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Research Article

Optimization of C/N Ratio and Inducers for Wastewater Paper Industry Treatment Using *Trametes versicolor* Immobilized in Bubble Column Reactor

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C/N ratio and $MnSO_4$ and $CuSO_4$ concentrations were optimized for decolorization and chemical oxygen demand (COD) removal of bleached Kraft pulp mill effluent by *Trametes versicolor* immobilized in polyurethane foam. Statistical differences ($P < 0.0001$) at high C/N ratios (169), 2 mM $CuSO_4$, and 0.071 mM $MnSO_4$ were determined. Decolorization of 60.5%, COD removal of 55%, laccase (LAC) 60 U/L, and manganese peroxidase (MnP) 8.4 U/L were obtained. Maximum of decolorization (82%), COD removal (83%), LAC (443.5 U/L), and MnP (18 U/L) activities at C/N ratio of 405 (6.75 mM $CuSO_4$ and 0.22 mM $MnSO_4$) was achieved in step 7 at 4 d. Positive correlation between the decolorization, COD removal, and enzymatic activity was found ($P < 0.0001$). *T. versicolor* bioremediation capacity was evaluated in bubble column reactor during 8 d. Effluent was adjusted according to optimized parameters and treated at 25°C and air flow of 800 mL/min. Heterotrophic bacteria growth was not inhibited by fungus. After 4 d, 82% of COD reduction and 80% decolorization were recorded. Additionally, enzymatic activity of LAC (345 U/L) and MnP (78 U/L) was observed. The COD reduction and decolorization correlated positively ($P < 0.0001$) with enzymatic activity. Chlorophenol removal was 98% of pentachlorophenol (PCP), 92% of 2,4,5-trichlorophenol (2,4,5-TCP), 90% of 3,4-dichlorophenol (3,4-DCP), and 99% of 4-chlorophenols (4CP).

1. Introduction

The paper industries generate significant quantities of wastewaters requiring around 15–60 m^3 per ton of pulp produced. Wood and the sugarcane bagasse are the main raw materials for the process. Kraft pulp is commonly bleached with chlorine and its oxides, with an initial oxygen bleaching step. Of the different waste streams, bleaching plant effluents are the most toxic, due to the chlorinated organic compounds, color, and COD generated [1]. The brown color of wastewater is due to various conjugated structures including quinones, benzoquinones, complexed catechols, chalcones, and stilbenes, which absorb visible light [2, 3].

Biological treatment using white rot fungi such as *T. versicolor* involves multiple biochemical and physical reactions that can be carried out simultaneously, like the breakdown of intermolecular bonds, demethylation, hydroxylation, dechlorination, and the opening of the aromatic ring [4]. All of these transformations are developed together through the combined action of several enzymes, for example, laccase, manganese peroxidase, lignin peroxidase, xylanases, veratryl alcohol oxidase, and so forth [5, 6]. Other mechanisms are related to physicochemical interactions such as adsorption, deposition, and ion exchange [7]. Several strategies have been successfully applied to enhance the efficiency of biological treatments including immobilization of the fungus in inert supports. This system has been shown to have more potential than the free cell because it reduces the problems of viscosity, oxygen transfer, and biomass recycling [8]. Several works had been made to assay different support, natural such as

maple woodchips, jute twine, wheat straw, and stone and synthetic as polyurethane, nylon mesh reporting colonization of the support by fungi and dye decolorization [9, 10]. Other alternatives consist on the supplementation of simple carbon sources that are used as a cosubstrate and addition of the inducers such as $MnSO_4$ and $CuSO_4$ to increase the levels of enzymatic activity MnP and Laccase [10–12]. Even though there are several reports related to the effect of these compounds, however, there are few reports about the effect of C/N ratio, Mn, and Cu on manganese peroxidase and laccase induction, during effluent decolorization under nonsterilized conditions.

The objective in this study was to evaluate the effect of the C/N ratio and addition of $MnSO_4$ and $CuSO_4$, on LAC and MnP activity induction by *T. versicolor* immobilized in polyurethane foam (PUF) and to determine *T. versicolor* capacity for color, chlorophenols, and COD removal from a wastewaters of bleaching Kraft mill paper industry in bubble column reactor under nonsterilized conditions. The strategies used were a first-order design and the path of steepest ascent method for optimization of nutritional conditions in the effluents.

2. Materials and Methods

2.1. Microorganism. *T. versicolor* was used in this study. The fungus was maintained on mineral oil at 4°C. For reactivation *T. versicolor* was grown in a wheat bran extract agar plates at 25°C for 8 d [13].

2.2. Immobilization of the Fungus. Polyurethane foam (PUF) cubes of 1 cm^3 were used as the supporting matrix for immobilization of the fungus. The surface area was 450 m^2/m^3 with a density of 18 Kg/m^3. Fifty cubes with ten agar plugs (8 days old) were inoculated in 500 mL Erlenmeyer flasks containing 200 mL of wheat bran extract medium [14]. After 9 d of incubation at 25°C and 120 rpm in an orbital shaker the biomass immobilized in the support was passed in the wheat bran extract agar for 2 d at 25°C. Then the cubes with abundant white mycelium were removal of the petri dishes and these biomaterials were inoculated into the reactors. For the experiments with the dead biomass, the cubes were sterilized at 121°C for 15 min.

2.3. Scanning Electron Microscopy (SEM). Thin-sliced sections of the matrix were obtained in order to observe the mycelial growth in the PUF. The thin sections were prepared and fixed for 1 h with glutaraldehyde at 2.5% (v/v) and were then washed with 0.1 M phosphate buffer (pH 7.0) and fixed with osmium tetraoxide at 1% (v/v) for 2 h. The fixed particles were dehydrated using a series of ethanol washings with increasing ethanol concentration (70, 85, 95, and 100% v/v) for 5 min. After 24 h, the specimens were mounted on stainless steel stubs, coated immediately with gold in an ion coater, and examined using a Jeol-JSM-6300.

