Optimized Atrazine Degradation by *Pleurotus ostreatus* INCQS 40310: an Alternative for Impact Reduction of Herbicides Used in Sugarcane Crops

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Abstract

The herbicide atrazine (2-chloro-4-ethylamine-6-isopropylamine-s-triazine) is extensively used for weed control in sugarcane crops. The application of fungi for the biodegradation of xenobiotics has been studied with promising results. Therefore, atrazine degradation mediated by *Pleurotus ostreatus* INCQS 40310 was evaluated, and the involvement of ligninolytic enzymes along with the degradation process was also investigated. To promote high degradation percentages and rates, a fractional factorial experimental design was first used to determine the most significant medium components for atrazine degradation. This strategy improved atrazine degradation from 39.0% to 71.0% after 15 days, with the formation of different metabolites. Afterward, a 3^2 full factorial design was performed using the variables selected in the first part of this study. The salts $FeSO_4$ and $MnSO_4$ showed significant influence in the percentages and the rates of atrazine degradation. The medium optimization resulted in 90.3% and 94.5% of atrazine degradation after 10 days and 15 days, respectively. Although laccase activity was measured during the degradation process, it was not possible to correlate laccase activity with atrazine degradation. The results demonstrated the efficiency of *P. ostreatus* INCQS 40310 for atrazine degradation, thus demonstrating the potential of this fungus as a bioremediation agent.

Keywords: *Pleurotus ostreatus*; Atrazine; Herbicide degradation; Experimental design; Sugarcane herbicides

Introduction

Pesticides have been extensively applied in agriculture over the past 30 years, especially in Brazil, which has become one of the largest consumers of these xenobiotics, followed by Japan and the United States [1]. According to recent data and including formulated products [2], 725,000 tons of pesticides were commercialized in Brazil in 2009, 59% of which corresponded to herbicides (429,693 tons), followed by 21% insecticides and acaricides (150,189 tons), 12% fungicides (89,889 tons) and 8% categorized as other (55,806 tons).

In Brazil, the 2.9% expansion of sugarcane-cultivated areas from the 2011/2012 harvest to the 2012/2013 harvest [3] was accompanied by an increase in the consumption of specific inputs for the crop. This expansion has been motivated by a growing demand for ethanol, a fuel that plays a significant role in the Brazilian energy matrix.

As reported by de Armas et al. [4], herbicides comprise the most widely used class of pesticides in the cultivation of sugarcane. Moreover, 45% of the total world's production of herbicides corresponds to the triazine class [5]. The most often used triazine-class herbicide is atrazine (2-chloro-4-ethylamine-6-isopropylamine-s-triazine), which is generally applied to control weeds during pre- or post-emergence, especially in sugarcane-, corn- and soybean-cultivated areas [5]. The chemical structure of atrazine is represented by a triazine ring that has been substituted with chlorine, ethylamine and isopropylamine, the combination of which makes it recalcitrant to biological degradation in the environment [6].

Atrazine has low solubility in water (33 mg L^{-1} at 27°C). However, it is soluble in many organic solvents (360 to 183,000 mg L^{-1}), such as acetonitrile and methanol [7]. Due to its low partition coefficient (octanol-water), it is not significantly adsorbed by soil. Hence, it is often found above the tolerable levels recommended by environmental agencies in surface water and groundwater [4]. The United States Environmental Protection Agency and the World Health Organization established limits of 2 μg/L and 3 μg/L of atrazine in drinking water, respectively [8]. The degradation and mineralization of atrazine can occur through physical-chemical processes or by microorganism action. Previous studies reported in the literature have identified more than 15 metabolites resulting from atrazine degradation [9-13]. The main atrazine degradation products are hydroxylated and chlorinated compounds, including desethylatrazine (2-chloro-4-amino-6-isopropylamine-s-triazine, DEA), desisopropylatrazine (2-chloro-4-ethylamine-6-amino-s-triazine, DIA), desethyldesisopropylatrazine (2-chloro-4,6-amino-s-triazine, DEDIA), desethylhydroxyatrazine (2-hydroxy-4-amino-6-isopropylamine-s-triazine, DEHA), desisopropylhydroxyatrazine (2-hydroxy-4-ethylamine-6-amino-s-triazine, DIHA) and hydroxyatrazine (2-hydroxy-4-ethylamine-6-isopropylamine-s-triazine, HA).

