of 2,4-dichlorophenoxyacetic and atrazine by *Trametes versicolor* (L.:Fr.) Pilát

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Received 8 February, 2018; Accepted 15 August, 2018

The degradation kinetics of different sources of 2,4-dichlorophenoxyacetic (2,4-D) and atrazine by the natural strain Mo008 of the basidiomycete fungus *Trametes versicolor* (L.:Fr.) Pilát was studied, knowing that in this process the strain used produces an enzyme complex composed of manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase, and the response on the global consumption rate and yield is dependent on the 2,4-D source, being more efficient with an analytical source and mixture with atrazine than amine salt; however, it was more efficient to degrade atrazine. The strain studied (Mo008) consumed 1000 ppm of 2,4-D analytical, 2,4-D amine, 2,4-D plus atrazine and atrazine in 750, 850, 650 and 550 h, respectively.

Key words: *Trametes versicolor*, degradation kinetics, biodegradation, 2,4-dichlorophenoxyacetic (2,4-D), atrazine.

INTRODUCTION

Lignin is one of the most abundant polymers in nature and the fungi that cause white rot of wood are responsible for initiating its depolymerization (Buswell and Odier, 1987; Gold et al., 1989; Kirk and Farrell, 1987; Szklarz et al., 1989). Ligninolytic fungi such as *Trametes versicolor, Phanerochaette chrysosporium* and *Pleurotus ostreatus*, have been extensively studied in the recovery of effluents from various industries due to the production of an enzymatic system capable of degrading lignin and phenolic compounds (Yateen et al., 1998; *Tortella* et al., 2008), which is composed of the manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Rothschild et al., 1999; Guo et al., 2000; Ullah et al., 2000; Gómez et al., 2005).

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Diverse species of basidiomicetes have been studied in recent years, due to their ability to degrade lignin and phenolic compounds (Córdoba et al., 2012; Betancur et al., 2013; Stamatiu et al., 2015). Studies in biotechnology and industrial applications of lignolytic fungi are focused on the species that cause white rot of the wood as a group in general, although this is taxonomically and physiologically heterogeneous, which has overestimated the importance of the best studied fungus, *P. chrysosporium* and has left aside other species with better qualities for biotechnological applications (Peralta et al., 1998; Nyanhongo et al., 2007).

T. versicolor is a basidiomycete that has the ability to degrade compounds with varying kinetics, which is attributed to the production of enzymes that degrade lignin, particularly laccase and MnP, which are excreted in amounts that increase during lignolytic activity (Hobbs, 2004; Lin et al., 2008; Lau et al., 2004; Sedarati et al., 2003). In this sense, it is reported that at an initial concentration of 50 mg L^{-1}, *T. versicolor* showed a complete removal of benzene after 14 h of exposure and by increasing it to 300 mg L^{-1} the removal took 42 h. Concentrations of 50 ml L^{-1} of toluene were removed in 4 and 36 h when increased to 300 ml L^{-1} (Demir, 2004). On the other hand, Megan et al. (2010) reported that this species degrades trifluralin and dieldrin in maximum amounts of 24.6 and 115 ml L^{-1} as well as the mixture of both in a concentration of 32.3 ml L^{-1} in a time of 480 h. Within this degradation process, *T. versicolor* excretes an enzyme that acts as a kind of Mn(II)-dependent peroxidase (Johansson and Nyman, 1987). MnP is able to catalyze the oxidation of phenanthrene, fluorene and other phenolic compounds (Collins and Dobson, 1996) suggesting that this enzyme is capable of degrading a large number of phenolic compounds as well as the LiP; however, the laccase enzyme, which is also present in this fungus, does not degrade phenanthrene and fluorene, but does have activity over a wide range of polyphenols (Collins and Dobson, 1996; Majcherczyk et al., 1998). Obtained data by Pozdnyakova et al. (2018) support the hypothesis that, the degree of degradation of the phenolic compounds can depend of the composition of the extracelular ligninolytic complex of strain used. In this regard, for the correct selection of fungal strains for remediation, it is necessary to study the activity of the basic ligninolytic enzymes. This will allow the development of a technological process to avoid the accumulation of toxic substances in the treated objects. On the basis of their degradative properties and the composition of the ligninolytic enzyme system, *T. versicolor* can be employed for detailed study and for the development of technologies of remediation of contaminated environments. The present research was carried out to determine the degradation kinetics of *T. versicolor* natural strain Mo008 as well as to know the enzimatic complex involved in the degradation process over acid 2,4-dichlorophenoxyacetic (2,4-D) and atrazine.

MATERIALS AND METHODS

Strain

In the present investigation, the native strain of *T. versicolor* (L.:Fr.) Pilát isolated from *Crescentia alata* in Jojutla, Mor., México was used (Mo008).

Detection of 2,4-D in liquid medium

To extract 2,4-D from a solution, a methodology proposed by Anonimo (1995) was used, the residues were collected from a steam rod at 50°C and 50 rpm and 50 mL of ethyl alcohol (95%) was added to concentrate the 2,4-D residues.