2.4. Effluent Characteristics. Effluent was provided by a pulp paper industry. pH of effluent was 8.5, COD was 8000 mg/L, C/N ratio (10), and color unit was 5800, 11.2 mg/L PCP, 18 mg/L 2,4,5-TCP, 12.4 mg/L 3,4-DCP, 14 mg/L 4CP, 85×10^2 CFU/mL heterotrophic bacteria, and 10×10^2 CFU/mL heterotrophic yeast. The color of the effluent was measured according to the APHA, 2005. The COD was determined by the open reflux titration method [15]. The chlorophenols were analyzed by HPLC, according to the methodology of Ríos and Calva [16]. The changes in the microbial populations in the effluent without treatment and the effluent treated with *T. versicolor* were investigated. Colony forming units per milliliter (CFU/mL) were measurement used counts of microtechnique using PDA agar and nutrient agar for yeast and bacteria. Most abundant bacterial colonies were characterized by performing Gram stain and ten isolated colonies were grown in King B agar plates for 48 h at 37°C. Then, they were examined under (366 ± 10) nm ultraviolet light for 10 s. The colonies that showed fluorescence were estimated as presumptive evidence of the presence of *Pseudomonas* spp.

2.5. Evaluation of Enzymatic Activity. The enzyme MnP (EC 1.11.1.13) was determined by the oxidation of phenol red 0.05% (w/v), in succinic buffer 20 mM, pH 4.5 [17]. LAC (EC 1.10.3.2) was determined through the oxidation of ABTS 0.5 mM in sodium acetate buffer 100 mM, pH 4.5 [18]. The enzymatic unit was defined as the formation of 1 μmoL of product per minute under the evaluated conditions.

2.6. Experimental Design

2.6.1. Factorial 2^2 with Central Points Experimental Design. Complete factorial design (replicates and with central point), which takes into account all degrees of freedom, was used. The general form of this design is 2^n, where n is the number of factors (in this case ratio C/N and inductor concentration) and 2 represents two levels of work. The levels are identified as low level (–1), high level (+1), and the central point (0). The conditions of factorial design were 10/1 and 169/1 for C/N ratio and 0.029/1 mM $MnSO_4$/mM $CuSO_4$ and 0.071/2 mM $MnSO_4$/mM $CuSO_4$ for inductor concentration. These values were chosen based on previous results (data not show). The treatments and the results of the experimental design are shown in Tables 1(a) and 1(b).

A first-order experimental design was treated with an empirical model which relates the response measured to the independent factors of the experiment. For a two-factor system, the model is

$$y = b_o + b_1 x_1 + b_2 x_2 + x_1 x_2, \quad (1)$$

where y corresponds to the predicted decolorization, COD removal, and enzyme activities (LAC and MnP); b_o is the intercept, b_1, b_2 are the linear coefficients, and X_1, X_2 are the factors. The evaluation of the coefficients determined the address in which the factors should move to obtain better results (Tables 2 and 3). The quality of the fitted model was evaluated analyzing the variance (ANOVA) and verifying if the model reproduces the experimental data in the studied range. The "Design Expert" (version 6.0) and "SAS" (version 5) software were used for analyses of the obtained data.

Experiments were carried out in 125 mL Erlenmeyer flasks containing 50 mL of the wastewater supplemented with

Table 1: Values of real values and coded levels used in the factorial designs.

(a)

Treatment	Factor X_1	Factor X_2	Decolorization (%)	COD removal (%)	Laccase volumetric activity (U/L)	MnP volumetric activity (U/L)
1	−1	−1	7.24	10.26	0	0.3
2	+1	−1	20.13	72.47	23.08	1.57
3	−1	+1	10.57	15.60	0	0.89
4	+1	+1	60.36	51.40	60.36	8.64
5	0	0	55.97	19.05	44.67	0.16
6	0	0	57.14	23.81	48.04	0.13
7	0	0	59.20	18.18	44.5	1.6

Numeric value equivalent to the code level (−1), (+1) *y* (0).

(b)

Factor level	C/N ratio (X_1)	Inductor concentration (X_2) mM $MnSO_4$/mM $CuSO_4$
Lower level	10/1	0.029/1
Upper level	169/1	0.071/2
Central point	90/1	0.05/1.5

Table 2: ANOVA results, model and coefficient validation for decolorization and COD removal in percentage.

Factor	Decolorization Percentage						Chemical oxygen demand COD removal (%)					
	SS	DF	MS	*F* value	Prob > *F*	RC	SS	DF	MS	*F* value	Prob > *F*	RC
Model	5390.19	3	1796.7	215.62	<0.0001	24.57	7914.67	3	2638.22	113.46	<0.0001	37.43
X_1	2946.13	1	2946.1	353.56	<0.0001	15.67	7205.92	1	7205.92	309.89	<0.0001	24.50
X_2	1422.90	1	1422.9	170.76	<0.0001	10.89	185.54	1	185.54	7.98	0.0180	−3.93
X_1X_2	1021.16	1	1021.1	122.55	<0.0001	9.22	523.20	1	523.20	22.50	0.0008	−6.60
Curvature	2596.72	1	2596.7	311.63	<0.0001		700.64	1	700.64	30.13	0.0003	
Pure error	83.33	10	8.33				232.53	10				
Cor total	8070.23	14					8847.83	14				
CV			9.27				CV		14.18			
R^2			0.9802				R^2		0.9629			
Adeq Precision			31.871				Adeq Precision		22.347			

different glucose and inducer concentration (according to experimental design). A total of ten cubes colonized with the fungus were inoculated and Erlenmeyer flasks were continuously shaken at 120 rpm for 4 d at 25°C. Control was carried out with the same number of Erlenmeyer flasks but inoculated with cubes colonized by fungal biomass inactivated with thermal treatment at 121°C for 30 min.