Bioremediation explores the genetic diversity and metabolic versatility of microorganisms and has been a very promising approach to convert environmental contaminants into less toxic products that

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can be integrated into natural biogeochemical cycles [14]. Within the soil, the degradation of atrazine by fungi usually follows pathways that involve the sequential removal of the aromatic ring substituents, beginning with dealkylation, which is the first step in the degradation of this compound [12,15]. The application of fungi for the biodegradation of recalcitrant chemicals has been widely studied [16-20]. This interest is based on the fungal capacity to degrade several molecules of organic pollutants and other persistent toxic substances using nonspecific enzymes [21-26]. Basidiomycetes, including the genus *Pleurotus*, can degrade lignocellulosic materials through extracellular ligninolytic enzymes, such as laccase, manganese peroxidase and lignin peroxidase. The oxidative activity and the low specificity of this enzymatic pool are generally responsible for the degradation of recalcitrant organic pollutants [27], which makes these enzymes important catalysts for environmental applications. In addition, previous studies with the white-rot fungus *Phanerochaete chrysosporium* also indicated the degradation of atrazine by P450 enzymes [15,28].

The purpose of this work was to apply an experimental design as a tool to improve atrazine degradation that is mediated by the well-known fungus *Pleurotus ostreatus*. This optimization strategy of promoting the increase of atrazine degradation aims to present a promising alternative for the degradation of sugarcane herbicides.

Materials and Methods

Fungus and culture media

The fungus *P. ostreatus* INCQS 40310 was kindly provided by the Federal University of Lavras and deposited in the Collection of Reference Microorganisms in Health Surveillance (Fiocruz-CMRVS). *P. ostreatus* INCQS 40310 was grown on potato dextrose agar (PDA) medium for 7 days and preserved at 4°C prior to freezing in glycerol at -70°C. The fungus *P. ostreatus* INCQS 40310 was selected from a previous study on atrazine tolerance [29].

For the initial atrazine degradation experiments, a basic nutrient medium at pH 6.0 was used, as previously described by Gorbatova et al. [30].

Atrazine degradation experiments

P. ostreatus INCQS 40310 was inoculated into PDA. After inoculation, the agar plates were incubated at 28°C. After 7 days of incubation, 3 mycelial plugs (5 mm diameter) from the colony margin were used as an inoculum. The mycelial plugs were transferred to conical flasks containing 300 mL of basic nutrient medium and atrazine at a final concentration of 10 mg L^{-1}. The degradation studies were performed in triplicate; therefore, 3 flasks for each condition studied were incubated while agitating at 200 rpm for 20 days at 28°C ± 2°C. Samples of 3 mL were collected from each flask every 5 days of incubation and extracted on Waters Oasis® MCX cartridges (a cation-exchange resin extraction). Atrazine and its derivatives were analyzed by high-performance liquid chromatography (HPLC). Control experiments were performed under the same conditions in the absence of the *P. ostreatus* INCQS 40310 or atrazine, as described above.

High-performance liquid chromatography analyses

After solid-phase extraction, the extracts were analyzed by HPLC using a Shimadzu LC-10AT chromatograph that was equipped with a UV-Vis detector, which was monitored at 221 nm and 230 nm. The flow was kept at 1 mL/min, the auto-injection volume was 20.0 μL and a Class VP 6.1 program was used for data acquisition and system control. The HPLC column used was a Shim-Pack C18 (250×4.6 mm, 4.6 μm), and the mobile phases were composed of ACN and phosphate buffer, pH 7.2, using a segmented gradient elution [29]. The atrazine degradation and product formation were analyzed by HPLC under the same conditions, as previously described. The standards of A (purity 98.4% w/w) and its derivatives, DIA (purity 98.0% w/w), DEA (purity 98.5% w/w), HA (purity 96.0% w/w) and DEDIA (purity 95.7% w/w); were obtained from Dr. Ehrenstorfer GmbH company. DEHA (purity 98.7% w/w) and DIHA (purity 98.0% w/w) were obtained from Riedel Company-de-Haën. All solutions were stored in a freezer.

Maximizing the degradation of atrazine through experimental design

To attain the optimum culture medium composition for the atrazine degradation, a factorial experimental design was used to evaluate the significance of each of the components. Physical variables, such as agitation speed, temperature, pH and incubation time, were not considered for the statistical design analysis. These conditions were selected from previous studies reported in the literature, as culture medium components have been demonstrated by Teixeira and co-workers to be more relevant than physical variables [20].