Calibration curve of 2,4-D, 2,4-D amine and atrazine

This was obtained using the methodology of Bhoi (2011), for which a standard solution of ethylic alcohol (95%) was prepared with 100 mg L^{-1} of 2,4-D analytical (SIGMA®) and 2,4-D amine (Hierbamina®). A solution in distilled water of 10 mg L^{-1} of atrazine was prepared too and from each standard solutions aliquot was taken to prepare a solution of 0, 2, 5, 10, 15 and 20 mg L^{-1} from both sources of 2,4-D and 0, 0.5, 1, 2, 3, 4, 5 and 10 mg L^{-1} of atrazine. The absorbance was measured in a spectrophotometer (Genesys 10uv®) at 287 nm for 2,4-D and 220 nm for atrazine.

Bioreactors for biodegradation of 2,4-D, 2,4-D amine and atrazine

PVC containers with 15 L^{-1} solution with 1000 ppm of 2,4-D (SIGMA®), 2,4-D amine (Herbamina®) and the mixture of 2,4-D amine plus atrazine (Atrazine 90®) at pH 5.0 and 25°C were used as bioreactors. Filters with steril polyurethane fiber support and 10 g of mycelium of *T. versicolor* strain Mo008 and previously developed for 15 days in malta-agar culture medium were constructed and adapted to each biorreactor. The strain was exposed for 1000 h at continuous flow. Every 50 h, 3 samples of 100 ml were collected from each reactor in amber glass bottles and stored at 0°C.

Degradation kinetics

The degradation kinetics was obtained by estimating yield of biomass-substrate and consumption global velocity (CGV) based on the methodology presented by Rubio (2005) and Marron et al. (2006). The yield represents the amount of mycelium produced by the fungus per each ppm consumed (Equation 1) and the CGV like decrease of the concentration of the substrate in a given time (Equation 2).

$$Y = \frac{(Xf - Xi)}{Si - Sf} \quad (1)$$

Where, Y is the yield; Xf is the final biomass; Xi is the initial biomass; Sf is the final concentration; Si is the initial concentration.

$$CGV = \frac{(Si - Sf)}{Tt} \quad (2)$$

Where, CGV is the consumption global velocity; Is is the initial substrate; Sf is the final substrate; Tt is the total time.

Enzymatic analysis

Preparation of enzyme expression fluid

An enzyme expression fluid was prepared according to the methodology of Penninckx and Jiménez (1996) and Jiménez et al. (1999) which contained 1% of glucose, 0.02% of ammonium tartrate, 0.05% of $MgSO_4.7H_2O$, 0.01% of $CaCl_2.12H_2O$, 0.05% of Tween 80, 0.1 mg of thiamine chloride, veratryl alcohol 2.5 mM, 70 ml L^{-1} of trace elements (which contains per liter: 1.5 g of nitriloacetic acid, 3 g of $MgSO^4.5H_2O$, 1 g of NaCl, 0.1 g of $FeSO_4.7H_2O$, 0.1 g of $CaCl_2.2H_2O$, 0.1 g of $ZnSO_4.7H_2O$, 0.01 g of $CuSO_4.5H_2O$, 0.01 g of ALK $(SO_4).12H_2O$, 0.01 g of HBO_3 and 0.01 g of $Na_2MoO_4.2H_2O$) and brought to pH 4.5 with sodium tartrate buffer. In 500 ml Erlenmeyer flasks, 70 ml of expression fluid was added and sterilized at 120°C for 15 min. A gram of mycelium of the Mo008 strain was added with 15 days of development (Jiménez et al., 1997) and it was placed on a shaker at 150 rpm at room temperature. At 48 h, 40 ppm of Mn^{+2} was added and held for an additional 48 h on the shaker.

Preparation of extracts

Mycelium (1 g^{-1}) developed in the enzyme expression fluid was centrifuged (Hermle®) at 5000 rpm for 5 min (Pennickx and Jimenez, 1996). The supernatant was stored at -15°C in total darkness for further use. This same methodology was used to obtain extracts of the mycelium developed in the filters of the bioreactors with the solution of 1000 ppm of 2,4-D amine after 1000 h of exposure in continuous flow.

Conditioning of extracts

The temperature was elevated to 20°C to each extracts, as each one of them was used for the detection of the enzymatic activity.

Calibration curves for the substrates used

To measure the enzymatic activity of LiP, a calibration curve was performed for concentrations from 0 to 5 mM of veratril alcohol in a sodium tartrate buffer 50 mM pH 4.5 and 25°C. For the case of Manganese Peroxidasse (MnP), the curve was estimated for concentrations from 0 to 0.5 mM of phenol red [0.01%] in sodium succinate buffer 0.1 M and for laccase, concentrations from 0 to 9 mM of ABTS 5 mM in 0.1 M sodium acetate buffer pH 5.0. The absorbance was recorded at 310, 610 and 420 nm for each substrate, respectively. From the absorbance data recorded for each of the enzymes studied, the concentration of the substrate (mM) was estimated from the equation obtained from the calibration curve, the extinction coefficient and the enzymatic activity (mM/min/ml). From these results, the Michaelis-Menten kinetic model was obtained as well as the Lineweaver-Burk representation to obtain the maximum reaction velocity (Vm) and the concentration of the substrate for which the reaction speed is half the speed maximum (km)