2.6.2. Optimization of Factors with the Path of Steepest Ascent Method. After the first-order experimental design was established, the concentration factors were optimization with the path of steepest ascent method. The selected value of origin was the C/N ratio and inducer concentrations used in the 2^2 factorial designs (C/N ratio: 90/1 with 5.5 g/L glucose to obtain 90/1 C/N ratio, 0.05 mM $MnSO_4$, and 1.5 mM $CuSO_4$). The delta selected to calculate the different ascents was 0.5 (C/N: 45, 0.025 mM of $MnSO_4$, and 0.75 mM of $CuSO_4$), the increased of ratio C/N was make for addition of glucose into effluent according to optimization methods reported by Montgomery [19]. Experiments and controls were carried out in same form to use in design factorial and the parameters were evaluated on the 4 d (Table 4).

2.7. Biological Reactor and Operational Conditions. The biological treatment was performed in reactors made up of glass tubing (6 cm inner diameter, 50 cm long) packed with 160 polyurethane foam cubes colonized with *T. versicolor* and 800 mL of nonsterilized bleaching effluent, supplemented with glucose up to a C/N ratio of 405 (glucose concentration 25 g/L) and inducers (6.75 mM $CuSO_4$ for LAC activity and 0.22 mM $MnSO_4$ for MnP activity). The reactors were maintained at 25°C, with an air flow rate of 800 mL/min (Figure 1). The process was evaluated over 8 d. The values in the figures correspond to the mean values of three replicates with a standard deviation of less than 20%.

Table 3: ANOVA results, model and coefficient validation for laccase an MnP volumetric activities.

Factor	Laccase volumetric activity (U/L)						MnP volumetric activity (U/L)					
	SS	DF	MS	F value	Prob > F	RC	SS	DF	MS	F value	Prob > F	RC
Model	7305.25	3	2435.0	237.19	<0.0001	20.86	136.50	3	45.50	72.22	<0.0001	2.85
X_1	5220.55	1	5220.5	508.50	<0.0001	20.86	61.05	1	61.05	96.90	<0.0001	2.26
X_2	1042.35	1	1042.3	101.53	<0.0001	9.32	43.95	1	43.95	69.77	<0.0001	1.91
X_1X_2	1042.35	1	1042.3	101.53	<0.0001	9.32	31.50	1	31.50	50.00	<0.0001	1.62
Curvature	1487.34	1	1487.3	144.87	<0.0001		11.81	1	11.81	18.74	0.0015	
Pure error	102.67	10	10.27				6.30	10	0.63			
Cor total	8895.25	14					154.61	14				
CV			12.40				CV		0.9426			
R^2			0.9820				R^2		33.01			
Adeq Precision			32.626				Adeq Precision		18.196			

Table 4: Path of steepest ascent, experimental design.

Ascent	Factor X_1 C/N ratio	Factor X_2 Inducer Concentration mM $MnSO_4$/mM $CuSO_4$
Origin	90/1	0.05/1.5
Delta 0.5	45/1	0.025/0.75
Origin + 1Δ	135/1	0.075/2.25
Origin + 2Δ	180/1	0.1/3
Origin + 3Δ	225/1	0.12/3.75
Origin + 4Δ	270/1	0.15/4.5
Origin + 5Δ	315/1	0.17/5.25
Origin + 6Δ	360/1	0.20/6.0
Origin + 7Δ	405/1	0.22/6.75
Origin + 8Δ	450/1	0.25/7.5
Origin + 9Δ	495/1	0.27/8.25
Origin + 10Δ	540/1	0.30/9.0
Origin + 11Δ	585/1	0.32/9.75
Origin + 12Δ	630/1	0.35/10.5

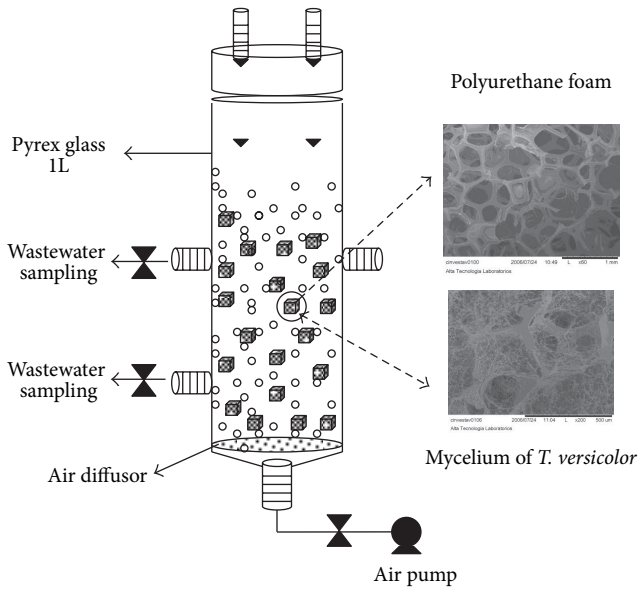

Figure 1: Bubble column reactor with polyurethane foam cubes colonized with *T. versicolor.*