It is important to study the actual effects of each compound in the medium and to also minimize the amount of each component to reduce costs. The 2_{IV}^{8-4} fractional factorial design was initially employed to investigate the main effects and their interactions with the factors on the percentage of atrazine degradation. In the first stage of the study, the concentration of salts was equal to zero. The following independent variables were tested: (X_1) zinc sulfate, (X_2) iron sulfate, (X_3) manganese sulfate, (X_4) magnesium sulfate, (X_5) copper sulfate, (X_6) glucose, (X_7) peptone, and (X_8) yeast extract. All these factors were evaluated at two levels, low (−1) and high (+1), which were combined according to a 2_{IV}^{8-4} fractional factorial design (resolution four), with 4 repetitions at the central point (0) and generating relationships I=1235, I=1246, I=1347 and I=2348. Subsequently, the 3^2 full factorial design was used for the optimization of the microbial process for atrazine degradation. The monitored responses were the degradation of atrazine (expressed as percentage) and laccase enzyme activity (expressed in UI mL^{-1}). Based on factorial design, samples were collected at 5, 10, 15 and 20 days of incubation and processed as described above during the experiments of atrazine degradation.

Software

In the present study, the software Statistica 7.0 (StatSoft, Tulsa, OK, USA) was used for the design of the experiment and analysis of the obtained data.

Determination of enzymatic activities

The presence and involvement of ligninolytic enzymes produced by *P. ostreatus* INCQS 40310 was also investigated. Laccase activity was determined spectrophotometrically according to the method of Niku-Paavola et al. [31] with slight modifications by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-ABTS at room temperature without agitation. The assay mixture contained 3 mM ABTS, 0.2 M sodium succinate buffer (pH 4.5) and 100 μL of reaction medium in a total volume of 2 mL. The oxidation of ABTS was monitored at 420 nm (ε=36,000 M^{-1} cm^{-1}).

Manganese peroxidase activity was determined spectrophotometrically according to the oxidation of phenol red in the presence of manganese and hydrogen peroxide [32]. The assay mixture

contained 0.25 mM sodium lactate, 0.5% bovine albumin, 2 mM $MnSO_4$, 500 μL of the reaction medium, 0.1% phenol red and 2 mM H_2O_2. The oxidation of phenol red was monitored at 610 nm (ε=44,600 $M^{-1} cm^{-1}$).

Lignin peroxidase activity was determined spectrophotometrically according to the oxidation of veratryl alcohol into veratryl aldehyde [33]. The assay mixture contained 50 mM sodium tartrate buffer, 50 mM veratryl alcohol, 1800 μL of the reaction medium and 10 mM H_2O_2. The oxidation of veratryl alcohol was monitored at 310 nm (ε=9,200 $M^{-1} cm^{-1}$).

Results and Discussion

Optimization of atrazine degradation through factorial design

Twenty assays were performed with different concentrations of each component of the culture medium to determine optimal conditions for the maximum degradation of atrazine, according to the data shown in table 1. The changes in the concentrations of the components in the culture medium promoted an increase in the percentage of atrazine degradation and the diversification of the metabolites formed.

Different results were obtained according to the compositions of the culture medium, revealing that the highest percentage of atrazine degradation occurred in assay 12 in which 71% of atrazine was transformed after 15 days. Moreover, after 10 days of cultivation, less than 50% of the atrazine had been consumed. It should also be noted that the maximum atrazine degradation was 39.0% prior to the factorial design [29].

The use of this statistical tool proved to be very efficient because it increased atrazine degradation by 1.8 times and diversified the metabolites that were formed. In addition to DEA and DIA, the metabolites DIHA, DEDIA and DEHA were also detected (Table 2).

According to the literature, atrazine is more toxic than its degradation products [34]. For example, atrazine presented higher chronic toxicity when compared to DEA towards rat endocrine activity. The degradation product DEDIA was also less toxic than s-triazine (the ring system of triazine) towards birds. For phototrophic microorganisms, the decreasing order of toxicity was: atrazine>DEA>DIA. Additionally, the degradation products HA and DEDIA were nontoxic towards most phototrophic microorganism cultures tested [35]. The herbicide atrazine was partly converted into polar dechlorinated and/or N-dealkylated metabolites. These results agreed with previous studies described in the literature [15] in which the dealkylated products appeared as the major metabolites during the microbial degradation of chloro-s-triazines. In general, soil fungi remove the ethyl group of atrazine before the removal of the isopropyl group [36,37].

Table 2 presents the most relevant results obtained from the fractional factorial design (2_{IV}^{8-4}), regarding not only the optimal percentage of degradation (4,11,12) but also the results that showed minimum medium composition (1) and unidentified metabolites (13,16). Another interesting result was obtained from assay 4, which promoted 60% atrazine degradation after 20 days with the formation of DIHA, DEDIA, DIA, HA and DEA.