Determination of enzymatic activity

The activity of LiP was measured using the method proposed by Tien and Kirk (1983), which records the increase in absorbance at 310 nm due to the oxidation of veratril alcohol to veratryl aldehyde. The reaction employs 2.2 ml of sodium tartrate buffer (50 mM, pH 4.5 at 25°C), 40 µl veratryl alcohol (2 mM) and 240 µl of the culture extract. The reaction is initiated by adding 20 µl H_2O_2 (0.2 mM) and the absorbance is measured at 310 nm. The activity of MnP was recorded following the methodology described by Glenn and Gold (1985). This method is based on the oxidation of Mn (II) to Mn (III) and uses as a substrate 2.5 ml of red phenol (0.01%) and $MnSO_4$ (0.1 mM) in sodium succinate buffer (0.1 M). The reaction mixture contains 2.5 ml of substrate and 200 µl of the culture extract. The reaction is started by adding 20 µl of H_2O_2 (0.1 mM), after incubation for 2 min at 30°C and the absorbance WAS measured at 610 nm. On the other hand, laccase activity was recorded using the method described by Bourbonnais et al., (1995) which records the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS substrate, 5 mM). After dissolving the substrate in 2.5 ml of sodium acetate buffer (0.1 M, pH 5.0), 100 µl of the extract was added, the mixture was incubated for 2 min at 30°C and after that time the absorbance was recorded at 420 nm. For all cases, the absorbance was measured at intervals of 30 s for 5 min.

All analysis had three replicates and the variance analysis and Tukey test (α=0.01) was made with SAS® Stadistical Software.

RESULTS AND DISCUSSION

Calibration curve for detection of analytical 2,4-D, 2,4-D amine and atrazine

The obtained curves are represented by a linear model with a correlation coefficient that allows to affirm that the detected absorbance is a direct function of concentration in each one of the samples (Figure 1).

Degradation kinetics of *T. versicolor*

Specifically, 2,4-D is one of the herbiides most used to control boradleaf weeds; the active ingredient has chronic health effects, and lethal on the soil because it has a residual effect. It has been shown that there are microorganisms that have the capacity to degrade these phenolic compounds, which is called bioremediation (Akintui et al., 2015). The degradation kinetics in bioreactors was determined according to the yield of the biomass-substrate, which expresses the increase of biomass of *T. versicolor* for each ppm of consumed substrate (Rubio, 2005), with a response differentiated by the source 2,4 -D (Table 1) as well as the mixture of this with atrazine (Figure 2), confirming a differentiation in the yield for each substrate (Figure 3A), which coincides with Field et al. (1992), Bhalerao and Puranik (2007) and Siddique et al. (2003) who attribute the development of filamentous fungi on different substrates which directly influence respiration and biomass as the only carbon source. Stamatiu et al. (2015) found that some strains exposed to chlorpyrifos and endosulfan outgrow the mycelial development of their respective controls after a period of inhibition (3 to 16 days). *Phanerochaete chrysosporium, P. ostreatus, Bjerkandera adusta* and *T. versicolor* are the most commonly used for the degradation of such compounds owing to their production of ligninolytic enzymes such as LiP, MnP and laccase (Pozdnyakova et al., 2018).