3. Results and Discussion

3.1. Scanning Electron Microscopy of Fungal Colonization in the Support. Polyurethane foam was used as inert matrix to favor the penetration of the fungus. This material has a surface area of 450 m^2/m^3 and density of 18 Kg/m^3. Probably these characteristics favored the colonization process reaching a maximum biomass per cube of 93 mg/cm^3 at 9 d. On the other hand, we observed by SEM that the colonization of the fungal biomass was inside the foam and an extensive mycelium network was recovered completely from the PUF surface (Figures 2(a) and 2(b)). In some sections of the PUF were also observed other microorganisms that were from the wastewater as Gram-negative bacilli and yeast (Figures 2(c) and 2(d)). These Gram-negative bacilli produced blue fluorescence and cytochrome C oxidases (oxidase test); these results suggest the presence of *Pseudomonas* spp. into wastewater. *Pseudomonas* spp. is a bacterium widely distributed in nature and produces multicomponent enzyme systems (mono- and di-oxygenase) that add one or two oxygen to the aromatic nucleos to form arene *cis*-diol. With these enzymes *Pseudomonas* spp. could biotransform some lignin byproducts and participates in the color and COD removal. Activities that could be done in cooperation with the yeast and *Trametes versicolor* could be immobilized into PUF support. A similar result were found for Tiku et al.. In their research *Pseudomonas* sp. and *Bacillus megaterium* were recovered from pulp and paper mill effluents, and its ability to remove COD and BOD was demonstrated [20].

3.2. Experimental Design for Decolorization by T. versicolor. The main effects of the two factors studied and the binary interaction effect involving these factors are shown in Tables 2 and 3. The effects that presented statistical influence, for a level of confidence of 95%, on the dependent variable (decolorization, COD removal, and LAC and MnP activities), are C/N ratio, inductor concentration, and their interaction inside the range of the experimental studies.

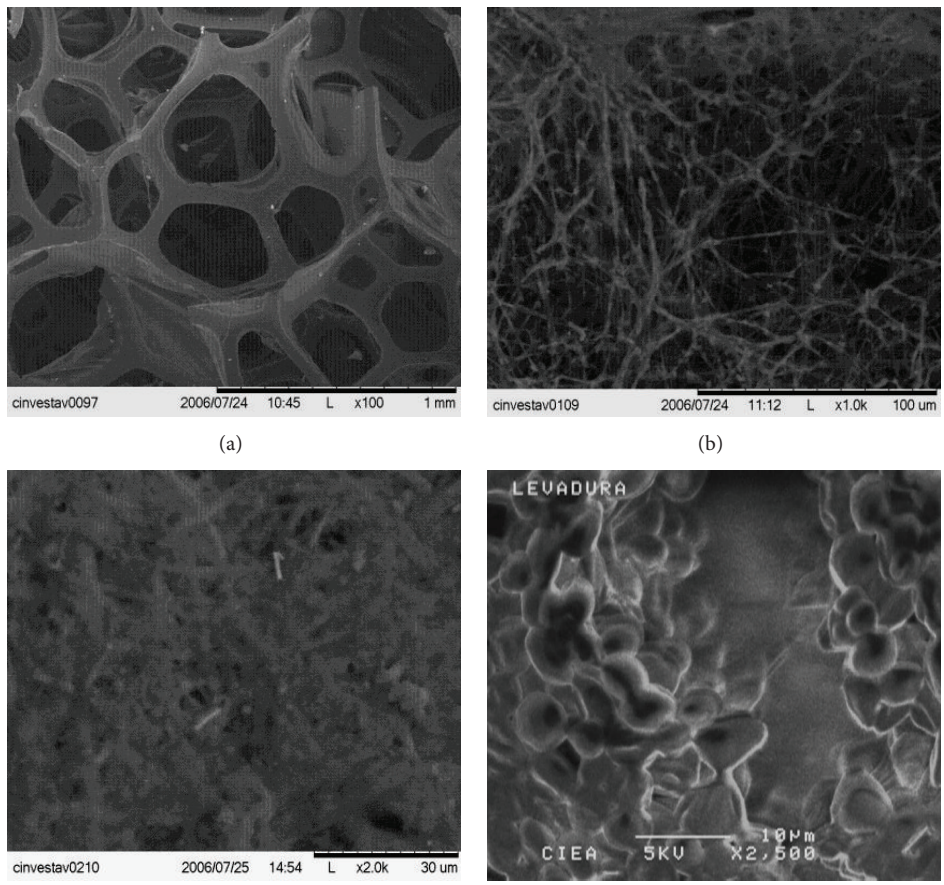

Figure 2: SEM microphotographs of polyurethane foam without colonization 100x (a). Polyurethane foam colonized with *T. versicolor* at 24 h of incubation 1000x (b). Mycelium of *T. versicolor* and Gram-negative Bacilli at 72 h of incubation 2000x (c). Yeast cells into the support at 72 h 2500x in bubble column reactor.

Analysis of variance (ANOVA) showed that X_1 factor, X_2 factor and their interaction X_1X_2 (high C/N ratio (169), and inductor concentration (0.071 mM $MnSO_4$/2 mM $CuSO_4$)) have a significant effect on decolorization, COD removal, and enzyme production. The values of the regression coefficients from the 2^2 factorial experimental design with central points (Tables 2 and 3) were calculated, and the first-order equations for decolorization, COD removal, and LAC and MnP activity (2), (3), (4), and (5) are shown:

$$\text{Decolorization (\%)}\,(y) = 24.5 + 15.66x_1 + 10.88x_2 + 9.21x_1x_2, \quad (2)$$

$$\text{COD removal (\%)}\,(y) = 37 + 24x_1 - 3.93x_2 - 6.6x_1x_2, \quad (3)$$

$$\text{Laccase activity U/L}\,(y) = 20.81 + 20.86x_1 + 9.32x_2 + 9.32x_1x_2, \quad (4)$$

$$\text{MnP activity U/L}\,(y) = 2.85 + 2.26x_1 + 1.91x_2 + 1.62x_1x_2. \quad (5)$$