Even though the atrazine degradation in assay 1 achieved only 38% after 10 days, this result should be emphasized because the culture

Assays	Contrast coefficients								Atrazine degradation (%)			
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	5 days	10 days	15 days	20 days
1	-1	-1	-1	-1	-1	-1	-1	-1	8.5	38.5	38.8	33.5
2	1	-1	-1	-1	1	1	1	-1	11.6	21.5	16.0	21.5
3	-1	1	-1	-1	1	1	-1	1	15.5	23.2	22.2	23.7
4	1	1	-1	-1	-1	-1	1	1	22.6	31.7	49.7	59.7
5	-1	-1	1	-1	1	-1	1	1	23.2	24.4	43.3	49.4
6	1	-1	1	-1	-1	1	-1	1	19.4	23.3	21.0	33.1
7	-1	1	1	-1	-1	1	1	-1	32.5	34.6	35.7	31.5
8	1	1	1	-1	1	-1	-1	-1	22.0	38.2	39.8	28.9
9	-1	-1	-1	1	-1	1	1	1	6.8	16.3	18.7	18.7
10	1	-1	-1	1	1	-1	-1	1	14.3	19.0	36.1	37.1
11	-1	1	-1	1	1	-1	1	-1	23.6	36.5	52.1	63.3
12	**1**	**1**	**-1**	**1**	**-1**	**1**	**-1**	**-1**	**4.9**	**52.5**	**71.0**	**71.0**
13	-1	-1	1	1	1	1	-1	-1	21.4	25.1	34.9	35.7
14	1	-1	1	1	-1	-1	1	-1	5.1	5.7	8.9	6.3
15	-1	1	1	1	-1	-1	-1	1	24.0	26.6	33.0	32.2
16	1	1	1	1	1	1	1	1	15.4	18.2	22.9	23.9
17	0	0	0	0	0	0	0	0	15.0	19.9	20.1	26.0
18	0	0	0	0	0	0	0	0	21.6	24.9	25.9	38.5
19	0	0	0	0	0	0	0	0	23.5	26.9	28.1	23.6
20	0	0	0	0	0	0	0	0	16.0	19.6	18.1	24.8

X_1 – $ZnSO_4$ (g L^{-1}): (-1): 0.0, (0): 0.001, (+1): 0.002
X_2 - $FeSO_4$: (g L^{-1}) (-1): 0.0, (0): 0.0005, (+1): 0.001
X_3 - $MnSO_4$ (g L^{-1}): (-1): 0.0, (0): 0.05, (+1): 0.1
X_4 - $MgSO_4$ (g L^{-1}): (-1): 0.0, (0): 0.5, (+1): 1.0
X_5 - $CuSO_4$ (g L^{-1}): (-1): 0.0, (0): 0.25, (+1): 0.5
X_6 – glucose (g L^{-1}): (-1): 2.0, (0): 5.0, (+1): 8.0
X_7 – peptone (g L^{-1}): (-1): 1.0, (0): 3.0, (+1): 5.0
X_8 – yeast extract (g L^{-1}): (-1): 2.0, (0): 5.0, (+1): 8.0

Table 1: Fractional factional design 2_{IV}^{8-4} with encoded variables and atrazine degradation.

medium from this assay contained the poorest medium composition established in the factorial design, level-1, as described in table 1. Therefore, considering the lowest concentration of constituents and also the absence of all salts, it is noteworthy that conditions in assay 1 simulated the low-cost medium composition in this study.

The results obtained after the fractional factorial design (2_{IV}^{8-4}) presented certain advantages, as mentioned above, including an increased level of degradation, medium composition reduction and diversification of the degradation products.

The first part of this study revealed the significance of the variables analyzed. Considering the results obtained from the 2_{IV}^{8-4} fractional factorial design (Table 1), only the variables X_2 (FeSO$_4$) and X_3 (MnSO$_4$) were significant within the 95% confidence interval for atrazine degradation (%) during the first 15 days. The other variables were not significant within the intervals investigated, according to the Pareto chart (data not shown). Regarding the Pareto chart, the effect bars that crossed the red dashed line were significant within the 95% interval. Additionally, FeSO$_4$ exhibited a positive significant effect, while MnSO$_4$ had a negative effect. The other variables were not significant within the concentration interval investigated. Thus, considering the results obtained from the 2_{IV}^{8-4} fractional factorial design and to improve the atrazine degradation, the concentration of no significant variables were maintained at low levels (-1), i.e., ZnSO$_4$=0 g L^{-1}, MgSO$_4$=0 g L^{-1}, CuSO$_4$=0 g L^{-1}, glucose=2 g L^{-1}, peptone=1 g L^{-1} and yeast extract=2 g L^{-1}. A 3^2 full factorial design with triplicate at the central point was then performed, considering just the variables FeSO$_4$ and MnSO$_4$. The studied concentration range of FeSO$_4$ was increased, while MnSO$_4$ was decreased based on the 2_{IV}^{8-4} fractional factorial design results, as shown in table 3.