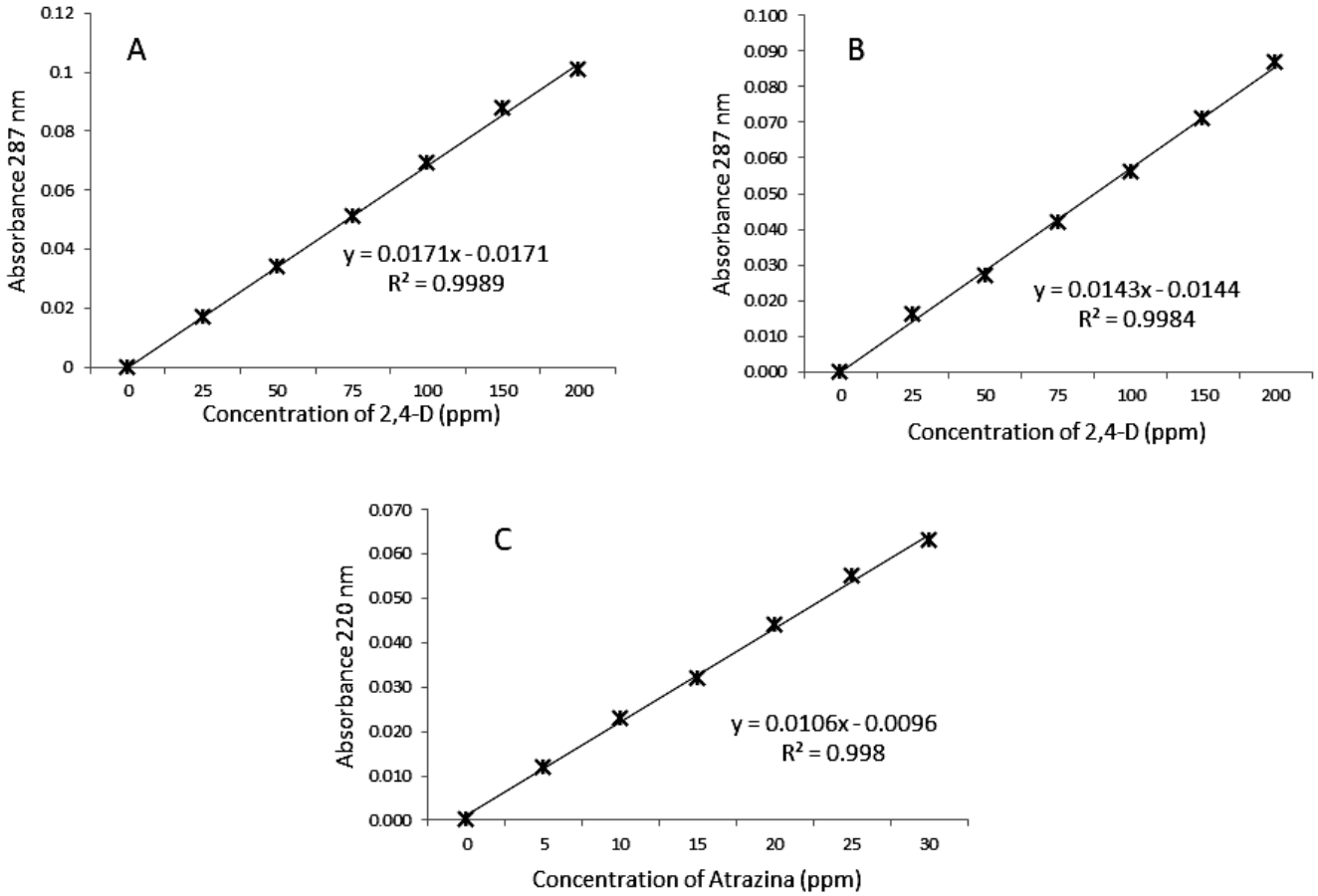

Figure 1. Calibration curves for the detection of analytical 2,4-D (A); 2,4-D-amine (B); and atrazine (C).

Table 1. Bioreactor degradation kinetics of *Trametes versicolor* (L.:Fr.) Pilát strain Mo008.

Parameter	Yield (g ppm^{-1})	GCR* (ppm h^{-1})	Filter biomass (g)	Consumption time (h)
Analytical 2,4-D	0.0121	1.333	12.113	750
2,4-D amine	0.0085	1.176	8.480	850
2,4-D amine + atrazine	0.0117	1.538	11.747	650
Atrazine	-	1.818	-	550

*Global consumption rate.

On the other hand, Dutta et al. (2010) reported a stimulus in basal respiration and in microbial biomass when chlorpyrifos like substrate is used in comparison with the control, whereas Das and Mukherjee (2000) and Eisenhauer et al. (2009) mentioned that soil micro-organisms treated with organophosphorus and organochlorine pesticides increase their population. The global consumption rate (GCR) that expresses the amount of ppm degraded by *T. versicolor* per hour indicates that when 2,4-D amine is mixed with atrazine, it tends to be higher (Figure 3B) than when only analytical 2,4-D and amine salt are used, and the studied strain is more efficient to degrade atrazine than 2,4-D; the 1000 ppm added to the bioreactor was degraded in a time of 550 h with a GCR of 1,818. These results indicate that *T. versicolor* (Mo008) can be used as a biodegradation tool for this type of compounds in high concentrations (1000 ppm) as compared to what has been reported by Demir (2004), Wu and Yu (2006), Srivastava et al. (2008), Kumar et al. (2009), Megan et al. (2010) and Pozdnyakova (2018).

Chlorophenols are the most common organic compounds which are widely used in agricultural industry and public health. The most important pollution sources