According to (2), (4), and (5), an increase in the concentration of the respective factors should have a positive effect for the dependent variables. However, the most influential factor was the C/N ratio (X_1 factor) with linear coefficients value of 15.6, 24, 20.86, and 2.26 for decolorization, COD removal, laccase, and MnP activities. These results suggest that *Trametes versicolor* increased the removal capacity and enzyme activities when de C/N ratio increases (ligninolytic conditions). For this reason the best results were obtained when the wastewater was supplemented with high concentration of glucose as simple carbon source (treatments 2 and 4); in contrast when the supplement with glucose were low (treatments 1 and 3), the removal and laccase activity were lower than T2 and T4. These results could be related to the production of proteolytic enzymes that cleave the ligninolytic enzymes when fungi have a deficit or low concentrations of carbon and nitrogen.

3.3. Optimization of Factors with the Path of Steepest Ascent Method. In this study we only used the first-order equation obtained for decolorization to optimization process using the path of steepest ascent methodology. The experimental results showed that, as the coefficient of the factors was increased, a positive effect was observed ($P < 0.0001$) on

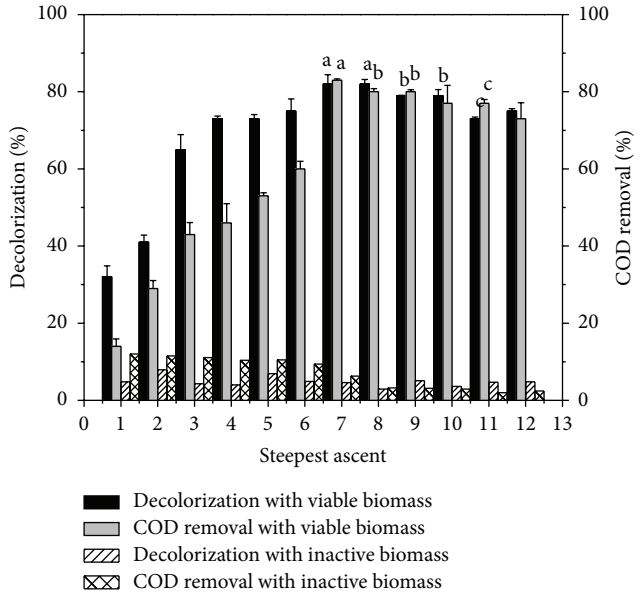

Figure 3: Decolorization and COD removal by *T. versicolor* in the path of steepest ascent method. 4 d, 120 rpm, and 25°C.

the dependent variables. The maximum values of 82%, 83%, 443 U/L, and 18 U/L, for color removal, COD removal, and LAC and MnP enzyme activities, respectively, were obtained (Figure 3). The highest value was obtained in step 7 (C/N ratio 405 and inducer concentrations 0.22 mM $MnSO_4$ and 6.75 mM $CuSO_4$), with a positive correlation ($P < 0.0001$) between decolorization and COD removal in this step. These results could indicate that the organic compounds such as quinones and stilbenes were degraded and their capacity to absorb light were progressively decreased [3]. On the other hand the requirement for a second carbon source during paper industry wastewater degradation by different white rot fungi has been widely reported by Kaushik and Malik, Elisashvili and Kachlishvili, and Justino et al. [21–23]. In these works it was shown that degradation capacity of Basidiomycetes increased when Kraft effluents were supplemented with a less-complex carbon source, such as glucose. These results agree with those reported by Belém et al. [2], who used two white rot fungi (*Pleurotus sajor caju* and *Pleurotus ostreatus*) for the wastewater treatment, finding that as the concentration increased in a simple co-substrate (glucose), the efficiency in the COD removal and absorbance reduction for several wave lengths increased at 14 days of incubation. However further experiments are needed to determine other carbon sources, which may allow a more efficient decolorization and COD removal from a technical and economic point of view. The glucose was a suitable carbon source to determine the optimization model but is expensive for environmental applications using reactor with major capacity.

The correlation between decolorization, COD removal, and enzymatic activity, LAC and MnP, was less ($P > 0.0001$). This result could be justified in terms that the capacity of bioremediation of these microorganisms is associated with biochemical processes and physical-chemical parameters such as biosorption, deposition, and ionic exchange with components of the cellular wall, like chitin and chitosan [10–25]. For these reasons the removal can be development for both mechanism and the correlation with the enzymes was different with respect to the decolorization and COD removal. In the controls it was determined 10% of decolorization and 15% of COD removal indicating that dead biomass and the support could be adsorbed color and organic matter.

When analyzing the effect of the inducers on the enzymatic activity in step 7, one can observe a significant difference ($P < 0.0001$) between the production of LAC (Figure 4(a)) and MnP (Figure 4(b)) with respect to others steps. Youshuang et al. [26] demonstrated that *T. versicolor* is producing a thermostable metal-tolerant laccase and this enzyme showed good decolorization of triphenylmethane and azo dyes in the experiments without mediators.

The enzymatic laccase activity increased when copper is present in the culture medium. It has been postulated by Guillén and Machuca and Rivera-Hoyos et al. [27, 28]. These authors suggest that manganese and copper regulate *mnp* gene transcription via a multicomponent system, perhaps involving signal receptors, a transducer, and an intercellular second messenger, such as cyclic AMP or a phosphorylation cascade, similar to the mechanism of metalloregulation in others eukaryotes. On the other hand, Álvarez et al. studied the effect of copper on the expression of genes encoding the ligninolytic enzymes, laccase (*lcs*) and manganese peroxidase (*mnp*) in *Ceriporiopsis subvermispora*. They showed that this metal increased transcript levels of *lcs*, *mnp1,* and *mnp2,* and they isolated and characterized an ACE-1 like transcription factor from *C. subvermispora* (Cs-ACE1) essential to laccase induction with copper addition [29].