Presented in table 3, the results obtained from the 3^2 full factorial design showed that the percentages of atrazine degradation were only as high as 4.1% among the 11 experiments performed after 5 days of incubation. However, after 10 and 15 days, the results were significant. Experiments 3, 8, 9, 10 and 11 were very similar for the period of 10 and 15 days, presenting atrazine degradation percentages of approximately 80%. Less favorable conditions were observed for atrazine degradation after 10 and 15 days when the concentrations of FeSO$_4$ and MnSO$_4$ were simultaneously maintained at the lowest level (77%, assay 1). However, the best results obtained simultaneously for the period of 10 (90.3%) and 15 days (94.5%) were observed when FeSO$_4$ and MnSO$_4$ were fixed at low and high levels, respectively (assay 7). Therefore, assay 7 showed the best results from this optimization study. In addition to maximizing the atrazine degradation level, which reached 90.3% within 10 days of incubation, it also allowed a 5 day reduction in incubation time. Very good results were also obtained with assays 5, 6 and 7 in which the atrazine degradation was greater than 90% after 15 days. It should also be noted that all the assays showed good percentages of atrazine degradation, which varied from 77% to 95% after 15 days of incubation. The period of 15 days presented the best set of results for the percentage of atrazine degradation. As a matter of fact, the 15 day period did not show a lack of fit for the model:

$$y' = 88.94(\pm\ 2.20) + 0.12(\pm\ 1.74)\ X1 + 2.77(\pm\ 1.74)\ X2 - 1.02(\pm\ 2.70)\ X12 - 2.40\ (\pm\ 2.70)\ X22 - 4.16(\pm\ 2.13)\ X1X2$$

within a 95% confidence interval, as the lack of fit test returned F$_{calculated}$ (mean square $_{lack\ of\ fit}$ / mean Square $_{pure\ error}$)=1.03, which was less than F$_{critical, n1:3, n2:3, a:0.05}$ 19.16. This result was corroborated by the corresponding response surface plot, which describes the atrazine degradation percentage for the system studied. It is important to

highlight that no model coefficients were significant for α equal to 0.05 (Figure 1).

The sequential strategy enabled the selection of a culture medium that was capable of promoting high atrazine degradation (90.3% and 94.5%) after 10 and 15 days, respectively. Table 4 clearly shows that, in addition to DEA and DIA, which were previously produced, DIHA, DEDIA and DEHA were also obtained. This result revealed the

Assays	Degradation (%)	Time (days)	Metabolites Formed
1	38.8	10	DIA, DEA
4	59.7	20	DIHA, DEDIA, DIA, HA, DEA
11	63.3	20	DIA
12	**71.0**	**15**	**DIHA, DEDIA, DEHA, DIA, DEA**
13	35.7	15	DIA, HA, DEA, U.M.*
16	24.8	15	DEHA, DEA, U.M.*

*U.M. – unidentified metabolites

Table 2: Percentage of atrazine degradation and its metabolites formed for the most relevant results after the fractional factorial design (2_{IV}^{8-4}). The best result, assay 12, is highlighted.

Assays	Contrast coefficients X$_1$	X$_2$	Atrazine degradation (%)		
			5 days	10 days	15 days
1	-1	-1	3.2	46.9	77.1
2	0	-1	4.1	48.5	88.4
3	1	-1	2.2	82.5	83.7
4	-1	0	2.9	48.7	86.9
5	0	0	3.6	82.6	93.0
6	1	0	2.4	72.7	91.1
7	**-1**	**1**	**3.9**	**90.3**	**94.5**
8	0	1	2.6	84.4	86.9
9	1	1	2.9	83.6	84.5
10	0	0	3.8	85.8	86.9
11	0	0	4.1	80.5	84.8

X$_1$- FeSO$_4$ (g L^{-1}):(-1):0.001; (0): 0.0015; (+1): 0.002
X$_2$- MnSO$_4$ (g L^{-1}): (-1):0.0 (0): 0.025; (+1): 0.05