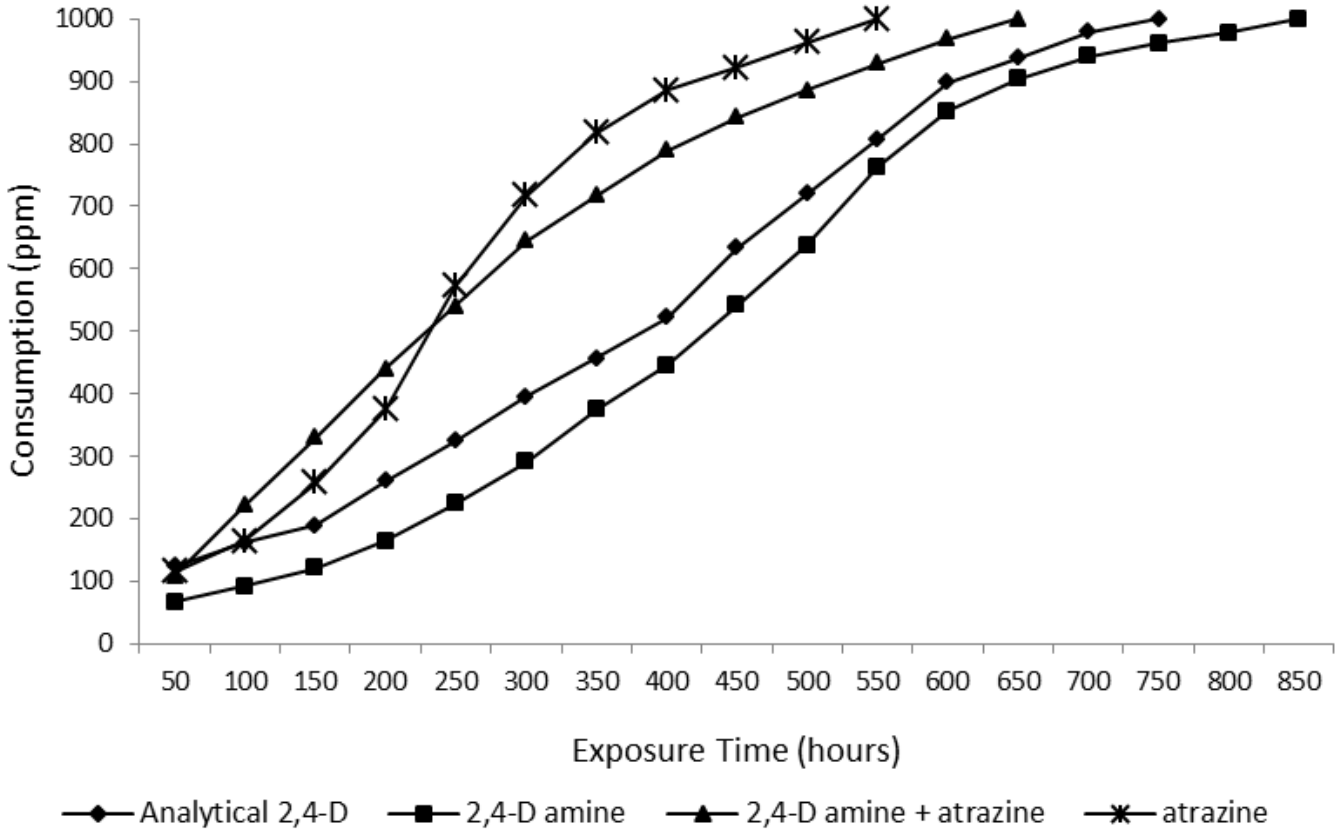

Figure 2. Bioreactor consumption by *Trametes versicolor* (L.:Fr.) Pilát strain Mo008 of each of the substrates evaluated.

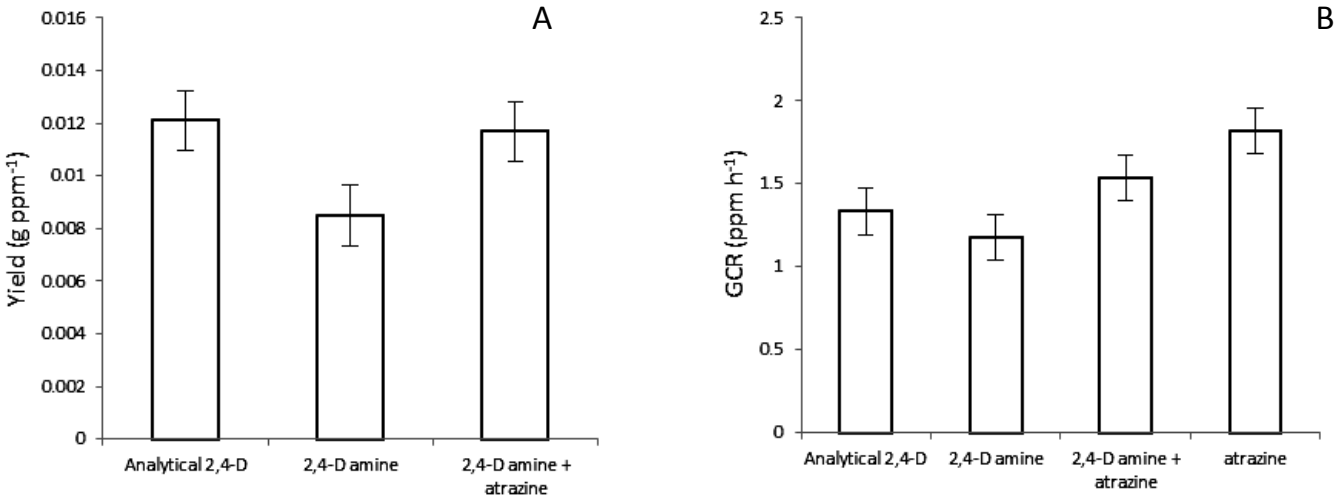

Figure 3. Bioreactor degradation kinetics of *Trametes versicolor* (L.:Fr.) Pilát strain Mo008. **A,** Yield biomass/substrate (g ppm^{-1}); B, global consumption rate (GCR) in ppm h^{-1}.

of chlorophenols are the waste waters from pesticide, paint, solvent, pharmaceutics, wood-preserving chemicals, paper and pulp industries and water disinfecting processes. Because these types of products are toxic, resistant to microbial degradation and can accumulate in the food chain, many countries have restricted or