In our study the induction by heavy metals only was observed at concentrations of 6.75 mM copper and 0.22 mM manganese. Above these values these metals had a toxic effect on *T. versicolor* growth, and a decrease in the enzyme activity was observed (data not show). The mechanism by which *T. versicolor* is active at high concentrations of metals is associated with two phases. The first is independent of the metabolism and concerns the sorption of the metal to the cellular wall, while the second is dependent on the energy [30]. The sorption of significant quantities of the metal to the fungal cell wall is a rapid process, which is visualized by the pigmentation of the mycelium. These pigments (melanin, polymers of L-DOPA, catechol, glutaminyl-3, 4 dihydroxybenzene and 1, and 8 dihydroxynaphthalene) were produced for LAC enzyme [31]. Copper-tolerant fungi produce oxalate which precipitates the metals in the form of Cu-oxalate crystals. This mechanism could be acting in our case since, with a C/N ratio of 405 *Trametes* sp. produced organics acid which led to a decrease in the pH to 4.1. This value is within the range reported for the two enzymes (between 4.0 and 4.5).

3.4. Biological Treatment in Bubble Column Reactor. In this study *T. versicolor,* under nonsterile conditions, decreased the color during the first 24 h, and a level of 80.1% decolorization was attained by 4 d (Figure 5(a)). The mechanisms

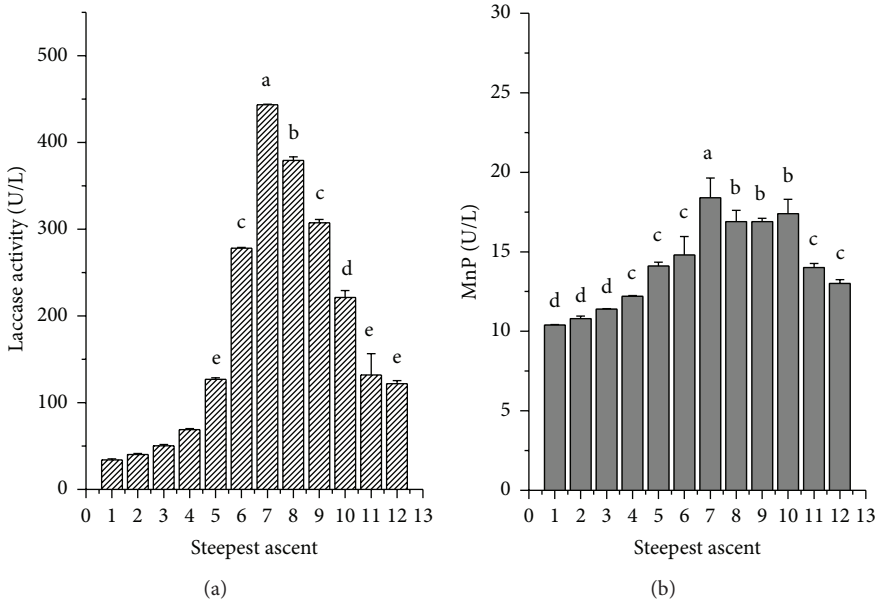

Figure 4: Enzyme activity during treatment with *T. versicolor* in the path of steepest ascent method, 4 d, 120 rpm, and 25°C. LAC activity (a) and MnP activity (b).

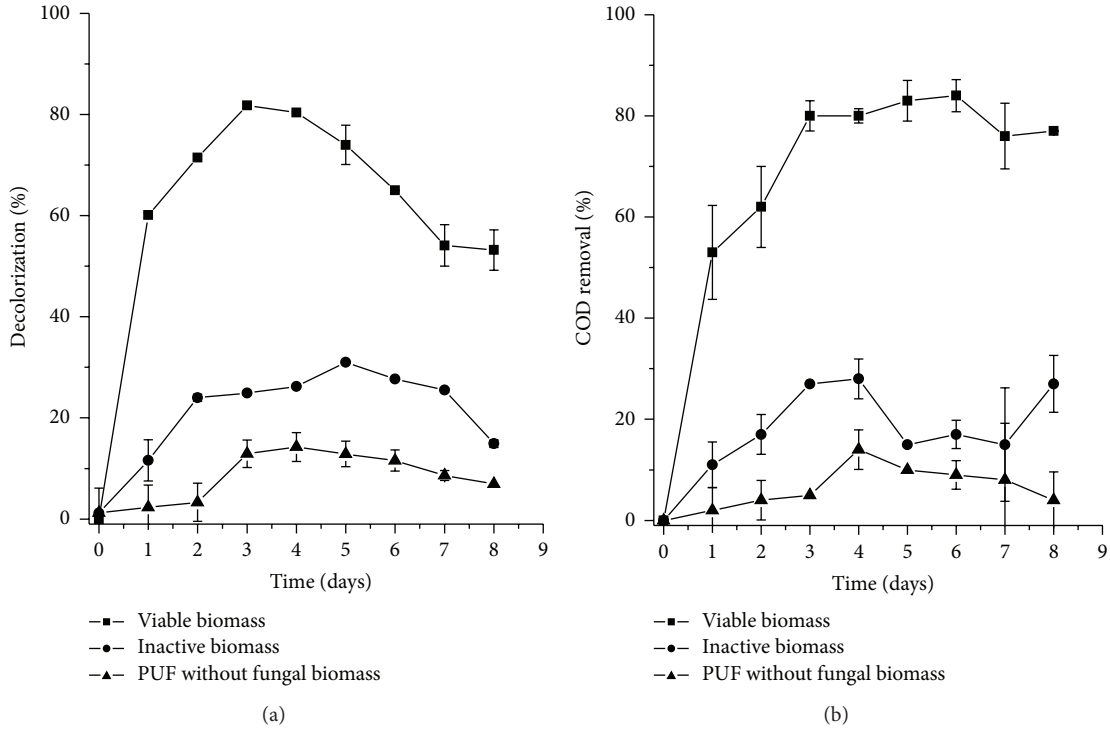

Figure 5: Biologic treatment with immobilized *T. versicolor,* 8 d, 25°C, 800 mL/min flow air, and nonsterile conditions in bubble column reactor. Decolorization (a) and COD removal (b).