Table 3: Experimental design (3^2) with encoded variables and atrazine degradation.
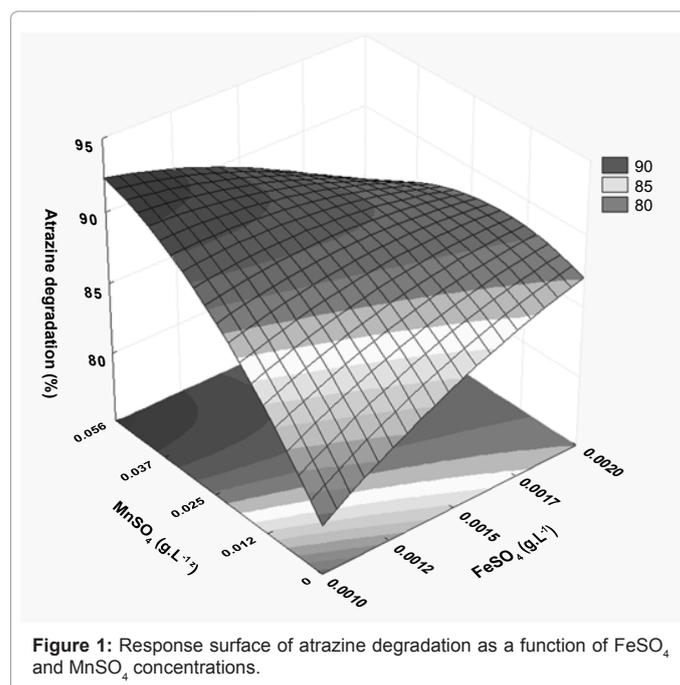

Figure 1: Response surface of atrazine degradation as a function of FeSO$_4$ and MnSO$_4$ concentrations.

relevance of the adopted strategy, as it allowed the diversification of the metabolites and a reduction in both the number and the concentrations of the components of the culture medium.

Compared to previous studies [15,28,38,39], the results obtained in the two stages of the experimental design (assay 12 and assay 7) of the current investigation were far superior (Table 5).

Determination of laccase activities

In the present study, the correlation between the ligninolytic enzymes and the atrazine degradation was also evaluated. The fungus *P. ostreatus* is widely known for its capacity to secrete several ligninolytic enzymes [40]. Laccase, lignin peroxidase and manganese peroxidase were evaluated during this optimization study; however, only very low laccase activity was determined, as LiP and MnP activities were not detected. Moreover, the laccase activity pattern revealed very similar behaviors both in the presence and absence of atrazine, which indicated that atrazine did not act as an inducer of the laccase production (data not shown).

Figure 2 shows the activity of laccase over 20 days in the 2_{IV}^{8-4} fractional factorial design, and Figure 3 shows the activity of laccase obtained from the 3^2 full factorial design.

From the fractional factorial design (Figure 2), the highest laccase activity was obtained in assay 11 (1.4 UI mL^{-1}), which exhibited 63% atrazine degradation after 20 days, while the highest percentage of atrazine degradation was found in assay 12 (71% after 15 days) (Table

2). Although it was not possible to correlate the laccase activity with the atrazine degradation, the medium from assay 11 tripled the production of laccase when compared with the results obtained before the experimental design (0.533 UI mL^{-1}). Assay 12, which showed the highest level of atrazine degradation, presented a non-significant laccase activity of 0.174 UI mL^{-1} after 10 days (Figure 2). Similar results were found in the 3^2 full factorial design, indicating low activity of laccase in assay 7 (highest percentage of atrazine degradation).

The literature also reported studies from which there was no correlation between pesticide degradation and ligninolytic enzymes, corroborating the present results. Hiratsuka et al. [41] demonstrated that *Trametes versicolor* IFO 30340 was able to degrade a series of diphenyl ether herbicides without the involvement of ligninolytic enzymes. However, intracellular enzymes, such as cytochrome P450 monooxygenases, were responsible for mediating the initial N-dealkylation of the herbicide. The involvement of the P450 enzymes in atrazine degradation was also studied in the presence of the white-rot fungus *P. ostreatus* [15,28].

Bastos and Magan [42] studied the degradation of the herbicide atrazine in soil by the fungus *Trametes versi color* over 24 weeks and found that the enzyme laccase played an insignificant role in the degradation process, which corroborates the findings of the current investigation.