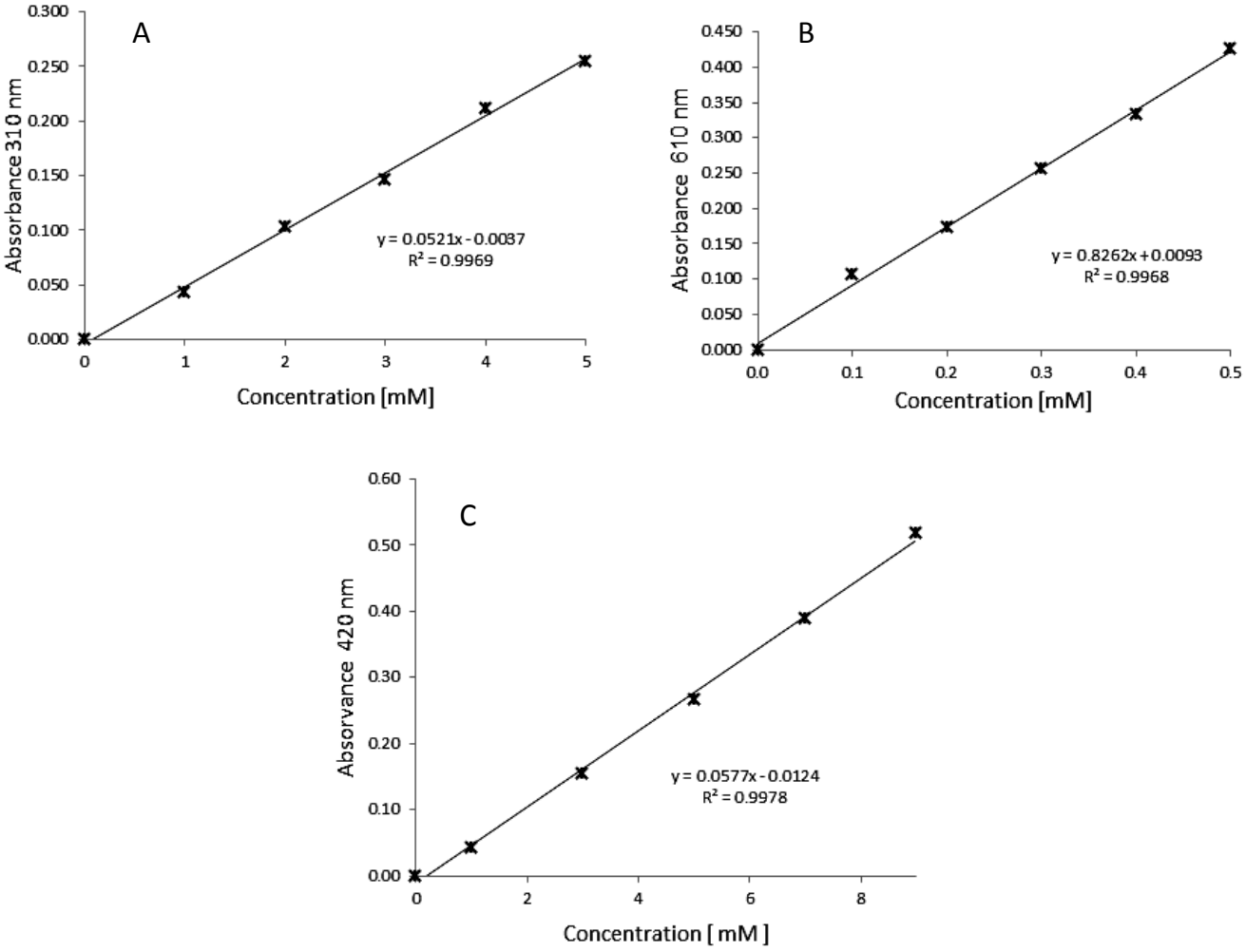

Figure 4. Calibration curve for each substrates to detection of enzymatic complex involved in degradation of 2,4-D by *Trametes versicolor* (L.:Fr.) Pilát strain Mo008. **A,** Lignine peroxidase; B, manganese peroxidase; **C,** lacase.

banned their production or use and have designated them as priority pollutants in their own list of hazardous wastes (Ruiying and Jianlong, 2007). *T. versicolor* has the ability to produce different enzymes that can degrade recalcitrant compounds, for this reason, it is used in biotechnology for bioremediation studies, likewise it can be use to remove Cu^{2+}, Pb^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+} from organic textile dyes (Congeevaram et al., 2007; Fu and Viraraghavan, 2001; Baldrian, 2003; Bayramoğlu et al., 2003; Solis et al., 2015).

Calibration curves for the measurement of enzymatic activity in degradation of 2,4-D amine and the mixture of 2,4-D amine and atrazine

The calibration curves for each extracts used to detect enzymatic activity are represented by linear models with correlation coefficients above 0.99 as shown in Figure 4.

Enzymatic activity in the degradation of 2,4-D amine and 2,4-D amine plus atrazine

The complex enzymatic involved in the degradation of phenolic compounds (LiP and MnP) was detected in the micelium extracts exposed to 2,4-D amine as well as to the mixture of this with atrazine in continuous flow bioreactor and phenol oxidases or polyphenolic oxidases as laccase. For each enzyme detected, the mycelial extract developed in the enzymatic expression fluid (Control) showed a higher affinity than the mycelial extract developed in the bioreactor (Table 2); however, the development of *T. versicolor* in 2,4-D amine as well as in the mixture with atrazine indicate that this fungus produces the enzymatic complex involved for the degradation of both compounds (Karam and Nicell, 1997; Duran, 1997; Duran and Esposito, 2000) and that the degradation process was greater when the 2,4-D amine was mixed with atrazine than when it was exposed

Table 2. Enzymatic activity of *Trametes versicolor* (L.:Fr.) Pilát detected in strain Mo008.