involved in the decolorization could be associated with physical adsorption followed by a biochemical mechanism. The first mechanism is not associated with metabolism but implies a biosorption through the formation of hydrophilic-hydrophobic interactions between the compound and the cell wall [25]. The second mechanism is associated with primary metabolism, in which the fungus uses a cosubstrate, like glucose, to degrade the most toxic compounds and to allow the color reduction [10, 32]. The decolorization could be carried out by oxidation (via removal of one or two electrons)

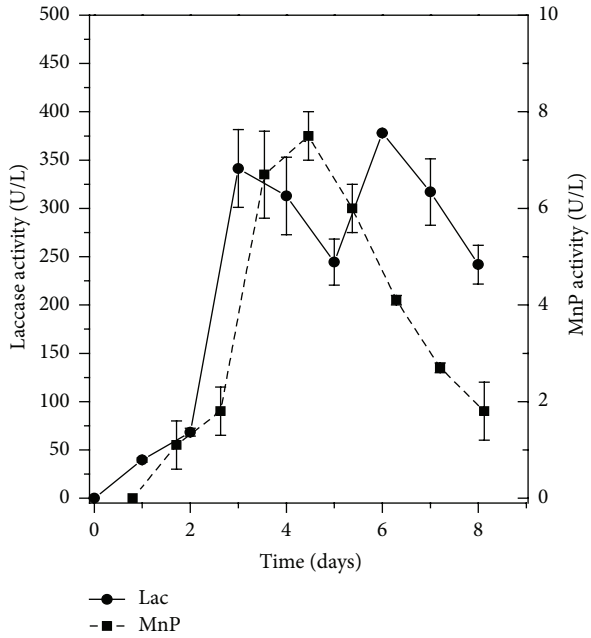

FIGURE 6: Enzymatic activity with immobilized *T. versicolor*. LAC activity (a), MnP activity (b), 8 d, 25°C, 800 mL/min flow air, and nonsterile conditions in bubble column reactor.

of the recalcitrant chromophoric groups of the lignin to form radical cationic species that can follow different degradation routes [32–34]. During this study enzymatic activity was observed to increase progressively until it reached maximum values between days 4 and 5 of 345 U/L LAC and 78 U/L MnP (Figures 6(a) and 6(b)). Statistically a correlation ($P < 0.0001$) exists between the percentage of decolorization, COD reduction, and enzymatic activity.

The results obtained are interesting, since the fungus carries out the bioremediation process of the wastewater from the pulping industry without requiring sterile conditions, reaching a high (80%) colour removal in 96 h with 25 g/L glucose as cosubstrate at acid pH (Figures 7(a) and 7(b)). In comparison, Sahoo and Gupta reported 60% decolorization of effluent at 72 h with a supplement of 20 g/L glucose using free cells of *T. versicolor* but under sterile conditions [35].

With regards to the reduction of the COD, the initial concentration was 50400 mg/L, and this progressively diminished, attaining 82% of up-to-date removal with a positive correlation (Figure 5(b)) between percentage of COD reduction and chlorophenols ($P < 0.0001$). Rocha-Santos et al. demonstrated that the biomass of different white rot fungi and *Rhizopus oryzae* can be used for the organic compounds removal (carboxylic acids, fatty alcohols, phenolic compounds, and sterols) present in Kraft pulp mill effluent [36]. We found similar results with glucose as a source of carbon, low nitrogen concentration, copper and manganese addition, and acidic pH.

With respect to the quantified chlorophenols, we observed during the biological treatment that removal started in the first 24 h with the biosorption processes and further degradation of the organic compounds. Maximum removal was attained after 4 d with 98% of PCP, 92% of 2,4,5-TCP, 90% of 3,4-DCP, and 99% of 4CP (Figure 8). The latter was possibly generated by the dechlorination of highly chlorinated compounds like pentachlorophenol and tetrachlorophenol. A positive correlation ($P < 0.0001$) between COD reduction, decolorization, chlorophenol removal, and LAC activity was obtained (Figure 6(a)).

The removal of chlorophenols may be associated with a cometabolism process, and it becomes more efficient in the presence of a simple cosubstrate. The use of fermentable sugar allows a decrease in pH up to 4.1 (Figure 7(b)). A similar behavior was observed by Ulla, who used *Coriolus versicolor* for PCP removal and found that pH 5.0 was associated with glucose consumption and a greater removal (90%) was obtained, showing a strong correlation with the levels of LAC expression [37]. The enzymatic degradation of aromatic chlorinated compounds starts with an oxidative dechlorination due to the LACs, resulting in the formation of *p*-quinone (2,6-dichloro-1,4-quinone or tetrachlorobenzoquinone) or cationic radicals that result in a further dechlorination, which could occur via a nucleophilic attack on a molecule of water, producing chloroquinones [38]. These compounds are strong oxidizers and can be transformed into the corresponding hydroxyquinones, eliminating the atoms of chlorine completely, so that the ring opens upon the action of MnP and LAC to form sour beta intermediary ketoadipic acid that enters into the Krebs cycle [39].