The aforementioned results and the previous studies reported in the literature support the choice of *P. ostreatus* INCQS 40310 for

Conditions studied	Culture medium Composition (g L^{-1})		Pergentage of degradation after 10 days	Pergentage of degradation after 15 days
Initial Condition [30]	ZnSO$_4$ FeSO$_4$ MnSO$_4$ MgSO$_4$ CuSO$_4$ Glucose Peptone Yeast Extract	(0.001) (0.0005) (0.05) (0.5) (0.25) (5.0) (3.0) (5.0)	22.8	**39.0**
Fractional factorial design (2_{IV}^{8-4}) (Best condition – assay 12)	ZnSO$_4$ FeSO$_4$ MgSO$_4$ Glucose Peptone Yeast Extractt	(0.002) (0.001) (1.0) (8.0) (1.0) (2.0)	52.5	**71.0**
Full experimental design (3^2) (Best condition – assay 7)	FeSO$_4$ MnSO$_4$ Glucose Peptone Yeast Extract	(0.001) (0.05) (2.0) (1.0) (2.0)	**90.3**	**94.5**

Table 4: Comparative analyses of culture media composition before and after the experimental design. The best results of atrazine degradation were also given.

Fungi	Time (days)	Concentration	Metabolites formed	Degradation (%)	Reference
Phanerochaete crhysosporium BKM-F-1767	16	2 µm L^{-1}	HA, DEA, DEHA and DIA	48	[15]
Pleurotus ostreatus	42	10 mg L^{-1}	U.M.*	15,5	[34]
Pleurotus pulmonaris	25	20 mg L^{-1}	DEA, DIA, DEDIA and 2-chloro-4-ethylamino-6-(1-hydroxyisopropyl) amino-1,3,5 triazine	50	[28]
Lentinula edodes	30	10 µg mL^{-1}	U.M.*	26	[35]
Agaricus subrufescens	30	10 µg mL^{-1}	U.M.*	35	[35]
P. ostreatus INCQS 40310	**15**	10 mg L^{-1}	**DEA and DIA**	**39**	**Present study; before factorial design**
P. ostreatus INCQS 40310	**15**	10 mg L^{-1}	**DIHA, DEDIA, DEHA, DIA and DEA**	**71**	**Present study; fractional factorial design (2^{8-4})**
P. ostreatus INCQS 40310	**10**	10 mg L^{-1}	**DIHA, DEDIA, DEHA, DIA and DEA**	**90**	**Present study; full factorial design (3^2)**