Km expressed in mM						
	Lignin Peroxidase		Manganese Persoxidase		Laccase	
	2,4-D[a]	2,4-D[b] + Atrazine	2,4-D[a]	2,4-D[b] + Atrazine	2,4-D[a]	2,4-D[b] + Atrazine
Control	0.1377	0.0878	1.7202	0.4092	0.9056	0.9393
Extract	0.1684	0.1217	1.0412	0.5337	0.9047	4.8181
a: amine; b: analytical						
Vm expressed in mM·min^{-1}						
	Lignin Peroxidase		Manganese Persoxidase		Laccase	
	2,4-D[a]	2,4-D[b] + Atrazine	2,4-D[a]	2,4-D[b] + Atrazine	2,4-D[a]	2,4-D[b] + Atrazine
Control	51.0204	67.5674	3.5958	0.5510	188.6792	303.0303
Extract	52.6315	64.1025	0.5605	0.4450	158.7301	416.6666
a: amine; b: analytical						

individually due to the values of speed maximum detected in the last process (Figure 5). Oxidative enzymes play an important role in the decontamination of effluents and soil and *T. versicolor* possesses a complex mechanism involving enzymes that attack lignin directly, like LiP, MnP and laccase (Córdoba et al., 2012); these enzymes can be used in the management of environmental pollutants such as textile effluents, pulp effluents, organochloride agrochemicals and crude oil residues (Kantharaj, 2017) which were detected in the present study. The genus *Trametes*, which belongs to the White-rot fungi, is assumed to be one of the main producers of laccases. *T. versicolor* produces laccase and MnP as major lignolytic enzymes; however, and in a particular case, the role of these enzymes in decolorization of azo dyes is not yet clear. Laccase and/or MnP activities in culture fíltrate of *T. versicolor* were not able to decolorize azo dyes, thus indicating a role of other enzymes or cell-bound components in azo dye degradation (Swamy and Ramsay, 1999). The use of enzymes for the treatment of contaminants has been proposed by numerous researchers, however, most of these investigations are focused on demonstrating the decrease of several contaminantsby biological organisms that produce the enzyme complex studied as a basis for future remediation engineering projects, a fundamental step for its implementation (Gianfreda et al., 1999; Heitzer, 1993; Aitken, 1993; Heitzer, 1998). This enzyme complex is mainly composed of the so-called lignolytic enzymes that include laccasa, MnP and LiP. These enzymes catalyze the oxidation of lignin, but its non-specific nature allows the degradation of xenobiotic compounds with a chemical structure similar to lignin (Dominguez et al., 2010). *T. versicolor* (L.:Fr.) Pilát is a basidiomycete that produces extracellular enzymes that participate in the degradation of lignin in a nonspecific way (García and Torres, 2003) and has the possibility of using it in a broad spectrum of recalcitrant substances that show structural similarities with lignin. However, Katharaj (2017) reports that MnP degrades the lignin mainly by attacking phenolic lignin component. In the presence of H_2O_2, this enzyme oxidizes the phenolic structures by converting Mn^{2+} to Mn^{3+}. Oxalato and malonate are the mediators that produce carbon centered radicals, peroxyl radicals and superoxide radicals which improve the effective lignin-degradings system.

MnP is an essential component to certain basidiomycetes and some wood decaying white-rot fungi, which secrete MnP in several forms into their environment. Laccases are the copper containing polyphenol oxidases which enable degradation of phenolic compounds and also reduce molecular oxygen to wáter (Arora et al., 2010; Divya et al., 2013). Laccases oxidize the phenolic units in lignin to phenoxy radicals, which can lead to aryl-C cleavage (Kawai et al., 1988). Laccase can also oxidize non-phenolic substrates in the presence of certain auxiliary substrates (Call and Muncke, 1997; Kantharaj et al., 2017). Compounds such as chloro-phenols, polychlorinated biphenyls (PCBs), DDTs, dioxins, polycyclic aromatic hydrocarbons (PAH's), alkyl halides, nitrotoluenes, azo dyes and polymers can be modified or degraded to varying extents (Linn et al., 1993; García, 2001; Raimbault, 1998; Gold and Allic, 1993; Karam and Nicell, 1997; Kuhad et al., 1997; Majcherezyk et al., 1998); in addition, 2,4-D amine and atrazine can be efficiently degraded by this species as shown in this investigation.