When using the biological reactor without sterilizing the wastewater, the bacteria (12×10^2 CFU/mL) and fungi (23×10^2 CFU/mL) present in them increased progressively until we obtained recounts of 50×10^7 CFU/mL for heterotrophic bacteria and 34×10^6 CFU/mL for heterotrophic fungi. A positive cooperation was possible between *T. versicolor* (biomass per cube of 193 ± 5.3 mg/cm^3 at 8 d) and the other microorganisms. This situation is very favorable since the system could be used in reactors of higher capacity without the need to control the sterility. This diminishes the risk of losing efficiency due to the inhibition or death of the Basidiomycete.

4. Conclusions

Finally under laboratory conditions, the decolorization and COD removal in paper industry wastewater using *T. versicolor* were successfully optimized using a 2^2 factorial experimental design with central points and a steepest ascent method. A high C/N ratio together with the addition of millimolar amounts of $CuSO_4$ and $MnSO_4$ to the actively growing *T. versicolor* cultures offers an easy and efficacious method for stimulating color, COD, chlorophenols removal, and in minor proportion LAC and MnP activity. This represents an attractive way to stimulate pollutants removal partial associated with enzyme production and adsorption process.

The fungus *T. versicolor* interacted with bacteria and yeasts (50×10^7 CFU/L heterothophic bacteria, 34×10^6 CFU/mL of heterotrophic fungi) diminishing the color (1147 UC), COD (10400 mg/L), and chlorophenols in 4 d in bubble column reactor.

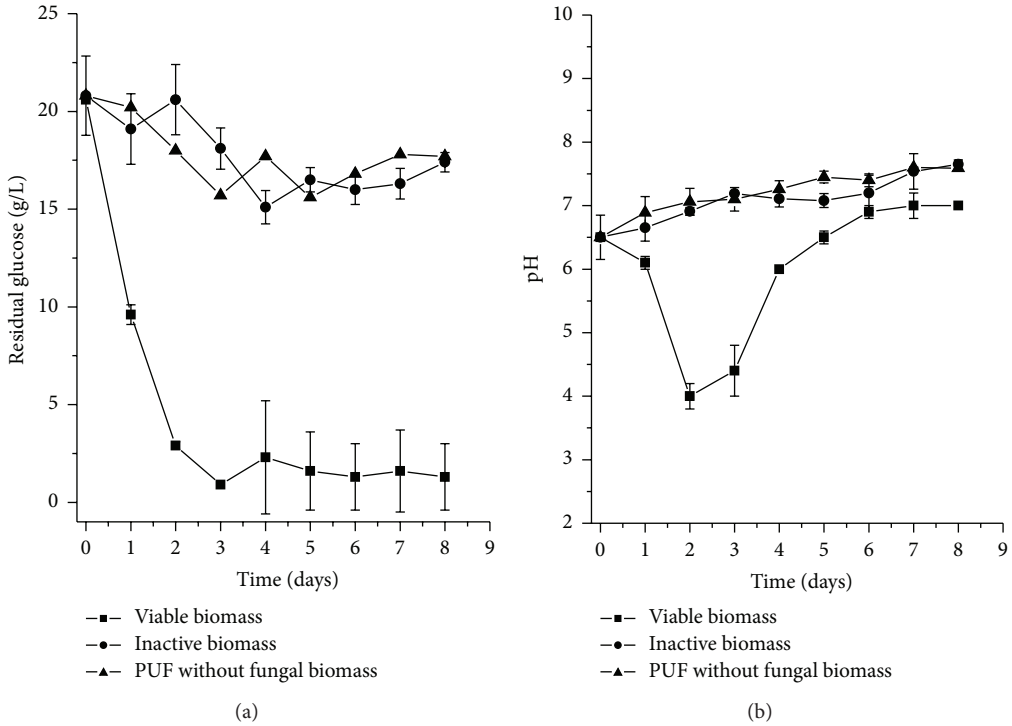

Figure 7: Glucose consumption (a) and pH (b) with immobilized *T. versicolor,* 8 d, 25°C, 800 mL/min flow air, and nonsterile conditions in bubble column reactor.

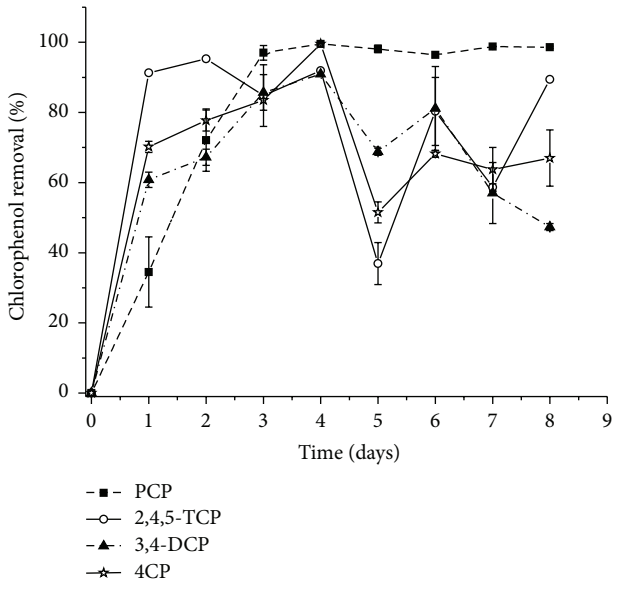

Figure 8: Chlorophenols removal with immobilized *T. versicolor,* 8 d, 25°C, 800 mL/min flow air, and nonsterile conditions in bubble column reactor.

Conflict of Interests

The authors declare that they do not have any conflict of interest.

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