Table 5: Studies on atrazine degradation mediated by fungi. Comparative description of experimental conditions and the metabolites obtained.
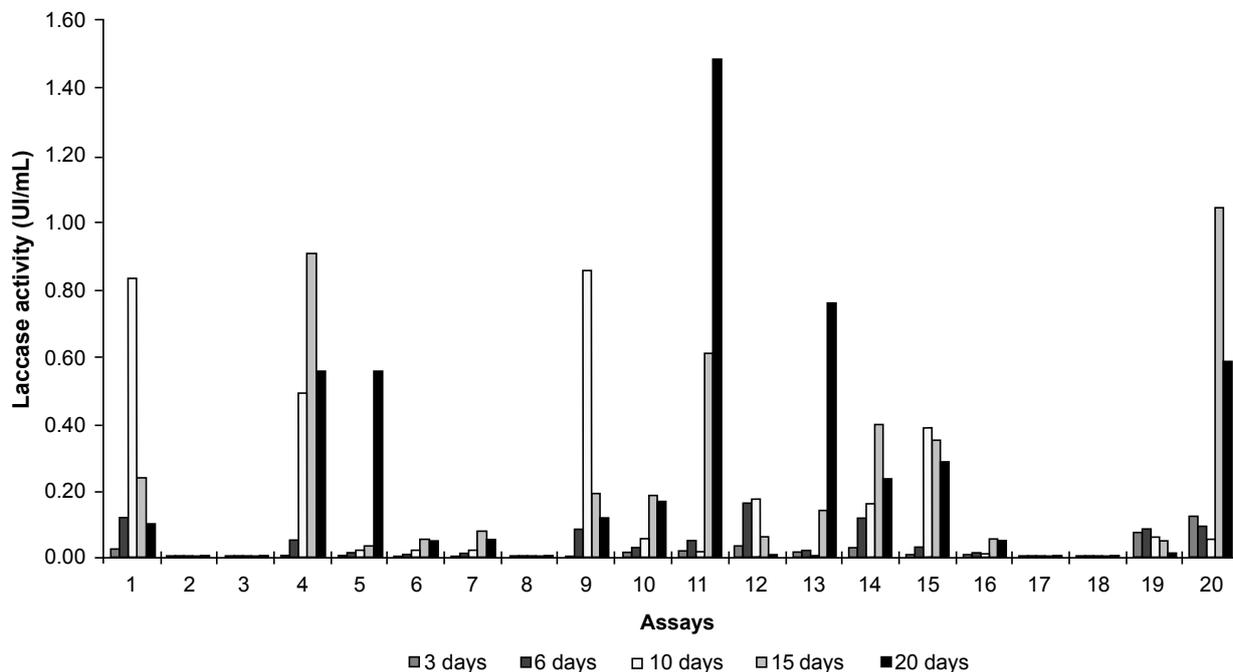
Figure 2: Laccase activity in all media studied in the 2_{IV}^{8-4} fractional factorial design (3, 6, 10, 15 and 20 days).
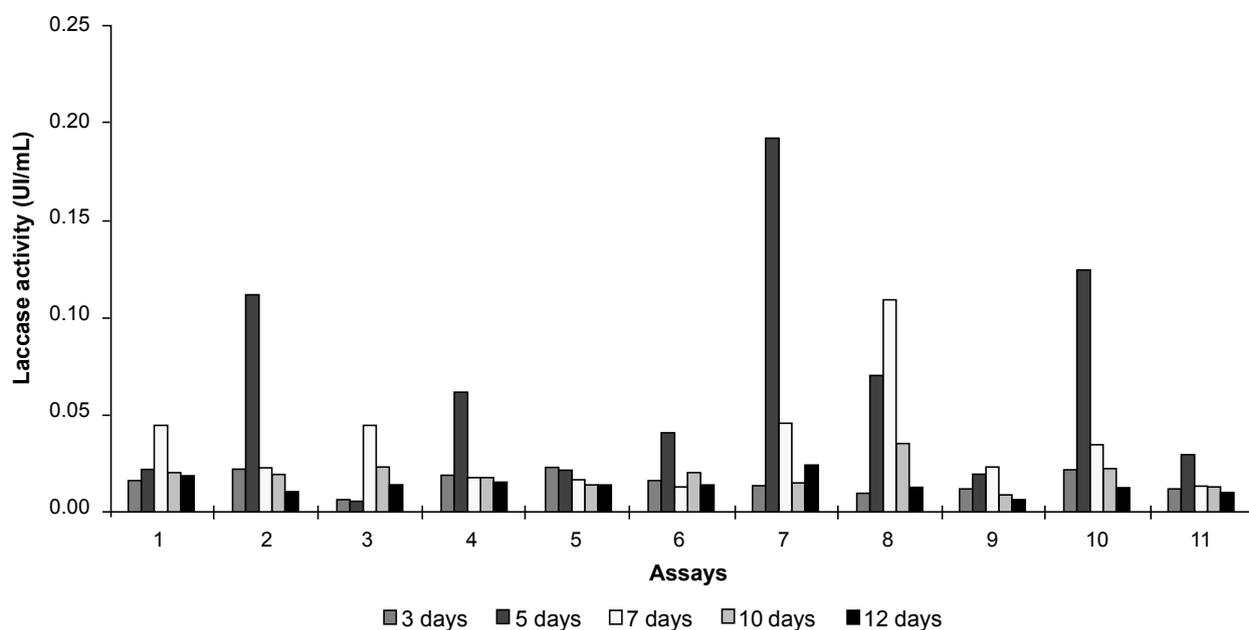

Figure 3: Laccase activity in all media studied in the 3^2 full factorial design (3, 5, 7, 10 and 12 days).

the degradation of atrazine. Indeed, the significance of the Fe_2SO_4 in the culture medium revealed during this study that the degradation process could be directly related to cytochrome P450 enzymes, which are well-known hemeproteins.

Conclusions

The optimization strategy enabled the selection of a culture medium capable of promoting high atrazine degradation, 90.3% and 94.5%, after 10 and 15 days, respectively, which increased atrazine degradation by a factor of 2.5 (39.0% to 94.5%). Our results agreed with previous studies described in the literature in which dealkylated products appeared as the major metabolites produced during the initial mechanism of microbial degradation of chloro-s-triazines.

Although *P. ostreatus* INCQS 40310 was able to degrade atrazine

and laccase was produced under certain conditions, it was not possible to correlate the production of this enzyme with atrazine degradation. Additionally, the significant presence of Mn in the optimized medium was not related to manganese peroxidase activity.

The results obtained in the optimization of the culture medium (salts of Fe and Mn as significant variables) suggested the involvement of other enzymes for atrazine degradation, such as P450 enzymes. The relationship between the involvement of extracellular/intracellular enzymes during atrazine degradation is now under investigation. Further work is necessary to clarify this question and the biochemistry of atrazine degradation by *P. ostreatus* INCQS 40310.

The present investigation demonstrates the high potential of the filamentous fungus *P. ostreatus* INCQS 40310 as a bioremediation agent. It is also important to mention that the use of factorial design was crucial for the improvement of the degradation levels.

Acknowledgments

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