The enzymes produced by the fungi *T. versicolor* was also employed for the detoxification of aromatic pollutants like agrochemicals and industrial effuents (Kantharaj et al., 2017). In recent years, the presence of micropollutants such as pharmaceuticals, industrial chemicals, personal care products and many other chemical compounds in the aquatic enviroment have become a significant problem worldwide (Doruk et al., 2018). Sahadevan et al. (2016) reported that lignin-degrading enzymes, LiP, MnP and laccase can be used like appropriate biological substitute to treat highly alkaline effluents like pulp, paper industry and waste water and various non-steroidal, anti-

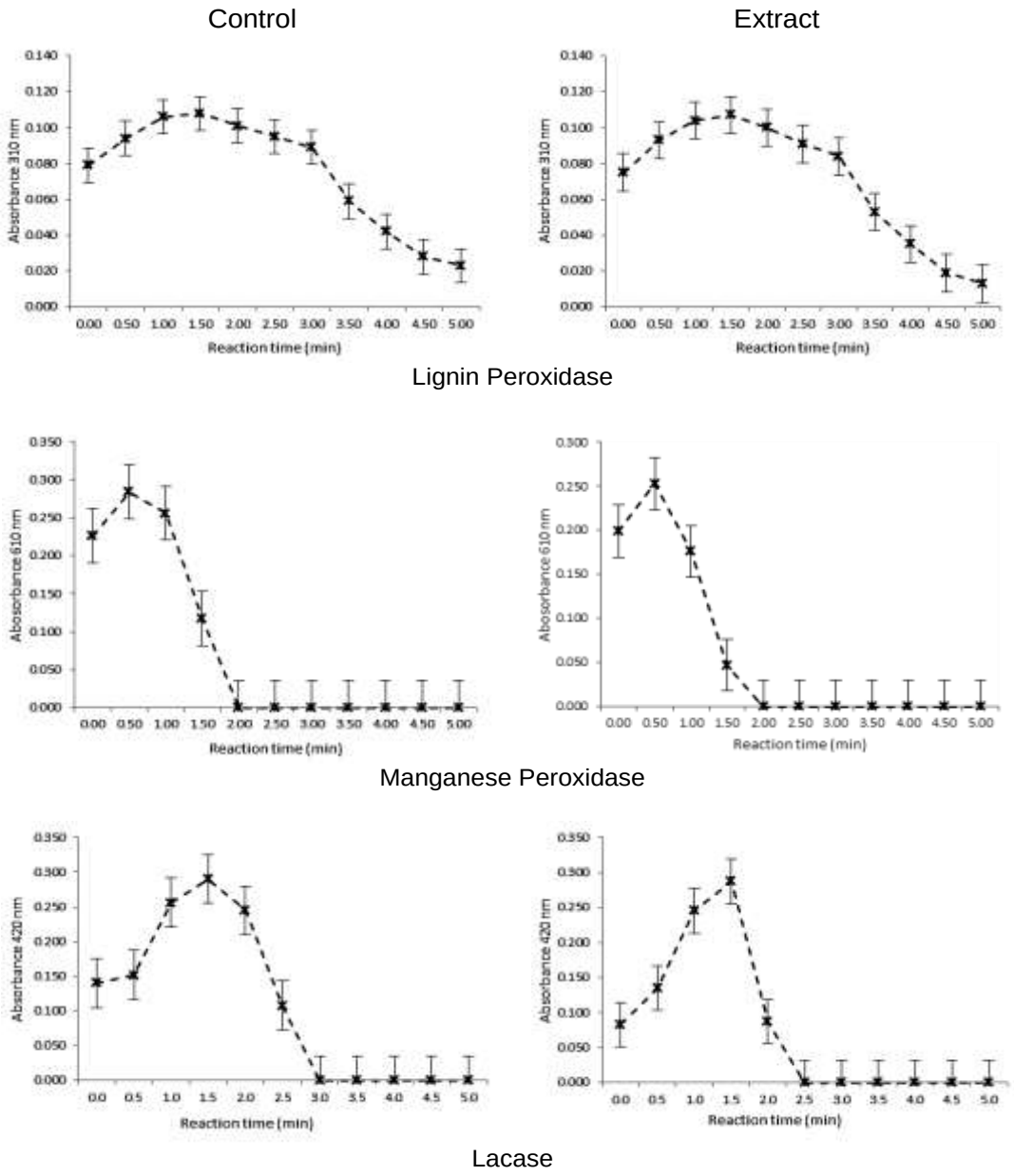

Figure 5. Average absorbance for each of the enzymes detected in the *Trametes versicolor* (L.:Fr.) Pilát strain Mo008 extract developed in the bioreactor filters exposed to analytical 2,4-D, 2,4-D amine, 2,4-D amine plus atrazine and atrazine.

inflammatory drugs such as naproxen, ketoprofen and ibuprofen (Marco et al., 2009; Marco et al., 2010a, 2010b).

Conclusions

T. versicolor (Natural strain Mo008) efficiently degraded atrazine and 2,4-D, being more efficient in the degradation of 1000 ppm of atrazine (550 h) than of 2,4-D (850 h); however, the mixture of both herbicides was consumed in a time of 650 h. Likewise, in the biodegradation of both, alone and as a mixture, the studied strain presented the activity of the enzymatic complex which was composed of laccase, LiP and MnP which have an important role in the degradation of phenolic compounds and other recalcitrants wastes due to their similiar structure to lignin. *T. versicolor* can be employed as a bioremediation tool for water contaminated with acid 2,4-D and atrazine and others phenolics compounds as well as pharmaceuticals wastes.

ACKNOWLEDGEMENTS

The authors thank the National Council of Science and Technology of Mexico (CONACyT), Department of Agricultural Sciences of the Autonomous University of Morelos State and Antonio Segura Miranda, Researcher of the Department of Agricultural Parasitology of the Autonomous University of Chapingo for the facilities granted to carry out the present research.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests in this research and its publication.

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